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Experimentele ecologie en de evolutie van microbiële diversiteit

De rol van spatiële structuur.

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Thesis abstract

In the light of the competitive exclusion principle, which states that complete competitors cannot coexist, many explanations have been sought to explain the high diversity found in nature. The most common explanation is the niche differentiation hypothesis: coexistence is obtained through differentiation of species in ecological niches. Spatial structure is thought to be a factor capable of providing opportunities for niche differentiation. We have focused on four aspects of spatial structure enabling genetic diversity to emerge and /or to be maintained.

First of all, population fragmentation, resulting from growth in spatially structured habitats, can increase diversity, because the resulting smaller subpopulations, due to their smaller population size, are more likely to adaptively diverge. By allowing small and large populations of *E. coli* to evolve for 500 generations in two different nutrient environments, we test this hypothesis. The results demonstrate higher variance in fitness among small populations, and consequently more heterogeneous adaptive trajectories for small populations, some of which surprisingly lead to higher fitness peaks than reached by even the best adapted large population.

In a short-term invasion experiment between a superior *E. coli* competitor and its inferior ancestor, we demonstrate that populations residing in structured environments experience slower invasion dynamics of beneficial mutations than well-mixed populations due to limited dispersal, and therefore local competition. Moreover, our results demonstrate a deceleration of invasion with increasing size of the invading subpopulation. This is caused by a decrease of inter specific competition relative to intra specific competition. Since inferior competitors are present in the community for a longer period of time, they can recombine with other persisting lineages or obtain new mutations, some of which might be beneficial. It is therefore possible that polymorphisms arise which would not have had the opportunity to emerge in a well-mixed environment. Even though both population fragmentation and slower competitive dynamics can increase the emergence of diversity, they do not provide a means for their maintenance.

Environmental heterogeneity on the other hand *can* cause maintenance of diversity. Environmental heterogeneity can be introduced by spatial structure, e.g. by providing gradients in biotic and abiotic factors, thereby increasing the number of niches. By allowing *E. coli* populations to evolve for 900 generations in either a well-mixed environment or two structured environments (with or without dispersal), we demonstrate stable coexistence of diversity in structured populations without dispersal. This can be attributed to negative frequency-dependent fitness interactions among niche specialists that either inhabit existing niches provided by the heterogeneous environment or new niches constructed by organisms inhabiting the environment.

In addition to examining aspects of spatial structure that provide means for populations to diversify, we examine a specific consequence of slower dynamics and environmental heterogeneity: the probability of mutators to hitchhike to fixation. Understanding the emergence of mutators is not only scientifically important, but also relevant for human health, since high frequencies of mutators have been found in bacterial populations and drug resistant mutants arise more often in mutator populations. *E. coli* mutator populations were introduced at different starting frequencies in a well-mixed environment and two structured environments differing in their dispersal rate. Contrary to expectations, we find an advantage in the rate of invasion for mutators in well-mixed environments. Faster competitive dynamics may allow a rapid increase of population size and hence a greater supply of mutations for subsequent adaptation. Due to a delay in mutator extinction in structured environments at low frequencies, mutators may gain from fluctuating conditions.

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1   Introduction

With species disappearing at a rate equalled by few mass extinctions, the preservation of biodiversity has become a matter of public urgency. However, the effectiveness of strategies for preserving diversity is constrained by our limited knowledge of the mechanisms giving rise to and maintaining diversity (Tilman 2000). Even though many hypotheses have been put forward to explain the great diversity found in ecosystems, empirical evidence is limited.

Diversity exists within species (genetic diversity), among species (species diversity) and at the level of the ecosystem (ecosystem diversity). These levels of diversity are not mutually independent; diversity within a species can be necessary for the coexistence between species and vice versa (Lankau & Strauss, 2007). In addition, it has been demonstrated that higher species diversity leads to greater ecosystem stability (McCann 2000). Studying all levels of diversity is essential to better understand ecosystem stability. In this thesis we will focus on the emergence and maintenance of diversity within species.

The competitive exclusion principle states that competitors occupying the same niche cannot coexist (Hardin 1960), since elimination of all but the superior competitor is expected. However, species do coexist; hence, clarifications are needed. The most common explanation for polymorphisms in populations and sympatric speciation is niche differentiation: heterogeneity of the environment gives populations the opportunity to diverge and specialize into the various niches provided by this heterogeneity (but see Maynard Smith & Hoekstra 1980). A well-described example of niche differentiation is the adaptive radiation in sticklebacks. A large benthic species feeds on large prey and lives in the littoral zone, whereas a smaller species feeds on plankton in the open water. Of a morphological intermediate form, feeding on both plankton and invertebrates, specialization in favour of one phenotype has been demonstrated experimentally, in the presence of strong competition by the opposite extreme (Schluter 1994).

Niche-differentiation is not the exclusive means by which diversity can arise and coexistence can be maintained (for a review see Scheffer *et al.* 2003). Other mechanisms that

have been proposed are for example oscillations and chaos caused by resource competition (Huisman *&* Weissing 1999), antibiotic interactions in microbial communities (Czárán *et al*. 2002), and neutral theory (Hubbell 2001). Unfortunately, empirical support for these models is, at present, limited.

Specialization to different niches is not enough for stable coexistence: *trade-offs* have to accompany these specializations, i.e. improvement in one trait leads to a disadvantage in other traits. Trade-offs may exist between any combination of traits, for example between competitive ability and dispersal ability (Tilman 1994), between the abilities to compete for different resources, or between competitive ability and susceptibility to toxins (Bohannan *et al*. 2002). Two different genetic mechanisms can cause trade-offs (Elena & Lenski 2003): mutations beneficial under certain conditions can be deleterious in other conditions (antagonistic pleiotropy), or mutations that are neutral in one niche can be deleterious in another niche (mutation accumulation).

Specialization and trade-offs lead to a higher intra specific competition versus interspecific competition (Chesson 2000), necessary for coexistence. However, even though trade-offs are necessary for stable coexistence, they are sometimes not sufficient. For stable coexistence it is necessary that populations can recover from low population densities. Therefore, the fitness of coexisting populations needs to be negatively frequency dependent (Turner *et al.* 1996; Chesson 2000).

The literature on adaptive radiation –the evolutionary divergence of members of a single phylogenetic line into a variety of adaptive forms (Futuyama, 1998) - is extensive. However, few examples are generally accepted to be adaptive radiations (Schluter 2000). Many other radiations lack evidence that they are adaptive. It is difficult to verify whether natural selection gave rise to a trait; using functional, experimental and genetic methods, hypotheses of adaptive radiations are tested, however, it is often difficult to go back in time and expressly determine why a trait evolved (Losos, 2000).

Selection experiments with animals have been performed that provide evidence for adaptive radiations (Schluter 1994; Losos *et al*.1997; Losos *et al*. 2001). However, due to the

complexity of these organisms and of their environment, it remains difficult to isolate the object of natural selection. In addition, even though maintenance of coexistence can often be confirmed (e.g. by perturbation experiments), demonstrating emergence of diversity remains complicated for higher animals.

Microbes have proven to be valuable model systems for investigating the genetic and ecological causes of diversity. Since the work presented here is embedded in the field of microbial experimental evolution, the introduction will mainly consist of examples from this field. Extrapolating results obtained from experiments using asexual, haploid organisms to sexual and / or diploid organisms must be done with caution. An important difference is that heterozygous advantage (Ford 1965), and sexual selection, contribute to the maintenance of polymorphisms in diploid and sexual organisms only.

Experimental evolution

Studying diversification processes using micro-organisms as a model system has proven to be fruitful for a number of reasons. Because experiments can be set up with high replication using genetically identical ancestors and a controlled selective environment, the evolutionary outcome can be directly attributed to the selective conditions in the experiment (Travisano & Rainey 2000). Aliquots of populations can be frozen at -80˚C, providing the opportunity to directly compare the fitness of any of the evolved types with the ancestor in competition experiments (Lenski *et al*.1991). An additional advantage is the relative small genome size of micro-organisms, making it easier to track down mutations than it is for higher organisms, especially for the many micro-organisms whose genomes have been sequenced. And lastly, owing to the relatively short generation time of micro organisms, adaptation can take place for up to thousands of generations per year.

Polymorphisms in homogeneous environments

Because experimental microbial populations tend to be quite large (in the order of millions), mutations are expected to arise every generation. In bacterial populations, Atwood and his colleagues (1951) found, that mutants with a selective advantage would arise and would replace the original bacterial population. This process would repeat itself, as soon as the

number of mutants would reach sufficiently high densities. Adaptation in asexual populations consists of these sequential substitutions of beneficial mutations within the same line of descendants (Koch 1974; Levin 1981). Even though several beneficial mutations will be present simultaneously and compete for fixation, it is expected that the largest beneficial mutations will win, thereby eliminating the pre-existing variation in a process called 'clonal interference' (Gerrish and Lenski 1998).

In accordance with the competitive exclusion principle, polymorphisms in homogeneous environments are not expected from this model of adaptation in asexual populations. Polymorphisms have nevertheless been found in a number of studies (Levin 1972; Helling *et al*. 1987; Rosenzweig 1994; Rozen & Lenski 2000; Maharjan *et al*. 2006). In a long-term evolution experiment of Helling and colleagues (1987), a stable polymorphism was found in a population in a simple glucose environment, founded by a single clone of *E. coli*. Data showed that the polymorphism was dependent on resource partitioning - the partitioning of a resource by multiple specialists diverged from a single ancestor- due to the excretion of secondary metabolites and cross feeding. It appeared that one evolved ecotype had become more efficient in utilizing the main resource component, whereas a second and third ecotype could grow more efficiently on a secondary metabolite excreted by the first one. In this case, the environment had become heterogeneous due to evolved metabolic changes in the organisms; a new niche was provided by evolution itself.

Polymorphisms in heterogeneous environments

A heterogeneous environment consists of multiple niches giving populations a chance to specialize on the different available niches. Niche opportunity can arise from e.g. diversity in resources, fluctuations in environmental factors or spatial structure (Rainey *et al.* 2000; Travisano & Rainey 2000; Kassen & Rainey 2004; Maclean 2005).

Resource heterogeneity

Using *Chlamydomonas* populations, it has been shown that pre-existing variance in fitness can be maintained in heterogeneous resource environments, but decreases in homogeneous environments (Bell 1997). Though this only demonstrates maintenance of diversity, later

experiments with *Pseudomonas* populations show a rapid diversification when populations consisting of isogenic genotypes are introduced into a complex resource environment (Maclean *et al*. 2005), but not in simple resource environments (Barrett *et al*. 2005). Diversification evolved at a greater rate in complex nutrient environments (Barrett & Bell 2006). Maintenance of diversity in these populations is established due to frequency-dependent selection (Maclean *et al*. 2005).

Temporal heterogeneity

Temporal variability of e.g. resource concentration, temperature and light, has been shown theoretically to promote coexistence of species (Dempster 1955; Dean 2005). This is evident in the seasons; competing species of higher organisms can survive unfavorable environments for which they are not adapted by having a life-history that avoids that particular environment, like seed dormancy or diapause (Hedrick 2006).

The serial- transfer method used in most microbial evolution experiments – and the feast-famine conditions micro-organisms experience in nature - resemble seasonal environments, where resources are abundant at the beginning of a transfer cycle and scarce after exponential growth has taken place (Turner *et al.* 1996). At least two studies have demonstrated the stable coexistence of two *E. coli* genotypes growing solely on glucose owing to the fluctuation of glucose during a transfer cycle (Turner *et al.* 1996; Rozen & Lenski 2000). In one study, one genotype was a superior competitor when glucose was abundant, the other when glucose was scarce (Turner *et al.*1996). However, the frequency dependence found was too strong to be explained by this demographic trade-off alone. In addition, cross-feeding was detected, which was necessary to explain strain coexistence. Another study found specialization to different parts in the growth cycle (D.E. Rozen, personal communication). One ecotype in the evolved population had become better at growing on cell debris during stationary phase, whereas the other ecotype had become competitively superior in growing on glucose during the exponential phase.

Differential ecological species responses preventing competitive exclusion are also found when temperature (Jiangl & Morin 2007; Descamps-Julien & Gonzalez 2005) or light

fluctuates. In aquatic systems, where light has a complex pattern of spatio-temporal variability, maintenance of diversity has been demonstrated for phytoplankton communities due to fluctuations in light intensity (Floder & Burns 2005) and light supply (Litchman1998). Using *Chlamydomonas*, Bell and Reboud (1997) revealed that in fluctuating light-dark environments diversification occurs due to a trade-off associated with adaptation to either light or dark environments. This genetic diversity is, however, only maintained in a spatially variable environment, not in a temporally fluctuating environment (Reboud & Bell 1997).

Complex interactions

Another contributing factor to the high diversity found in nature is predation (Chesson 2000) or parasitism. Laboratory experiments have shown the stable coexistence of sensitive and resistant *E. coli* bacteria in the presence of a phage (Levin *et al.* 1977; Chao *et al.* 1977; Bohannan & Lenski 1997) due to a trade-off between competitiveness for the primary resource (i.e. glucose) and phage resistance (Lenski 1988). Experiments using species of Pseudomonas have demonstrated diversification into multiple resistant forms (Buckling & Rainey 2002; Brockhurst *et al.* 2004; Brockhurst *et al.* 2005).

Many other factors and interactions have been shown to promote diversity, like productivity (Kassen *et al.* 2000), disturbance (Connell 1978; Gallet *et al.* 2007; Buckling *et al.* 2000) and allelopathy (Czárán *et al.* 2002; Kerr *et al.* 2002; Kirkup & Riley 2004). In many of these, spatial structuring has been implied to be essential for the maintenance of diversity because it provides for example refuges free of predators, or environmental heterogeneity (Kerr *et al.* 2002; Buckling *et al.* 2000; Kassen *et al.* 2000; Kirkup and Riley 2004; Schrag & Mittler 1986; Brockhurst *et al.* 2006). Few investigations however, have examined the role of spatial structure on diversification isolated from other factors (Rainey & Travisano 1998; Korona *et al.*1994; Korona 1996). In this thesis I focus on the role of spatially structured environments on the diversification of *E. coli* populations.

Spatial structure

Spatial structure could be a major factor explaining diversity, given that for example marine fauna, living in the relatively unstructured environment of the sea, lacks the astonishing

diversity of terrestrial fauna despite its large geographic range (Thorson, 1957). Spatial structure can enhance diversity in several ways.

First of all, through the presence of physical-chemical gradients, spatial structure provides additional niches to the environment (Korona *et al.* 1994; Korona 1996; Rainey & Travisano 1998). Rainey & Travisano showed that *Pseudomonas fluorescens* diversified rapidly in spatially heterogeneous microcosms (no shaking), but not in homogeneous microcosms (constantly shaken). In the homogeneous environment, only the original morph was found, whereas in the heterogeneous environment two additional ecotypes emerged; the wrinkly spreader and the fuzzy spreader (referring to the morphs they produce on agar plates). The wrinkly spreader occupied the air-broth interface by forming a biofilm, while the original morph occupied the broth. Maintenance between these types was ensured by an advantage for the wrinkly spreader when rare, due to oxygen limitation in the broth phase, together with a disadvantage when common, due to the destruction of the mat by its own weight. Coexistence of the fuzzy spreader seemed to depend on the interaction of the other two ecotypes.

Environmental heterogeneity can also be caused by organisms' ability to modify their surroundings. For example, by exploiting resources, fluctuations in resource supply can arise; light intensity can vary on the soil due to plant growth; and secondary metabolites can be excreted into the environment. In this manner, subpopulations may change abiotic and biotic gradients in their vicinity, creating new niches (Laland *et al.* 1999). As described before, in homogeneous environments organisms can change their environment in such a way that coexistence between two genotypes is possible (Helling *et al.* 1987); however, the ways to do this are more limited than in a structured environment because firstly, in a homogeneous environment, gradients cannot occur, and secondly, because if the population is not subdivided, the chances of interactions to occur are smaller simply due to a lower number of (sub)populations.

A third factor increasing the emergence of diversity in spatially structured environments is the segregation of the population into many small, semi-isolated subpopulations. Small populations are more likely to diverge due to a larger role of genetic drift (Wright 1931) and due to their tendency to follow more diverse adaptive routes owing to their preferential access to beneficial mutations conferring smaller benefits (Orr 1998; Burch

& Chao 1999; Miralles *et al*. 1999; deVisser and Rozen 2005), which are more abundant (Rozen *et al*. 2002; Orr 2005). Another consequence of fixing smaller mutations is a decrease in the rate of adaptation (Burch & Chao 1999; Miralles *et al*. 1999; deVisser *et al*. 1999).

Not only fixing smaller-effect beneficial mutations will lead to a slower adaptation rate in a structured environment, so will limited dispersal. Even though an invading mutant may have a higher competitive ability, limited dispersal and associated local resource competition will reduce its invasion rate (Crawly 1990; Burke & Grime 1996). This is a possible fourth factor influencing the emergence of diversity in structured environments. If the adaptation dynamics are slower, several mutants can coexist temporarily, possibly opening the way for a further increase in genetic diversity through recombination and the appearance of mutants able to coexist with others.

Fragmentation and slower invasion dynamics can increase diversity in populations, but only transiently. They do not provide a mechanism for coexistence of evolved diversity. For maintenance of the generated diversity, either heterogeneity of the environment is necessary or trade-offs have to exist between competitive ability and mechanisms preventing competition, like dispersal. The latter is an important aspect of coexistence in communities, but not one that will be examined in this thesis.

Aims and outline

The aim of the work presented in this thesis is to disentangle the various means by which spatial structure influences genetic diversity in ways described above. To investigate whether fragmentation of the population increases diversification, we first examined whether small populations follow more divergent adaptive routes than large populations (**chapter 2**). To this end, small and large *E. coli* populations were serially transferred for 500 generations in a simple and a complex (unstructured) nutrient environment. After evolution, fitness trajectories were compared.

Chapter 3 describes the influence of environmental heterogeneity, provided by spatial structure, with or without fragmentation and slower competitive dynamics, on the diversification of *E. coli* populations. The divergence of clones from *E. coli* populations that

were serially transferred in either a homogeneous environment (shaken liquid culture) or a spatially structured environment (agar surface), with or without dispersal was examined. In the structured environment without dispersal, we expected the population to be maximally fragmented, and therefore have the slowest competitive dynamics.

In **chapter 4**, we describe an experiment carried out to study the rate of invasion of a beneficial mutation in a spatially structured environment. This was done to test the hypothesis that adaptation would slow down in structured environments due to limited dispersal leading to inefficient local resource competition. While this is not a direct test of the causes of diversification within populations, slower dynamics increase transient diversity thereby possibly increasing the probability of stable coexistence of evolved ecotypes. To verify slower competitive dynamics in structured environments, a superior *E. coli* competitor was competed against an inferior one for five days in various environments that differed in the rate of dispersal.

A specific consequence of slower dynamics in structured environments is described in **chapter 5**. The fate of an *E. coli* mutator subpopulation was studied in both a homogeneous and a structured environment. Due to a difference in structure in the environments, we expected the mutator population to have different competitive dynamics. Understanding conditions that favour the emergence of mutator phenotypes is relevant for human health. High frequencies of mutators have been found in clinical bacterial populations (Leclerc *et al.* 1996). Mutator populations can cause treatment failure of antibiotics because resistant mutants arise more often in mutator populations. Even though it has been shown that in highly compartmentalized lungs of cystic fibriosis patients many mutators are present (Oliver *et al.* 2000), costs and benefits have been investigated solely in homogeneous environments (but see Giraud *et al.* 2001).

In **chapter 6**, I summarize and discuss the work presented in this thesis. I have tried to fit the data presented here in a broader ecological context. In addition, some future studies are suggested.

2 Heterogeneous adaptive trajectories of small populations on complex fitness landscapes

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Abstract

Small populations are thought to be evolutionarily handicapped, not only because they suffer more from deleterious mutations but also because they have limited access to new beneficial mutations, particularly those conferring large benefits. Here, we test this widely held conjecture using both simulations and experiments with small and large bacterial populations evolving in either a simple or a complex nutrient environment. Consistent with expectations, we find that small populations are adaptively constrained in the simple environment; however, in the complex environment small populations not only follow more heterogeneous adaptive trajectories, but can also attain higher fitness than the large populations. Large populations are constrained to near deterministic fixation of rare large-benefit mutations. While such determinism speeds adaptation on the smooth adaptive landscape represented by the simple environment, it can limit the ability of large populations from effectively exploring the underlying topography of rugged adaptive landscapes characterized by complex environments. Our results show that adaptive constraints often faced by small populations can be circumvented during evolution on rugged adaptive landscapes.

Introduction

It is widely held that the efficiency of natural selection is positively related to the size of an evolving population (Willi *et al*. 2006; Gerrish & Lenski 1998; Wilke 2004; deVisser *et al*. 1999; Reed 2005). This intuition derives from the expectations that small populations are more subject to the chance fixation of deleterious mutations by genetic drift (Muller 1964; Lande 1998; Lynch *et al*. 1993; Lynch *et al*. 1995; Chao 1990; Silander *et al*. 2007) and that fewer beneficial mutations arise in small populations compared to large ones (Fisher 1958; Muller 1932; Burch & Chao 1999; Miralles *et al*. 2000; Orr 1998; Rozen *et al*. 2002; Zeyl *et al*. 2003). An unexplored factor that could mitigate these constraints in small populations arises from the fact that beneficial mutations are unevenly distributed, with few mutations causing large fitness benefits and most causing more modest gains (Burch & Chao 1999; Orr 1998; Rozen *et al*. 2002; Barrett *et al*. 2006; Kassen & Bataillon 2006; Imhof & Slotterer 2001). This skewed distribution implies that smaller populations will substitute a more diverse set of beneficial mutations (deVisser & Rozen 2005), with the consequence that they may follow more heterogeneous adaptive trajectories than large populations (Jain & Krug 2007), particularly if mutations interact epistatically. While the effects of genetic drift on the fixation of deleterious mutations are well appreciated and studied, for example in Phase 1 of Wright's Shifting Balance Theory (Wright 1932), the effects of stochasticity on the fixation of beneficial mutations have not been considered in any experimental context. The aim of the present contribution is to examine conditions where heterogeneity in the fixation of beneficial mutations enables the constraints associated with a limited population size to be overcome.

Using the fitness landscape metaphor of Wright (1932), we consider the evolution of populations on two distinct fitness landscapes, one that is "smooth" with a single fitness peak, and another that is "rugged" with several peaks. We imagine that populations begin their evolutionary trajectories from fitness valleys; they will have already drifted, or been otherwise displaced, from a local fitness peak in Phase 1 of the Shifting Balance process (a process that is thought to be more efficient in small populations (Goodnight 2006), but see Weinreich *et al*. (2005)) and are awaiting the appearance and fixation of new beneficial mutations that will bring them into the domain of attraction of other, perhaps higher, peaks (Wright 1932). The

adaptive route taken by any given population is likely to be a function of the underlying topography of the fitness landscape (Colegrave & Buckling 2005; Burch & Chao 2000; Korona *et al.* 1994). On smooth adaptive landscapes with only a single peak, locating the fittest solution, or global optimum, is a matter of successively substituting the largest available beneficial mutations. It is assumed that this process will be slower in small populations as a result of their diminished access to (large) beneficial mutations (Silander *et al.* 2007; Burch & Chao 1999; Miralles *et al.* 2000; Orr 1998; Rozen *et al.* 2002). In contrast, reaching the global optimum on rugged landscapes is expected to be a function of the specific mutations that become substituted, because fitness on complex landscapes is determined by the epistatic interactions among combinations of mutations (Colegrave & Buckling 2005; Weinreich *et al.* 2006; Whitlock *et al.* 1995). Therefore, on rugged fitness landscapes small populations, owing to increased variability in the fitness effects of beneficial mutations that become substituted (deVisser & Rozen 2005), may locate a more diverse set of adaptive peaks, and on occasion ascend higher adaptive peaks than large populations. In contrast, by deterministically substituting only the largest beneficial mutations (DeVisser & Rozen 2005; Hegreness *et al.* 2006), large populations will be limited to fewer adaptive routes that climb the nearest fitness peak with the steepest slope, but not necessarily the highest peak. We note that this general prediction is consistent with Wright's conjecture that small populations (subdivided demes) offer the best opportunity to allow the Shifting Balance process to proceed (Wright 1932; Whitlock *et al.* 1995). However, the solution we outline suggests that small populations are not only more likely to drift away from local fitness peaks in Phase 1, but also that they are more efficient seekers of distant, and occasionally higher, fitness peaks under the influence of natural selection in Phase 2 of the Shifting Balance process due to their broader sampling from the distribution of beneficial mutations.

Here we first test these suppositions experimentally using evolving bacterial populations, and then explore the generality and limitations of our results using simulations. Twenty-four small and six large populations initiated with a single clone of *E. coli* were allowed to evolve for 500 generations in either a simple or a complex nutrient environment. The simple environment is a glucose minimal medium (DM) that has been shown to lead to considerable

adaptive parallelism (Cooper *et al*. 2003; Pelosi *et al*. 2006; Woods *et al*. 2006), consistent with a relatively smooth fitness landscape. The complex environment, Luria-Bertani Broth (LB), contains a variety of carbon sources and other nutrients that offer a broader array of adaptive options, consistent with a more rugged fitness landscape (Habets *et al*. 2006). Population size was manipulated by adjusting the culture volume, causing a ~ 50-fold difference (5×10^5 versus 2.5×10^7). The extent of adaptation of each population was determined by measuring the relative fitness of population samples against a differently marked ancestor in head-to-head competition in the same environment in which they had evolved.

Materials and Methods

Bacteria and media

The *Escherichia coli* B strains used in this experiment, REL606 and REL607, have been used extensively in experimental evolution and are described elsewhere (Lenski *et al.* 1991). These ancestral strains are genetically identical except for a difference in their ability to catabolize L-Arabinose, which can be used as a marker to distinguish both strains when plated on tetrazolium-aribinose (TA) indicator plates.

Two nutrient environments were used for the serial transfer experiment: a simple medium -with glucose as sole carbon source- and a complex medium. The simple medium is Davis' minimal broth supplemented with $2* 10^{-6}$ thiamine hydrochloride and 0.25 g glucose per liter (DM250) and the complex medium is a 1/10 dilution of Luria-Bertani broth (1/10LB). The contrasting environments were chosen following experiments showing that *E. coli* maintained in LB evolved more phenotypic and genetic heterogeneity than populations evolved in DM, consistent with more niches and alternative adaptive peaks in the former environment (Habets *et al.* 2006). The population density at stationary phase for both media types is $5*10^8$ cells/mL.

Evolution experiment

Populations derived from both ancestral strains were maintained for 500 generations by serial transfer in unshaken tubes (4.8 mL) or in wells of a 96-well plate (100μL), representing large and small populations, respectively. Every 24 hours the populations were diluted by a 1,000-fold dilution into fresh medium, and then incubated at 37ºC; for the small populations $5*10^4$ cells were transferred ($N_e = 5*10^5$) using a 96-pin replicator (Boekel Scientific), while for the large populations this was $2.5*10^6$ cells ($N_e = 2,5*10^7$). Each culture underwent roughly 10 generations of daily growth. Every 100 generations, the populations were stored in a 15% glycerol solution at -80 ºC. Large populations were replicated six-fold, small populations were replicated 48-fold, in two separate 96-well plates. For reasons of experimental tractability, following the 500 generations fitness assays were conducted on a randomly sampled subset of 24 from the 48 small populations from each medium type. Fitness trajectories of small populations were estimated for a random set of 12 populations from the original 24, and for all six large populations.

Fitness assays

The relative fitness of evolved populations was measured according to previous protocols (Lenski *et al.* 1991) by competing populations against the reciprocally marked ancestral clone for 10 generations. Conditions in competitions were equivalent to those during serial transfer. Prior to the competition, competitors were separately grown for 24 hours in the appropriate medium, to insure that both were in equal physiological states. At the beginning and the end of the competition, the frequency of both competitors was determined by plating onto TA. From these frequencies, relative fitness was estimated as the ratio of each strain's Malthusian parameter. Competitions for mean fitness of the populations were replicated three-fold; all other competitions were replicated six-fold.

Statistical analyses

To account for the difference in sample size between small and large populations, we tested the robustness of the results of our analyses by using a bootstrap procedure (Manly 1991). This was achieved by resampling with replacement from the original replicate fitness

estimates to generate 10,000 sets of 24 versus six pseudo-populations (of 12 versus 6 pseudo-populations for the analysis of fitness trajectories). For each set, an *F*-test on the among population variation in fitness of the 24 versus the six pseudo-populations was calculated. This distribution of *F*-values was then used to calculate the proportion of test values higher than the *F*-value corresponding to the real data, which reflects the probability that the higher among-population variation in small than large populations arose from random processes (or an asymmetry in sample size) (Orr 2006). A similar bootstrapping approach was employed to carry out *t*-tests comparing the rate of adaptation in small versus large populations in both environment types.

Fitness data were analyzed using *t*-tests with unequal variances; the greater fitness variation of small versus large populations precludes the use of standard ANOVA. Repeated-measures ANOVA were used to examine the adaptive trajectories of small or large populations. In order to avoid the problem of heterogeneous variances, we applied this ANOVA to small and large populations separately. We were particularly interested to see whether small populations showed evidence for significant heterogeneity in their respective adaptive dynamics, which would be apparent as a significant interaction between population and time of the repeated-measures ANOVA on individual adaptive trajectories.

Simulation design

The simulations were designed to approximate critical features of our experiments. The digital bacteria grow by dividing at rates determined by their fitness. The population starts at size $N0$ and growth continues until the population reaches carrying capacity, at which point serial transfer, modelled as multinomial sampling, reduces the population size back to $N0$ which initiates another round of exponential growth. This procedure is iterated until the desired number of generations is reached. Effective population sizes, N_e, calculated as $N0$ * (generations grown between transfers)(Gerrish & Lenski 1998), are equivalent to those used during the bacterial experiments ($5e^5$ or $2.5e^7$ for small and large populations, respectively). Initial populations are clonal, but at division, each clone generates mutants at a rate μ that differ in fitness from the parent clone. By convention, the ancestral clone is assigned a fitness

value of 1 and offspring a value of 1+s, where values of s are drawn from an exponential distribution $f(s) = \alpha e^{-\alpha s}$, with $\alpha = 42.5$ (Silander *et al.* 2007). Because recent simulations have suggested that higher fitness peaks can be reached by first going through an intermediate step with reduced fitness (Cowperthwaite *et al.* 2006; Lenski *et al.* 2003), we also performed simulations that included only a small fraction of mutations that lead to fitness larger than that of the ancestral strain, while the majority of mutations reduced fitness (i.e.: deleterious mutations >> beneficial mutations). However, due to the strong bottlenecks imposed by the repeated serial passages, these less fit mutants never survived long enough to produce consecutive, fitter mutants. Therefore, their inclusion had no effect on the outcome of the simulations. Because including deleterious mutations significantly increased computational demands but did not affect our conclusions, the results presented here are for simulations that only included beneficial mutations. At division, offspring remain unchanged or attain the state of any of L single mutant neighbours. Once a mutation occurs, a new mutant is created with fitness drawn randomly from the L possible neighbourhood values. Additionally, the new mutant obtains its own one-step neighbourhood of L mutants. To generate a smooth landscape, the newly created mutant is assigned a mutant neighbourhood which is identical to that of the ancestral strain, leading to a landscape with only a single global optimum. To approximate a rugged fitness landscape, we consider the other extreme, where 100% of the neighbourhood is replaced, with values for the possible fitness increase resampled from $f(s)$. This leads to a completely rugged landscape, where a single mutation changes the fitness effects of all other possible mutations. The mutation rate μ was set to 5e^{-6} (Perfeito et al. 2007) and the total number of 1-mutant neighbours, L, to 500. While these two parameters are chosen rather arbitrarily, we found that the results remain qualitatively unchanged for different parameter values, as long as the effective mutation supply rate for small populations is significantly smaller than the mutant neighbourhood, i.e. Ne* μ <<L, and Ne* $\mu \approx$ L for large populations. Such a situation allows the small populations to evolve stochastically, while the large populations will evolve in an essentially deterministic manner. The model was written in Matlab and will be provided upon request to A.H.

Results and Discussion

Fitness estimates of populations taken after 500 generations support the prediction that adaptation is more heterogeneous in small populations (Fig 1); significant among population variation for fitness was found for small populations in both environments (simple: $F_{21,44}$ = 4.21, $P < 0.001$; complex: $F_{22,45} = 3.58$, $P < 0.001$), while large populations show no apparent heterogeneity for fitness in either one (simple: $F_{5,12} = 0.80$, $P = 0.57$; complex: $F_{5,12} = 0.85$, $P = 0.54$). Furthermore, we found that the among-population fitness variation was higher for small than large populations in the complex nutrient environment ($F_{22,5} = 5.20$, $P = 0.038$), but did not differ in the simple environment ($F_{21,5} = 2.10$, $P = 0.21$). To account for the asymmetry in sample size between small and large populations, we tested the robustness of this *F*-test by using a bootstrap procedure (Manly 1991) and found results that were consistent with the original test (complex environment: $P = 0.043$; simple environment $P = 0.45$). As expected, given that large populations have increased access to beneficial mutations conferring large benefits, we found that large populations adapted faster than small ones in the simple environment ($t_{11,5} = -3.36$, 2-tailed $P = 0.0060$). However, the reverse was found in the complex environment ($t_{11,5} = -3.36$, 2-tailed $P = 0.0060$). As earlier, this result was confirmed using a bootstrap approach ($P = 0.0039$ and $P = 0.0073$, respectively). These data reveal that although adaptive heterogeneity is increased in small populations, the evolutionary consequences of this variation are highly dependent upon the topography of the adaptive landscape, because only on the rugged fitness landscape are benefits to this heterogeneity realized.

To explore the interactions between population size, environment, and fitness gain in more detail, the adaptive trajectories of a subset of small and large populations during evolution in the complex resource environment were obtained (Fig. 2). Whereas large populations showed parallel fitness gains ($F_{10,36} = 1.90$, $P = 0.077$), small populations explored the rugged adaptive landscape in different ways ($F_{22,46} = 4.06$, $P < 0.001$) indicating that they have followed divergent adaptive trajectories. The effect of this heterogeneity is particularly evident for three small populations with final fitness significantly higher than even the most
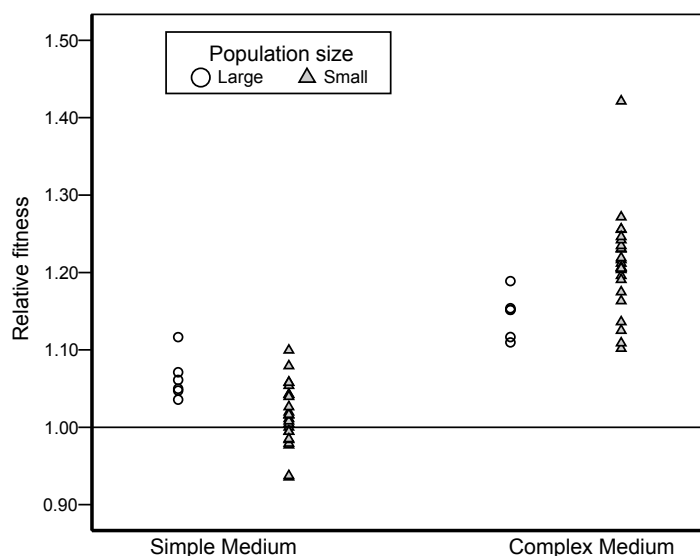
Figure 1: Relative fitness of large and small bacterial populations after evolution on either a simple or complex nutrient environment. A value of 1 indicates no change.

fit large population (t_2=3.90, 1-tailed, P = 0.03) (dotted lines Fig 2a). The fitness trajectories of these three populations were significantly different from those of the other nine small populations ($F_{1,33}$ = 11.83, P = 0.0016). Moreover, while they were more fit after 500 generations (t_{10} = 5.13, 2-tailed P < 0.001), at 100 generations their average fitness improvement was significantly lower than that of the other nine small populations (t_{10} = 2.79, 2-tailed P = 0.019).

Our experimental data demonstrate that the dynamics of fitness gain in complex environments depend on the topographical details of the fitness landscape. We show that this dependence is a function of the adaptive routes followed by evolving populations, and that this varies significantly between small and large populations. Finally, we show that adaptive walks that ascend "steep hills" do not always climb the highest peaks. Indeed, only those small populations that initially substituted smaller beneficial mutations obtained the largest fitness gains.
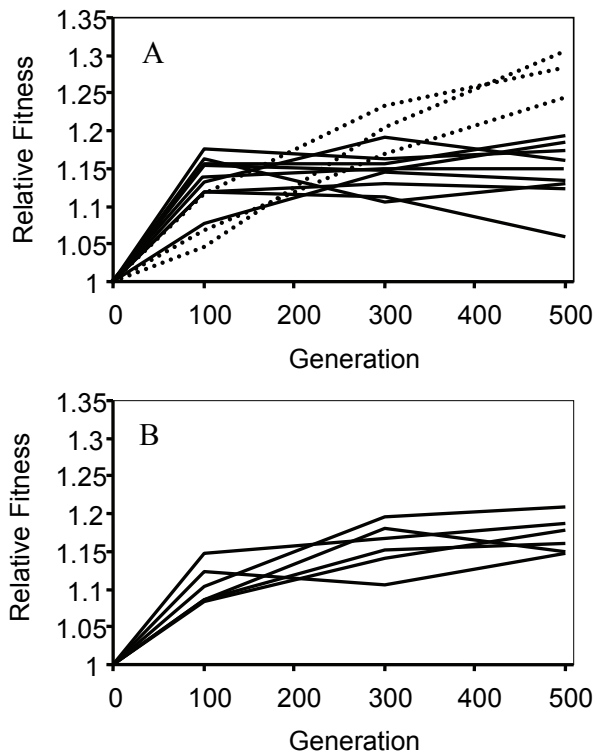
Figure 2: Fitness trajectories of 12 small (A) and six large (B) populations evolving in the complex environment. Dotted lines highlight small populations that have attained higher fitness than other small and even the most fit large populations (see text for details).

An assumption of our experimental model is that simple and complex resource environments correspond to adaptive landscapes that are "smooth" and additive, and "rugged" and epistatic, respectively. Although our data are consistent with this interpretation and there is precedent for this approach (Colegrave & Buckling 2005), it is not feasible to experimentally determine *a priori* the epistatic contingency of evolution in any experimental environment. In order to overcome this limitation, we performed computer simulations of populations evolving on adaptive landscapes where the levels of mutational epistasis could be explicitly defined. The simulations also enabled us to consider the evolutionary response of small and large populations over a vastly extended time scale that would not have been experimentally feasible.

In our computer simulations, digital bacteria undergo iterated cycles of exponential growth and serial dilution. Every clone grows according to a fitness value that is initially scaled to 1. Additionally, every clone has a fitness neighbourhood of fixed size *L*, which corresponds to the number of possible 1-step mutations the clone can reach. During growth, mutant offspring arise at a rate, μ, and thereby obtain a new fitness value that corresponds to one of the *L* neighbouring fitness values. Each mutant clone can either retain a fraction of the fitness neighbourhood of its parent, or obtain an entirely new fitness neighbourhood. If the parental fitness neighbourhood is retained, the result is a smooth fitness landscape with few maxima among the *L* fitness values and no epistasis. At the other extreme, if all fitness neighbours are replaced, the result is a maximally rugged fitness landscape with complete epistasis and many local optima. In both cases, the landscapes we utilize are likely to be exaggerated versions of what might be found in nature. Our use follows earlier pioneering fitness landscape simulations (Orr 2006; Perelson & Macken 1995; Macken & Perelson 1989; Kauffman & Levin 1987), and is intended to establish the simplest boundary conditions and not to faithfully reproduce the experiment.

Broadly, the simulations provide strong qualitative support for our interpretation of the experimental results. Figures 3a and 3b show the fitness trajectories for fifty individual small or large simulated populations evolving on either a smooth (Fig. 3a) or rugged (Fig. 3b) fitness landscape. In a manner consistent with our experimental results, a number of small populations on the rugged landscape, but not on the smooth landscape, obtain higher long-term fitness than even the most fit large populations. That this result is only found on the rugged landscape supports the idea that the dynamics of fitness gain are highly dependent on the topography of the underlying fitness landscape, with epistatic interactions among mutations providing a critical advantage to small populations. We next calculated variation over time in fitness among small and large populations as a function of landscape topography (Fig. 3c), from which we draw two conclusions. First, this analysis shows that among population heterogeneity is higher for small than large populations irrespective of landscape complexity. Secondly, it reveals that variance in evolutionary response is increased for both small and large populations during adaptation on rugged adaptive landscapes relative to their

behaviour on the smooth landscape. This latter effect is likely the result of the fact that rugged landscapes contain more fitness peaks, while the former is a consequence of the fact that small populations follow more heterogeneous adaptive trajectories. Most interestingly, these simulation results show that the benefits that accrue to small populations by following diverse adaptive trajectories are only realized when fitness is determined by epistatic interactions among beneficial mutations. Otherwise, small populations remain adaptively constrained.
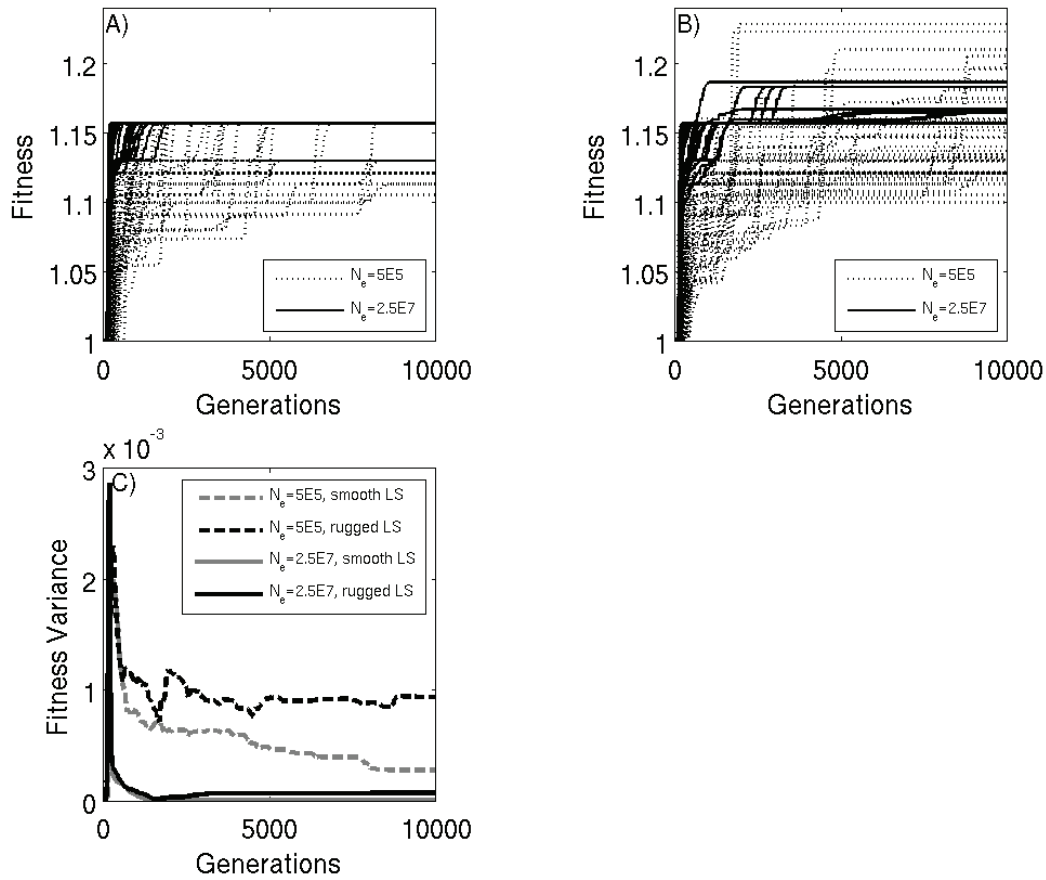

Figure 3: Simulation results of fitness gain in 50 small (dotted line) and large (unbroken line) populations on either a smooth (a) or complex (b) fitness landscape. The number of 1-step neighbours, *L,* is 500 and the mutation rate, μ, is 5e-6. Variation in fitness across treatments and population size is shown in Fig 3c.

In summary, our data provide experimental and theoretical evidence that limits to adaptation in small populations can be overcome during evolution on complex fitness landscapes. Furthermore, we show that the topography of the fitness landscape is an important

determinant of this outcome, because those small populations with the greatest final fitness improvement were ones that initially ascended relatively shallow slopes. It is important to note that benefits from more effective landscape searching are far from assured in small populations. Indeed, many small populations, both in the experiment and in the simulations, faced handicaps consistent with their diminished access to beneficial mutations of large effect. However, whereas the outcome of adaptation in large populations is nearly deterministic, adaptation in small populations can generate unpredictable results and unexpected benefits.

Although our experiments were not designed to specifically test Wright's Shifting Balance Theory (Wright 1932), it has not escaped our notice that our results are of particular relevance to Phases 2 and 3 of the theory. In Phase 2, populations previously displaced from their original adaptive peaks via genetic drift in Phase 1, are envisioned to ascend new peaks via the accumulation of beneficial mutations. Genetic drift in small populations, in Phase 1, and epistatic interactions among mutations, in Phase 2, are thought to facilitate this process. The results here are consistent with this view in two ways. First, we find that small populations are better able to locate a diverse range of fitness peaks than large populations, and second that advantages to this diversity are only realized on landscapes where epistatic interactions are expected to be common. In Phase 3, migration from the fittest populations causes demes resident on lower fitness peaks to cross fitness valleys in order to shift towards higher fitness peaks. Because our experiments did not include a migration treatment we lack experimental support for this final phase; however, it seems likely that appropriate rates of migration among small populations would have the effect of causing more efficient peak shifts among small than large populations. Tests of this conjecture are currently in progress.

Two further implications emerge from our data. First, although our experimental results partly depend on the specifics of the environments applied in our study, it is likely that our complex nutrient treatment actually underestimates the complexity of most environments, because it lacks both spatial structure (Korona 1996; Rainey & Travisano 1998) and interactions with other organisms such as predators and parasites (Brockhurst *et al.* 2005; Brockhurst *et al.* 2004; Meyer & Kassen 2007). If natural fitness landscapes are actually more rugged than

those used here the potential evolutionary advantage to small, marginal, or fragmented populations may be further enhanced, and this may somewhat mitigate risks to threatened populations of animals and plants. Second, we note that our experimental population densities are consistent with the population bottlenecks experienced by many microbial pathogens during initial infection (Rubin 1987). Such bottlenecks are thought to be costly to microbial populations due to the accumulation of deleterious mutations by Muller's ratchet, which may in turn serve to diminish microbial virulence (Bergstrom *et al.* 1999). However, the emergence of heterogeneous populations following infection in small inocula may serve as an important diversifying factor for microbes, perhaps providing microbes with an adaptive edge in the co-evolutionary "tug-of-war" between pathogens and their hosts. These implications await further testing.

3   The effect of population structure on the adaptive radiation of microbial populations evolving in spatially structured environments

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Abstract

Spatial structure is thought to be an important factor influencing the emergence and maintenance of genetic diversity. Previous studies have demonstrated that environmental heterogeneity, provided by spatial structure, leads to adaptive radiation of populations. In the present study, we investigate not only the impact of environmental heterogeneity on adaptive radiation, but also of population fragmentation and niche construction. Replicate populations founded by a single genotype of Escherichia coli were allowed to evolve for 900 generations by serial transfer in either a homogeneous environment, or a spatially- structured environment that was either kept intact or destroyed with each daily transfer. Only populations evolving in the structured environment with intact population structure diversified: clones are significantly divergent in sugar catabolism, and show frequency-dependent fitness interactions indicative of stable coexistence. These findings demonstrate an important role for population fragmentation, a consequence of population structure in spatially structured environments, on the diversification of populations.

Introduction

A central focus in evolution and ecology is the study of the relationship between adaptive radiation and ecological opportunity (Futuyma and Moreno 1988; Schluter 1996; Rainey *et al*. 2000; Travisano and Rainey 2000). Ecological opportunity can take the form of niche differences or niche partitioning (Amarasekare 2003). In a homogeneous environment, niche partitioning can occur when different genotypes specialize on different available resources (resource partitioning) or when they are limited by the same resources but at different times, e.g. when species grow at different times of the year (temporal niche partitioning). In the absence of these mechanisms, coexistence between diverged phenotypes can still occur if populations evolve in spatially structured environments (spatial niche partitioning; Amarasekare 2003).

Several consequences of spatial structure may enhance opportunities for adaptive radiation. First, spatial structure introduces environmental heterogeneity, e.g. by providing gradients in abiotic or biotic factors. When the fitness of different genotypes depends on spatially varying physical factors and trade offs are associated with specialization on these factors, coexistence is possible. The influence of environmental heterogeneity provided by spatial structure on the evolution of diversity has been studied in a variety of experimental systems using microbes (Korona 1994; Korona 1996; Rainey and Travisano 1998).

Second, in a spatially structured environment, the population is effectively subdivided into a number of more or less independently evolving subpopulations. Since these subpopulations are relatively small they are more likely to adaptively diverge, mediated by two mechanisms. First, genetic drift plays a larger role in small subpopulations (Wright, 1931). Second, small populations tend to fix beneficial mutations conferring smaller benefits, because they do not have access to the rare beneficial mutations of large effect that large populations have access to (Orr 1998; Burch & Chao 1999; Miralles *et al*. 1999; de Visser & Rozen 2005). Since smaller-effect beneficial mutations are more abundant than those causing substantial fitness increases (Rozen *et al.* 2002, Orr 2005), the small subpopulations of a spatially subdivided population are likely to follow diverse adaptive routes, an effect that may be amplified by epistatic interactions between beneficial mutations (D.E. Rozen, M.G.J.L. Habets, A. Handel and J.A.G.M. de Visser, unpublished results). Another consequence of

fixing smaller mutations is a decrease in the rate of adaptation (Burch & Chao 1999; de Visser *et al*. 1999; Miralles et al. 1999). Because the small, isolated subpopulations individually adapt more slowly, the population as a whole will consequently adapt more slowly than a well mixed population.

Third, the effect of population fragmentation may allow for additional diversity if the diverging subpopulations provide novel niches by modifying environmental conditions. They may do so in two ways: they can change (or provide a gradient in) abiotic or biotic factors, increasing heterogeneity of the environment (e.g. cyanobacteria can change the structure of the soil thereby providing increased access of desert plants to water; Jones 2005), or they may provide new resources (e.g. metabolic byproducts on which new specialists may feed). This modification of environmental conditions by organisms already inhabiting the environment has been called 'niche construction' (Laland *et al*. 1999) or 'ecosystem engineering' (Jones *et al*. 1994). Niche construction can occur in unstructured environments, but the likelihood may be increased in structured environments due to limited dispersal and therefore increased abundance of relatively independent subpopulations. Simply due to a larger number of (sub) populations, there are a larger number of potential niche constructors. In addition, gradients in (a)biotic factors cannot be generated in an unstructured (well-mixed) environment.

While the role of spatial structure in the emergence of genetic diversity has been well considered from a theoretical standpoint (Tilman & Kareiva 1997; Chesson 2000; Amarasekare 2003), experimental evidence is scarce (Korona *et al*. 1994; Rainey and Travisano 1998). The present study seeks to explore systematically how spatial structure affects both the emergence and the maintenance of diversity with the aim of disentangling the various consequences of spatial structure that are responsible for adaptive radiation. To this end, we propagated replicate populations of Escherichia coli, founded by a single genotype, for 900 generations in three environments that contained identical nutrients but had different structures: one unstructured environment (shaken liquid culture), and two structured environments (the agar surface of a Petri plate): one where the population structure was kept intact (no dispersal) and one where it was destroyed each day (maximum dispersal). This allowed us to distinguish the effects of environmental heterogeneity by itself, and heterogeneity in combination with maintained population structure (fragmentation and

possibly niche construction) on the diversification of the populations.

Overall, we find support for the hypothesis that adaptive radiation is facilitated in populations with intact population structure, in contrast to populations evolving in spatially structured environments but lacking population structure; significant diversity in catabolic change among clones was only found in populations evolved in spatially structured environments with intact population structure. Moreover, we find that this diversity is maintained by frequency dependent fitness interactions, resulting from trade-offs between adaptations either to newly constructed niches or to existing niches that are filled more efficiently by spatially-structured, fragmented populations.

Materials and methods

Strains, media and experimental design

The ancestral strains (Escherichia coli B, REL606 and REL607) used in this study have been used extensively in experimental evolution and have been previously described by R.E. Lenski and coworkers (1991). The two ancestral strains only differ in their ability to catabolize L-arabinose, a difference that can be distinguished on Tetrazolium Arabinose indicator agar (TA). On this medium Ara$^-$ cells grow as red colonies, while Ara$^+$ cells produce white colonies. TA plates contain per liter: 10 g tryptone, 1 g yeast extract, 5 g NaCl, 16 g agar, 10 g arabinose, and 1 ml of a 5% stock of tetrazolium (2,3,4-triphenyltetrazolium chloride).

To obtain a general view of the role of spatial structure on diversity, two nutrient environments were used for the evolution experiment: a minimal medium–with glucose as the sole carbon source- (simple environment) and a rich medium with multiple carbon sources (complex environment). For the simple environment we used Davis' minimal broth (Lenski *et al*. 1991) supplemented with $2* 10^{-6}$ thiamine hydrochloride and 0,25 g glucose per liter (DM250) and for the complex medium 1/10 Luria Broth (1/10 LB). The agar used for the structured environments (Agar bacteriological No.1, OXOID) had a mineral content that was insufficient to support replication on the agar alone. Final densities at stationary phase were equal for tubes and plates ($F_{1,9} = 2.330$, $P = 0.161$), while the complex environment had an

approximately 1.7 higher density than the simple nutrient environment ($F_{1,9}$ = 8.221, P = 0.019). The latter result is not likely to influence the effect of spatial structure and population structure on diversity, as our conclusions rely on comparisons within the same nutrient environment.

Populations derived from either ancestral genotype were propagated for 900 generations in three environments that differed in spatial structure: an unstructured environment (liquid cultures in shaken tubes), a structured environment (agar plate) with a population structure that was kept intact, and an intermediate treatment with a structured environment (agar plate) but a population that was mixed before each transfer. We shall refer to those treatments as tubes, structured plates and mixed plates, respectively. We transferred the mixed plates by scraping the cells off the plate into 10ml of saline and plating a sample of this on a new plate. A velvet cloth was used as a stamp for transferring the structured plates to keep the population structure intact. The number of cells transferred in all treatments was equal.

In the evolution experiment and the fitness assays we used small plates (diameter 60mm) and 20ml tubes. Tubes were shaken at 225 rpm. Culture volume for all treatments is 10 ml (either liquid or agar). Every day, 50μl (containing ~2,5* 10^7 cells) of a stationary phase culture was transferred to fresh medium and incubated for 24 hours at 37°C. The populations grew approximately 200-fold each day until they reached a density of about 5*10^8 cells per ml, which represents ~8 generations. Every hundred generations, a sample from all populations and three isolated clones from each population were stored in 15% glycerol at -80°C.

Each treatment was replicated six-fold. Due to contamination in some plate populations, only two mixed plate populations of each medium type and three structured plates of the simple nutrient environment were analyzed. To balance the analysis, we investigated only four populations of all other treatments.

Fitness assays

Relative fitness of the evolved populations was measured by competing three randomly isolated clones from each population against the ancestral clone. Competitions took place in the same environment and medium in which the clones had evolved. Both competitors were grown for 24 hours prior to competition in that particular environment to make sure that they were in the same physiological state. At the beginning and the end of the one- day competition, the frequency of the competitors was estimated by plating dilutions on a TA plate. To distinguish both competitors, competitions were always conducted between reciprocally marked strains. For each competitor, their Malthusian parameter was computed ($m = \ln[N_i(1)/ N_i(0)/1d]$ where $N_i(0)$ is the density at the start of the competition and $N_i(1)$ the density after one day of competition). Fitness relative to the ancestor was computed as the ratio of the Malthusian parameters (Lenski *et al*. 1991). Competitions were done with four-fold replication. Fitness of clones with a coefficient of variation higher than 10% were replicated an additional four times. All assays that had fewer than 20 colonies of one of the competitors on either day were excluded from analysis, because low colony numbers can lead to inaccurate fitness measures.

To investigate the specificity of adaptation, the three clones from each population at generation 900 were also competed collectively against the ancestor, with 3-fold replication, in their own and the alternative environment (tubes and plates).

Phenotypic MicroArray plates

Commercially available Phenotypic MicroArray plates (BIOLOG[TM], Hayward CA, USA) were used to measure within-population genetic diversity. These 96-well plates are designed to quantitatively measure numerous phenotypes of the cell at once. The wells of the PM1 plates each contain a different carbon source; respiration of each carbon source is quantified by reading the OD_{595} of a reduced tetrazolium dye.

From two random populations of each treatment, evolved for 900 generations in the complex medium, eight clones were isolated and examined in duplicate; the ancestor was assayed six fold. This amounts to 102 PM1 plates total. Protocols were according to BIOLOG. Briefly, clones were preconditioned for 24 hours in 1/10 LB tubes and plates

respectively after which each well of a PM1 plate was inoculated with 100 µl of a cell suspension (85% turbidity) of one clone. The PM1 plates were incubated for 24 hours at 37ºC after which the OD was measured with a 96-well plate reader. For analysis, we first divided performance on each of the 95 carbon sources (one well is the blank) by eye into three groups: no growth, partial growth and full growth. Carbon sources that either showed no growth or full growth for all clones and for the ancestor, were excluded from analysis. Next, the OD of the blank well was subtracted from the ODs of the remaining 48 carbon sources. Afterwards, the catabolic divergence of each evolved clone from its ancestor was measured by its deviation from a perfect correlation between mean performance on these 48 carbon sources of evolved clone and ancestor (see Cooper *et al*. 2003).

Frequency dependence

Negative frequency dependence for fitness of the evolved clones was measured by competing clones against the rest of the population from which they were sampled. For this purpose, spontaneous Ara$^+$ colonies were isolated from the clones of all Ara$^-$ populations of both simple and complex medium, in order to distinguish the clone during competition from the rest of its population. Since it is not feasible to isolate Ara$^-$ colonies from Ara$^+$ populations, only Ara$^-$ populations were investigated for frequency dependence. By having the clones compete at two different frequencies, 1:1 and 1:10, with four-fold replication, we could calculate the advantage when rare (AWR) as the difference between mean fitness when initiated at low frequency versus mean fitness when started at high frequency (Elena and Lenski 1997). If the AWR is significantly higher than 0, and both competitors, when rare, have a higher fitness than the common competitor, there is mutual invasibility, indicating a negative frequency dependent interaction.

Results

Relative fitness differs between treatments and media

Population mean fitness was measured by competing three clones from each population against the ancestor at two different time points (300 and 900 generations); overall, all treatments exhibited significant fitness gains (Fig.1; $P < 0.05$). Populations evolved in the complex nutrient environment gain higher fitness than populations evolved in the simple nutrient environment (repeated measures ANOVA: $F_{1,13} = 318.414$, $P < 0.001$), which can be attributed to several factors, including a dissimilarity in pre-adaptation of the ancestor to the environments, a slightly higher effective population size (see Methods), or a difference in the number of available niches. An increase in the number of niches in the complex nutrient environment could lead to an increase in the number of potential beneficial mutations and may include additional larger-effect beneficial mutations. In the homogeneous tube environment we find support for the assumption that the complex nutrient environment contains more niches, because the among- population variance in fitness is significant only in the complex nutrient environment ($F_{3,7} = 8.339$, $P = 0.01$), and not in the simple nutrient environment ($F_{3,8} = 0.618$, $P = 0.623$).

Irrespective of nutrient environment, rates of adaptation were significantly different between the three treatments with different levels of spatial structure ($F_{2,13} = 5.242$, $P = 0.021$). Mixed plates show significantly higher fitness than structured plates in both the simple ($F_{1,3} = 15.548$, $P = 0.029$) and the complex nutrient environment ($F_{1,4} = 9.641$, $P = 0.036$). This is consistent with expectations, because population fragmentation on the structured plate is expected to decrease the rate of adaptation. Additional experiments have been done to explain these differences in more detail and will be reported elsewhere.

Overall, we find significantly higher fitness of populations evolving on plates compared to tube populations ($F_{1,15} = 5.253$, $P = 0.037$), in spite of the fact that resources are identical. To further explore this difference, we examined the specificity of adaptation to these treatments.

Specificity of adaptation

The observed differences in fitness between plates and tubes could result from a difference in number or composition of niches across treatments. The plate environments may either provide additional niches relative to the tube environment (as a consequence of spatial structure) or both environments may provide different niches altogether. The presence of distinct niches in the two environments may be inferred if specialization in these niches involves trade-offs that affect performance in the alternative environment.
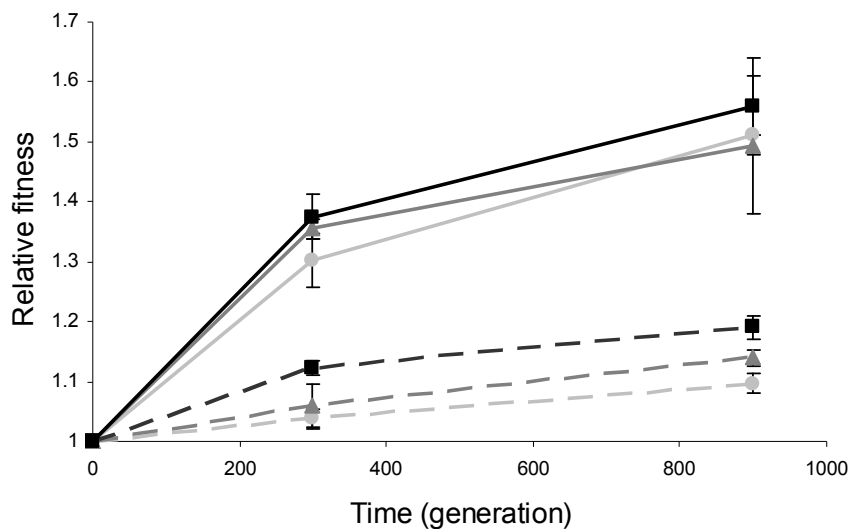

Figure 1. Mean relative fitness (± S.E.M.) of populations evolved in either tubes (circles), structured plates (triangles) or mixed plates (squares). Dashed lines represent populations evolved in the simple nutrient environment, solid lines the ones evolved in the complex nutrient environment. Fitness was measured by competing three clones of each population against the ancestor.

We tested this by competing three evolved clones from populations at generation 900 together against the ancestor in both environments (tubes and plates). Populations evolved in tubes did not show a difference in fitness when competed versus the ancestor on plates (Fig 2; paired t-test for the simple nutrient environment: $t_3 = -0.019$, two-tailed $P = 0.986$; complex nutrient environment: $t_3 = 1.282$, $P = 0.290$). Populations evolved on plates, however, had significantly lower fitness in tubes (simple nutrient environment: $t_4 = 4.313$, $P = 0.013$; complex nutrient environment: $t_5 = 10.721$, $P < 0.001$). One possible reason for the observed asymmetry in the

specificity of adaptation to plate and tube environments is that, in addition to niches shared by the tube and plate environments, plates harbor one or many additional niches that may contribute to the potential for adaptive radiation.
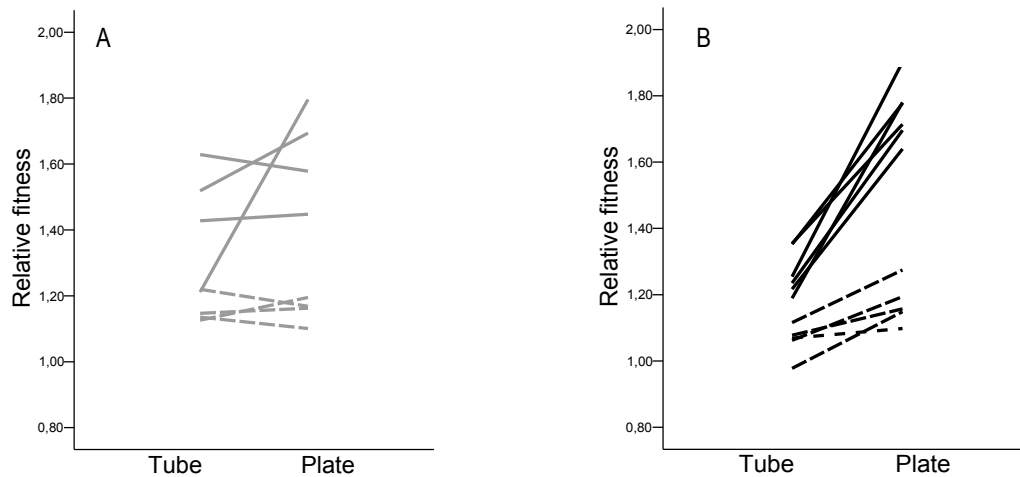

Figure 2. Relative fitness of the evolved populations at generation 900 when competed versus the ancestor in tubes and in plates. A. Tube-evolved populations; B. Plate-evolved populations. Dashed lines show the populations evolved in the simple nutrient environment, solid lines the populations evolved in the complex nutrient environment.

Diversity within populations

Fitness

As a first method to examine the emergence of genetic variation within populations we examined fitness variation among the three clones that were isolated from each population at 900 generations. In this first analysis, we found no significant fitness variation among clones for any population (results not shown). Extra replicates were conducted for those clones that had a coefficient of variation higher than 10%, which may obscure relevant and genuine genetic variation. However, this only minimally increased our ability to detect within-population variance for fitness: only the mixed-plate populations in the simple nutrient environment showed marginally significant variation among clones from the same population ($F_{4,26} = 2.777$, $P = 0.048$). Relative fitness is, however, expected to be a poor indicator of variation within populations, since convergence for this trait is anticipated.
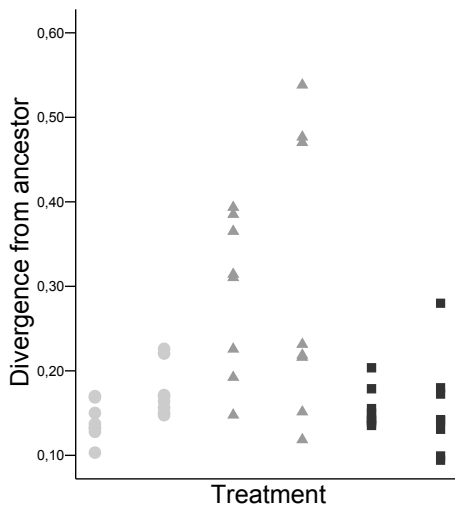
Figure 3. Divergence from the ancestor of eight isolated clones of six populations evolved in the complex nutrient environment, at generation 900. Tube populations are represented by circles, structured plate populations by triangles and mixed plate populations by squares.

Carbon catabolism

As a more sensitive method to detect within- population genetic variation, we assayed changes in the ability of evolved clones to catabolize 48 arbitrary carbon sources. The catabolic phenotypes of eight clones from two populations of each treatment, evolved in the complex nutrient environment, were determined using PM1 BIOLOG plates. These were used to estimate the catabolic divergence of each clone from its ancestor as the deviation from a perfect correlation. We tested the variation in catabolic divergence of clones from their ancestor between and within populations. The tube populations diverged from each other, but we found no significant variation within these populations (Fig. 3; nested ANOVA: between: $F_{1,16} = 10.359$, $P = 0.005$; within: $F_{14,16} = 1.476$, $P = 0.226$). The mixed-plate populations showed neither significant between-population nor within-population divergence (between: $F_{1,16} = 0.002$, $P = 0.963$; within: $F_{14,16} = 1.518$, $P = 0.210$). Only the structured-plate populations showed significant variation between clones from the same population, while no between-population divergence was found (between: $F_{1,16} = 0.645$, $P = 0.434$; within: $F_{14,16} = 24.219$, $P < 0.001$). When all populations were independently considered, significant variation between clones in their catabolic divergence from the ancestor was again only found for both

structured plates (population ara +1: $F_{7,8} = 25.372$, $P < 0.001$; population ara +2: $F_{7,8} = 23.875$, $P < 0.001$). These results support our hypothesis that maintained population structure adds considerably to the diversification of a population.
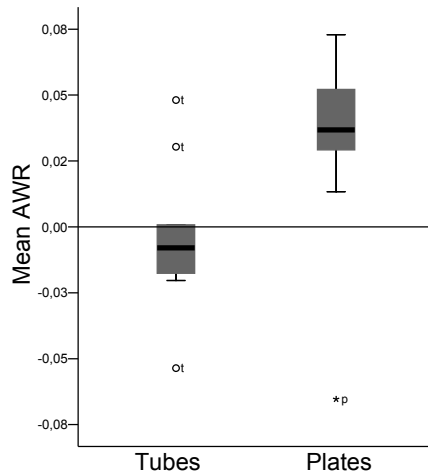

Figure 4. Box plots for the overall Advantage When Rare (AWR) for Ara- populations evolved in tubes or plates. Three clones were competed versus the remainder of the population at two different frequencies (1:1 and 1:10). Negative frequency dependence is evident when AWR > 0, indicating the ability for clones to invade from initial rarity.

Frequency dependence of fitness

We next tested whether the variation found in the populations evolved on plates was transient or sustained. One of the primary mechanisms maintaining genetic variation within populations is negative frequency-dependent selection (Levin 1988; Elena & Lenski 1997). We tested for negative frequency dependent fitness interactions by competing three clones from each ara+ population, of both the simple and complex environment, against the remainder of the population at two different frequencies (1:1; 1:10). One problem with this assay is that any advantage when rare (AWR) found in the structured plates is expected to be weak, because clones are competed against the whole population instead of against the particular subpopulation with which they evolved the putative frequency-dependent interaction. Before storing population samples in the freezer, the structure is necessarily destroyed, thus finding signs of frequency-dependent fitness interactions is conservative evidence for their existence. Nevertheless, when we conduct an overall ANOVA to test the difference in mean AWR of

clones evolved on plates and in tubes, we found a highly significant difference (Fig. 4; $F_{1,18}$ = 8.560, P = 0.009): clones from plates exhibit significant AWR (t_{11} = 3.256, one-tailed P = 0.004), whereas clones evolved in tubes do not (t_9 = -0.487, P = 0.319). These data show that diversity in the populations evolved in spatially structured environments was at least partly sustained by negative frequency-dependent fitness interactions between clones from the same population.

To further examine whether the populations evolved in a structured habitat with a structured population show higher AWR than those without population structure, we conducted separate t-tests for the four plate populations for which the analysis was possible (see methods). Only clones from the structured-plate populations exhibit significant negative frequency- dependent fitness (Table 1). Despite limitations in the number of populations we examined, it is encouraging that both the BIOLOG data and the frequency-dependence data reveal a significant impact of population structure on diversity.

Table 1: Estimates of the Advantage When Rare (AWR) for the 4 plate populations. Three clones were competed versus the remainder of the population at two different frequencies. P-values are based on a one-tailed t-tests of the hypothesis that AWR > 0.

Population	Average AWR	SD	T	d.f.	P-value
Structured plate Simple: ara-1	.0418	.0148	4.89	2	.020
Structured plate Simple: ara-2	.0471	.0130	6.25	2	.012
Structured plate Complex: ara-2	.0246	.0098	4.35	2	.024
Mixed plate Complex: ara-1	.0180	.0734	.42	2	.356

Discussion

This study attempted to assess the impact of several consequences of spatial structure on the adaptive radiation of asexual populations of bacteria. The results demonstrate a positive effect of population structure on the diversification of the population. Not only did we find more diversity in structured populations (Fig. 3), we also found negative frequency-dependent interactions indicative of stable coexistence of this diversity (Table 1). Structuring of a population leads to population fragmentation and this can increase the opportunities for new genotypes to arise and persist, in part because organisms have a local impact on their environment.

A fragmented population consists of many smaller, relatively independently evolving subpopulations. Because smaller populations tend to fix beneficial mutations with higher variance, but with smaller impact on fitness (Orr 2005), and because genetic drift plays a larger role in small populations, fragmentation is expected to facilitate adaptive radiation. However, fragmentation will not necessarily lead to stable coexistence, because stable coexistence is defined by mutual invasiveness: individual genotypes must be able to increase from rarity in the presence of the remainder of the community. Fragmentation, by itself, does not provide a mechanism by which this can occur.

In addition to increasing diversity, the fixation of smaller-benefit mutations decreases the rate of adaptation of the subpopulations (Burch & Chao 1999; de Visser *et al*. 1999; Miralles *et al*. 1999) and therefore of the population as a whole. Consistent with this expected effect of population fragmentation, we found slower adaptation in the structured-plate populations relative to the mixed-plate populations in both nutrient environments (Fig. 1).

The second consequence of population structure is the localization of the effects organisms have on their environment. Organisms not only depend on environmental conditions, but are also able to transform these conditions through their own physical or biochemical actions. Some organisms can modify their environment in such a way that they provide ecological opportunities for new genotypes or for other species, adding new niches to the environment (Jones *et al*. 1994; Laland *et al*. 1999). For example, the secretion of secondary metabolites can add a new niche to the environment allowing cross-feeding interactions to evolve; cross-feeding has been shown to lead to the emergence of stable

polymorphisms in bacterial populations (Helling *et al.*1987; Rozen and Lenski 2000). If niche construction occurs, genotypes become interdependent, because it is the genotypes themselves and not the environment that provide the conditions for coexistence. Fragmentation alone cannot provide a mechanism for stable coexistence, thus our data that show maintained diversity for populations evolving with a fixed population structure (Table 1) are consistent with the hypothesis that niche construction facilitates adaptive radiation.

However, the fact that we found significant diversity on the structured plate and not on the mixed plate may also be explained by an alternative mechanism, resulting from an interaction between environmental heterogeneity and fragmentation: large (mixed) populations may not be able to fill all niches available in the plate environment, because, as a consequence of more rapid substitution of beneficial mutations, variation is purged more rapidly by periodic selection. Furthermore, adaptation to a specific niche can constrain the population's ability to diversify in other niches because of antagonistic pleiotropy (Buckling *et al*. 2003). Because small populations, in contrast, have a greater adaptive exploratory ability, fragmentation can facilitate exploration of the various niches present in a heterogeneous environment (D.E. Rozen, M.G.J.L. Habets, A. Handel and J.A.G.M. de Visser, unpublished results). Additional experiments will need to be done to examine the nature of the negative frequency-dependent interactions we found, to distinguish between both hypotheses.

Despite evidence that diversification resulting from competition for resources in complex environments lacking spatial structure can occur (MacLean *et al.* 2005; Barrett *et al*. 2005), our system does seem to require spatial structure for adaptive radiation. The cause of this discrepancy in study outcome is currently unclear and may be the result of system specific differences in species used, resource base, or interactions between these two factors. In our system, environmental heterogeneity by itself doesn't facilitate the emergence of diversity either. Our mixed plate treatment was designed to test the effect of environmental heterogeneity, absent population structure, and as we have seen, neither variation in catabolic divergence, nor frequency dependence of fitness was found. Both *Ralstonia* sp (Korona *et al*. 1994) and *Pseudomonas fluorescence* (Rainey and Travisano 1998) populations diversify into several morph types when propagated in a structured, but not in a homogeneous habitat. The

structured environment used in the *Ralstonia* experiment resembles our structured environment with daily mixing of the population, and hence only environmental heterogeneity, provided by spatial structure, could have an influence on diversification (Korona *et al*. 1994). For the structured environment of the *Pseudomonas* experiment, non-shaken flasks were used, thus providing spatial structure as well as fragmentation of the populations. The diversity found by Rainey and Travisano (1998) might therefore also depend on fragmentation.

Conclusion

Our study is the first to systematically explore the impact of several aspects of spatial structure on the diversification of populations. We found that spatial structure can slow down the rate of adaptation by fragmenting populations into many smaller subpopulations. Further research will be directed towards finding causes and consequences of this decreased rate of adaptation. In addition, fragmentation facilitates the diversification of the population. Because fragmentation by itself cannot provide a mechanism for stable coexistence, we attribute the observed stable coexistence of subpopulations to the construction of new niches by organisms already inhabiting the environment or to the more diverse adaptive trajectories of effectively fragmented populations. Our results show the importance of not only ecological factors, but also of population genetic factors for understanding the role and consequences of spatial structure in adaptive radiation.

Acknowledgements

4 Spatial structure inhibits the rate of invasion of beneficial mutations in asexual populations

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Abstract

Populations in spatially structured environments may be divided into a number of (semi-) isolated subpopulations due to limited offspring dispersal. Limited dispersal and, as a consequence, local competition could slow down the invasion of fitter mutants, allowing short-term coexistence of ancestral genotypes and mutants. We determined the rate of invasion of beneficial mutants of *Escherichia coli,* dispersed to different degrees in a spatially structured environment during 40 generations, experimentally and theoretically. Simulations as well as experimental data show a decrease in the rate of invasion with increasingly constrained dispersal. When a beneficial mutant invades from a single spot, competition with the ancestral genotype takes place *only* along the edges of the growing colony patch. As the colony grows, the fitness of the mutant will decrease due to a decrease in the mutant's fraction that effectively competes with the surrounding ancestor. Despite its inherently higher competitive ability, increased intra-genotype competition prevents the beneficial mutant from rapidly taking over, causing short-term coexistence of superior and inferior genotypes.

Introduction

Adaptation in asexual populations consists of sequential substitutions of beneficial mutations within the same line of descendants via periodic selection (Koch 1974; Levin 1981). Several beneficial mutations that are simultaneously present will compete with each other for fixation (Muller 1932; Gerrish and Lenski 1998). It is expected that the largest beneficial mutation will sweep through the population following exponential dynamics until it is fixed, thereby eliminating pre-existing variation. The substitution rate of beneficial mutations will be lower in a spatially structured environment than in a homogeneous population, where purging is expected to be a relatively rapid process (Gordo and Campos 2006), because of the slower invasion of beneficial mutations due to limited offspring dispersal and localized competition – i.e., due to spatial constraints of population dynamics (Czárán 1998).

In a spatially structured environment, dispersal will often be limited to a certain degree, causing the population to become structured. Without dispersal, a beneficial mutant will invade as a single growing colony. This has a two-fold disadvantage: first, since growth mostly happens at the perimeter of the colony, the radius of a colony increases at a constant rate, which means a quadratic increase in time of the number of cells; although colonies exhibit vertical growth, this is expected to be at least an order of magnitude smaller than radial growth at the centre of a colony (Grimson & Barker, 1993). Comparing this to the – faster – exponential increase of cells in a well-mixed environment, population structure can be said to slow down growth of cells, independent of a competitor, because of limited expansion ability. Second, if nutrient diffusion is limited in a spatially structured environment, cells influence the availability of nutrients only for their immediate neighbors. Despite a difference in competitive ability, the mutant/ancestor ratio will change slowly, because most ancestral cells are not affected by the mutant. The mutant essentially limits its own growth, because most competition takes place among mutant clone mates (Pacala, 1986; Ives 1988; Hanski and Cambefort, 1991), and it can only take advantage of its competitive superiority at the perimeter of the mutant colony. Moreover, as the invasion process progresses, the invading strain is expected to lower its own fitness due to changes in the ratio of inter- to intra-specific competition. Thus, even though the invading strain has a higher competitive ability, limited dispersal and local resource competition will reduce the invasion rate (Crawley, 1990; Burke

& Grime, 1996).

Because of the slower dynamics of exclusion, several mutants can coexist temporarily, possibly opening the way for further increase in genetic diversity, e.g. through recombination and/or the appearance of mutants that can coexist with other mutants. With respect to adaptation to changing environments, higher genetic variation may be of great advantage to the populations (Boles *et al*. 2005). Since most species grow in spatially structured environments and are limited, to different degrees, in their dispersal rate, the implications of our study are not restricted to micro-organisms.

In a previous study, we have demonstrated experimentally that the long-term *rate of adaptation* slows down due to limited dispersal in a structured environment (Habets *et al.* 2006). Here, we investigate the reason for this decrease; we study how the *rate of invasion* and the fitness of a beneficial mutant are affected by dispersal in a structured environment directly, both experimentally and theoretically. This was done by measuring the rate of fixation of two *E. coli* mutants, each with an approximate 50% fitness advantage relative to its ancestor. To manipulate dispersal, we used a non-motile *E. coli* strain, which we dispersed to different degrees at the beginning and during the invasion assay. We show both experimentally and theoretically a conspicuous divergence in fixation dynamics as a function of spatial structure, the proximate reason for which is the decline of the mutant fitness due to spatial constraints on population interactions.

Materials & methods

Bacteria, media and experimental design

The invading strains (representing a beneficial mutant) were obtained in a previous experiment. Thirty-six populations derived from the *E. coli* B strains REL 606 or REL 607, were propagated for 900 generations in either a homogeneous environment or two spatially structured environments; one where the population structure was kept intact by using velvet to replicate the populations and one were the population was mixed before each daily transfer (Habets *et al*. 2006). From each structured environment, we isolated a clone from one

population at generation 900; these were used for the invasion assays against an ancestral clone. Due to a difference in the ability to use L-arabinose, ancestral cells grow as red colonies on indicator plates, whereas the invading strains (which originated from an isogenic strain except for the marker) produce white colonies. Indicator plates (Tetrazolium Arabinose indicator agar) contain per liter: 10 g tryptone, 1 g yeast extract, 5 g NaCl, 16 g agar, 10 g arabinose, and 1 ml of a 5% stock of tetrazolium (2,3,4-triphenyltetrazolium chloride).

The invading strains competed versus the ancestor for five days in an environment identical to the one they had adapted to: Petri plates (diameter 60mm) containing 10 ml of 1/10 Luria-Broth agar. Prior to the assay, each competitor was preconditioned to the same environment they would compete in, to make sure both clones were in the same physiological state. At the beginning of the invasion assay the ancestor ($2.5 *10^7$ cells) was spread on the agar surface, while the invader ($5 *10^4$ cells) was introduced in one of three different ways: in the first treatment (A), cells were introduced in one spot in the middle of the plate (1μl), in the second treatment (B) cells were introduced in 20 different spots (1 μl each), and in the third and fourth treatment (C and M) we uniformly mixed both competitors before spreading them evenly on the plate. The total number of cells of both competitors at the start of the experiment was the same for all treatments: 500:1. Every day, the populations were transferred to new plates containing fresh medium; populations of the first three treatments (A, B, and C) were transferred using velvet; in the fourth treatment (M) cells were scraped off the plate, diluted and mixed in 10 ml of saline before transfer. Fifty μl (containing ~$2.5* 10^7$ cells) of a stationary phase culture was transferred to fresh medium and incubated for 24 hours at 37°C. The populations grew approximately 200 fold each day, until they reached a density of about $5*10^8$ cells per ml, which represents ~8 generations.

At day 1, 2, 3 and 5 of the invasion assay, the relative frequency of the invader was estimated by plating a dilution of the population on indicator plates. For this, cells were scraped of the plates after the population was transferred, diluted in saline and mixed. Because it is not possible to accurately estimate the frequency at the start of the experiment (because cells cannot be scraped off without ending the experiment), experimental values are represented beginning at generation 8 (day 1).

In order to exclude the possibility that the results would be influenced by pre-

adaptation of the evolved clone to the transfer method (velvet versus mixing) invading strains with a fitness advantage of ~ 50% relative to the ancestor were used from each of the structured environments (Habets *et al*. 2006). Both clones were competed in all four treatments; every treatment was replicated 6 fold. No significant differences between clones were found (Table 1).

Rate of diffusion

Fifty µl of a 10-fold diluted stationary phase LB culture of the ancestral clone (~$2.5*10^7$ cells) was spread out on an agar plate (100 * 15 mm) partitioned into 20 ml of nutrient free agar and 5 ml of concentrated LB agar. The total nutrient concentration was the same as used for the invasion experiment. Cells were spread either *on* the nutritious agar, *in close proximity to* the nutritious agar, or *at some distance from* it. After 24 hours, the density of the populations was tested. This was done by scraping the cells off the plate.

Model

We used a simple stochastic cellular automaton (CA) model to imitate the 4 different treatments of the experiment. The CA space is a 1000 x 1000 square grid of sites representing the surface of the agar medium in a Petri dish. Each site is assumed to be capable of harboring a single bacterial cell, so growth is purely two-dimensional. Of the 10^6 sites, 0,5 % were inoculated at time 0 by bacteria at a mutant to ancestor ratio of 1:500, equal to the experimental design. The ancestral strain was dispersed on the plate at random. For treatment A, the invading cells were clumped into a single spot in the middle of the plate; in B the same number of cells was distributed into 20 spots and the spots spaced out evenly on the plate; in C and M the invaders were dispersed over the plate at random. A generation consists of 10^6 independent, random updating steps, so that each site is updated once on average every generation. An updating step starts with the random choice of a focal site and one of its neighboring sites. If the focal site contains a single cell and the neighboring one is empty, then the cell may put a copy of itself into the empty site with a probability equal to its basic fitness f_i, a parameter of the strain the focal cell belongs to. We used $f_{ancestor} = 0.67$ and $f_{mutant} = 1.00$ to maintain a relative fitness of 1.5 for the invader. Every 8th generation, a 0,5 % sample of

the bacteria present on the plate is transferred to a new plate. In treatments A, B and C the bacteria in the sample keep their previous site on the new plate; in treatment M the sample is reshuffled and dispersed on the new plate at random. Using this updating algorithm we have recorded the relative frequency and the relative fitness of the invading strain over 40 generations (5 days, 8 generations per day).

Note that the number of simulated cells is two orders of magnitude smaller than that in bacterial experiments described above, because the fates of enormous numbers of cells are impossible to follow even with the most powerful of recent computers. Therefore, a quantitatively correct simulation of the dynamics on a Petri dish is not feasible; rather the simulation model is intended to provide a qualitative explanation to the experimental data.

Results

Rate of invasion in model

We investigated the rate of invasion of a superior competitor in a spatially structured environment when mixed to different degrees with its well-dispersed ancestor. Simulations show that the superior clone can invade in all four treatments (Figure 1), but the rate at which this happens depends on the degree of mixing, with daily mixing (treatment M) leading to the quickest invasion. A rigid spatial population structure with a single invasion center (treatment A) delays the invasion process considerably. Keeping the rigid spatial structure but increasing the number of invasion centers (in treatments B and C) provides the invader with an additional competitive advantage, so that a relatively short time is sufficient for it to increase towards fixation.

Rate of invasion in experiment

In accordance with the simulations, the experimental results show that the superior competitor invades in all four treatments (Figure 2), and that the rate of invasion depends on the degree of mixing (Table 1), with daily mixing (treatment M) leading to the fastest invasion.

Table 1: Repeated-measures ANOVA testing the effect of four different degrees of dispersal on the rate of invasion of two evolved clones.

Between subjects

Source	Df	MS	F	P
Dispersal (treatment)	3	1.820545	418.034	< 0.001
Invading strains	1	0.001042	0.239	0.628
Dispersal*Invading strains	3	0.007549	1.733	0.179
Error	34	0.004355		

Within subjects

Source	df	MS	F	P
Time	3	2.43519	1165.930	< 0.001
Time*Dispersal	9	0.44386	212.515	< 0.001
Time*Invading strains	3	0.00441	2.112	0.103
Time*Dispersal*Invading strains	9	0.00442	2.115	0.035
Error	102	0.00209		

A potential dissimilarity between simulations and experimental data is a difference in diffusion rates of nutrients. A difference in diffusion rates would affect the rate of invasion, because diffusion of nutrients, together with the rate of uptake of nutrients by cells, determines the local nutrient concentration in a structured environment. If diffusion of nutrients is fast, cells can exhaust nutrients over a larger area, increasing the impact they have on their environment (Huston and DeAngelis, 1994). Whereas no diffusion takes place in the simulations, the dominant competitor in the experiment might rapidly obtain nutrients from a larger area, thereby preventing not only cells in close proximity, but also cells further away from growing. With a high rate of nutrient diffusion, the dynamics of invasion in a spatially structured environment would resemble the dynamics in a well-mixed environment.

We found that the rate of nutrient diffusion was negligible relative to the rate of nutrient uptake, because growth after 24 hours differed significantly between cells that grew either *on* nutrients, *in close proximity* to nutrients or *at some distance from* the nutrients in a

Petri plate that was partly nutrient-rich and partly nutrient-free ($F_{2,6} = 50{,}975$, $P < 0.001$). We can therefore conclude that diffusion was roughly similar for simulation and experimental conditions, consistent with the general similarity in invasion dynamics produced by simulations and experiment.
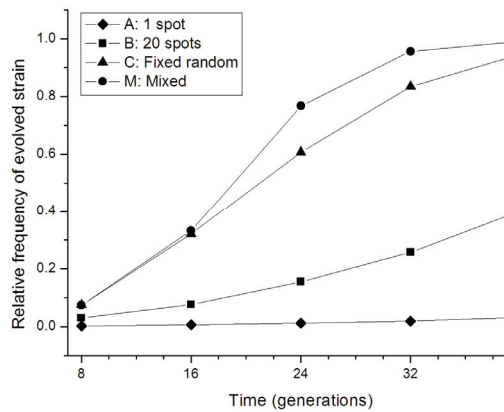
1

2

Figure 1: Simulated relative frequencies of the superior competitor over time. Parameters: Basic fitness: $f_{ancestor} = 0.67$, $f_{mutant} = 1.00$; Initial ancestor to mutant frequency ratio: 500:1; Transfer dilution: 200-fold; Transfer frequency: every 8th generation.
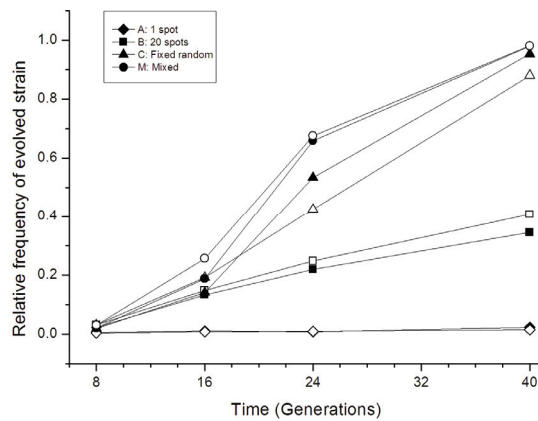
Figure 2: Relative frequency over time (generation) of the superior competitor during the competition experiment. Closed symbols represent the clone evolved on structured plate of our long-term experiment; open symbols represent the clone evolved on the mixed plate. No significant fitness difference is found between the clones. The different treatments represent the different degrees of mixing. The difference in density of the various starting "spots" of the superior competitor is negligible (the density on the plate is everywhere about $8.9 * 10^5$ per cm^2) and does not influence the competition.

Fitness of the superior competitor

We next tested whether the rate of invasion of the superior competitor is dependent on its frequency. The previous results suggested that with an increase in the number of invaders, intra- specific competition increases relative to inter- specific competition, reducing the rate of invasion. We tested this hypothesis by calculating the fitness of the invading strain, relative to its ancestor, at three different time points during invasion in both the simulation as well as the experimental data. The relative fitness is calculated by the ratio of the Malthusian

parameters (m) of the competitors; $m = \ln[N_i(1)/ N_i(0)/1d]$, where $N_i(0)$ is the density at the start of the competition, $N_i(1)$ the density after one day of competition assay, and d is the number of days (Lenski *et al*. 1991).

The simulation data show that the fitness of the superior clone depends on two aspects: the initial distribution of the superior competitor and the amount of mixing during invasion (Figure 3). Fitness is lowest if the invader is clumped in one spot, and highest when mixed at the start of the experiment. If besides mixing at the start, the population is mixed in between transfers (treatment M), an increase in fitness is observed. A conspicuous decrease in fitness of the invader was found in all other treatments (Figure 3). The results of the model confirm the hypothesis that reproduction of the invading cells is constrained by the increasing within-strain aggregation, preventing the bulk of invaders from directly interacting with the inferior competitive ancestral population, and therefore inhibiting their increase in relative frequency. The unexpected increase in fitness in the mixed treatment (M) of the simulations, which we did not see in the experimental data (see Figure 4), remains unexplained.

The experimental data exhibit the same dependence on both initial distribution of the invader and dispersal during invasion. Like the theoretical results, there is a significant difference in treatments (repeated measures ANOVA for differences in treatments: between subjects $F_{3, 35} = 80.697$; $P < 0.001$); the better the superior competitor is distributed over the plate at the start of the experiment, the higher the fitness at all time points (Figure 4).

When the competitors are not mixed daily (treatments A, B and C), there is a significant fitness decrease over time between generations 16 and 40 (Figure 4; A: $F_{2,27} = 4.449$, $P = 0.021$; B: $F_{2,27} = 30.981$, $P < 0.001$; C: $F_{2,27} = 6.923$, $P = 0.004$; M: $F_{2,24} = 0.466$, $P = 0.386$). This fitness decrease is significantly different between the treatments (time-treatment interaction: $F_{3,35} = 4,307$, $P = 0.011$) and appears to be largest in treatment B. The more dispersed the invader is at the beginning of the experiment, the faster the ratio of inter- to intra-specific competition changes, and thus the larger the predicted fitness decrease; we would therefore expect treatment C to have the largest fitness decrease. However, in treatment C the invasion progresses faster and towards the end of the invasion experiment, the population mainly consists of mutants with well-dispersed small islands of ancestral cells.

This leads to a deceleration of the rate of fitness decline. Because we measure over the same time period for all treatments, this deceleration in fitness decrease leads to a slower overall fitness decrease in treatment C than treatment B, even though all treatments demonstrate this fitness decline over time (Figure 4).
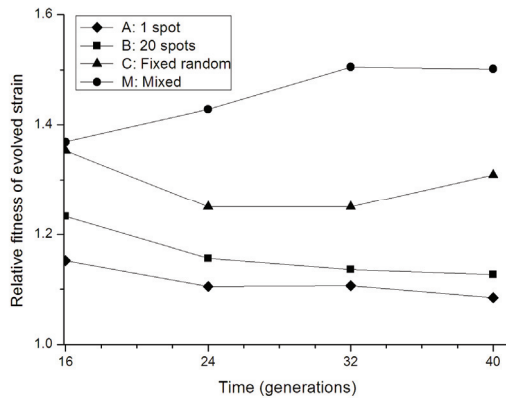

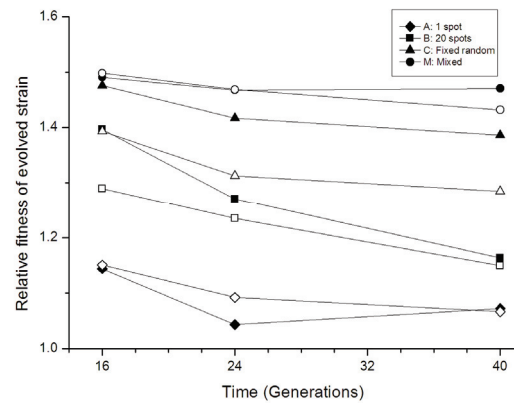**Figure 3**: Relative fitness of the superior competitor over time (generation) in the simulation. Parameters as in Figure 1.

Figure 4: Relative fitness of the superior competitor over time (generations) during the competition experiment. Closed symbols represent the clone evolved on structured plates; open symbols represent the clone evolved on mixed plate.

Discussion

We have assessed the rate of invasion of a beneficial mutant under the spatial constraints of competitive interaction. In a well-mixed environment, the difference in fitness between an ancestral strain and a new beneficial mutant is constant throughout competition, i.e., the mutant can realize its fitness advantage at a maximum level. When population growth occurs in a spatially-constrained fashion with limited dispersal and local interactions, however, the fitness of any genotype will depend on the fitness of neighboring genotypes, since nutrients are limiting in each spot. Moreover, space also counts as a limiting factor for sedentary organisms. Without dispersal, a mutant with higher fitness will not be able to exploit its advantage to the full, because it is mostly in competition with its clone mates. The rate of invasion will consequently be reduced if the dominant competitor is constrained spatially.

This finding helps to understand our previous findings, that populations evolving in spatially structured environments that were not mixed had a lower rate of adaptation than populations that were mixed regularly (Habets, *et al.* 2006). In contrast to these results, previously published theoretical data show that mixing in a spatially structured environment leads to a *decrease* in the rate of fixation of beneficial mutations due to an increase in the role of drift (Perfeito *et al.* 2006). By spatial reshuffling, the mutants are scattered which spoils the protective clumps they would form without mixing, thus increasing the chance the mutant will be lost by drift. Although a direct comparison with our model cannot be made – since we study the rate of *invasion* of a single beneficial mutant and not the rate of *fixation* (the number of beneficial mutants fixed within a certain time frame) – there is a clear difference in the role of demographic stochasticity due to a much larger population size in our study.

Due to slower invasion of adaptive mutants, we expect not only the ecology, but also the evolution of the community to be affected. Since inferior competitors are present in the community for a longer time, they can recombine with other persisting lineages or obtain new mutations, some of which might be beneficial. It is therefore possible that genotypes arise which would not have had the opportunity to emerge in a well-mixed environment. In short, spatial constraints can cause the maintenance of more genetic variation, which might help the population to adapt to changing environments (Boles *et al.* 2005). In addition, stable coexistence may arise between certain genotypes in a population, because a higher standing genetic variation provides more opportunities for such interactions. Thus we arrive at the conjecture that by slowing down the exclusion dynamics between competing genotypes, spatial constraints may increase evolutionary diversification in populations.

In an evolution experiment with *E. coli* in environments with varying degrees of spatial structure, we found support for this view. We observed a lower rate of adaptation *and* higher maintained diversity in bacterial populations evolved in a spatially structured environment with intact population structure relative to populations evolved in mass action or a structured but regularly mixed environment (Habets *et al.* 2006). Spatial structure has also been proposed as a crucial factor for the maintenance of diversity in communities with non-transitive competitive interactions (Czárán *et al.* 2002; Kerr *et al.* 2002). The associated

slower dynamics that we observe here may be an additional factor causing the short-term coexistence of competitors in such communities.

Our findings show that improving resource competitive ability (i.e. growth rate) in spatially structured environments is not as advantageous as in a well mixed environment. This suggests the importance of evolving other strategies, such as increased dispersal or interference competition to reduce local competition. We are currently exploring these proposals.

Acknowledgement

We would like to thank Daniel E. Rozen for his help in the (experimental) design of the project and the many useful discussions and comments.

5   Mutator dynamics in spatially- structured environments

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Abstract

The probability that mutators will invade populations of wild-type individuals by hitchhiking with beneficial mutations depends on the extent of adaptive opportunities. Consequently, mutators are expected to benefit from fluctuating or heterogeneous environments consisting of multiple ecological niches. However, experimental data on the dynamics of mutator populations in heterogeneous environments are scarce. Here, we investigate whether mutator populations of *E. coli* benefit from spatial structure of the environment by considering two features of such environments, i.e. environmental heterogeneity and slower competitive dynamics. Contrary to expectations, we found an increased invasion probability for mutators in the well-mixed environment only. We also tested the possibility that local conditions in structured environments may give rise to a higher mutation rate and hence a higher production rate of mutator mutants, but no such effect was found. A possible explanation for the observed mutator advantage in well-mixed environments is that the faster competitive dynamics among coexisting beneficial alleles allows a rapid increase of population size of each mutant subpopulation, and hence their supply of mutations for subsequent adaptation, in situations where the mutator fixes the first beneficial mutation. Our results only apply to constant environmental conditions, because the slower invasion dynamics associated with spatial structure cause a delay in mutator extinction at low frequencies, possibly giving them an advantage with changes in environmental conditions, such as the addition of antibiotics.

Introduction

Mutators are mutants with defective DNA-repair functions, and consequently have a higher mutation rate. A higher production of deleterious mutations appears to give mutators lineages an immediate slight growth disadvantage (Chao & Cox 1983). However, because mutators produce not only more deleterious but also more beneficial mutations, mutators can increase to high frequencies in populations by hitchhiking with the (genetically linked) beneficial mutations they produce. This indirect selective benefit depends upon the occurrence of beneficial mutations, and hence on opportunities for adaptation (Sniegowski *et al.* 2000; de Visser 2002); a well-adapted population presents fewer opportunities for a mutator to hitchhike to fixation than a poorly-adapted population. Mutators are thus predicted to be found in either rapidly changing environments (Oliver *et al.* 2000; Travis & Travis 2002) or spatially heterogeneous environments with multiple niches to which adaptation is possible (Oliver *et al.* 2000; Travis & Travis 2004).

Understanding the conditions that favor the emergence of mutator phenotypes is not only important scientifically, but is also relevant for human and animal health. High frequencies of mutator have been found in clinical bacterial populations (Leclerc *et al.* 1996; Oliver *et al.* 2000; Björkholm *et al.* 2001). Because drug resistant mutants arise more often in mutator populations, antibiotic treatment of clinical bacterial populations may directly select for the rise of mutator phenotypes present in low frequencies in most wild type populations (Oliver *et al.* 2000; Blázquez 2003) which could lead to clinical treatment failure (Giraud *et al.* 2002). In order to develop strategies to limit this complication it is critical to develop an understanding of the ecological conditions that lead to increases in the frequency of mutator cells.

So far, the evolutionary costs and benefits of hypermutability have been investigated in simple unstructured environments only (but see Giraud *et al.* 2001), despite the environmental structure present in almost any natural environment. We examined the invasion dynamics of a mutator strain of *E. coli* in structured and unstructured environments. We are particularly interested in the effects of three features of structured environments on the fate of a mutator subpopulation. First, the high number of niches arising from physico-chemical gradients in a

spatially structured environment will allow more opportunities for adaptation (Rainey & Travisano 1998), and hence for mutators to hitchhike with selected beneficial mutations. Second, the slower competitive dynamics among newly arising beneficial mutations caused by local resource competition in structured environments (Gordo & Campos 2005; Habets *et al.* 2007) may prevent mutants from being driven rapidly extinct when at low frequency. Persisting in a population may give the mutator strain the possibility of fixing additional beneficial mutations, which increases the likelihood of fixation. This might be especially important if the environment changes, for example due to the addition of an antibiotic (Tanaka *et al.* 2003). Third, it is possible that mutators arise more often in structured environments due to a higher mutation rate imposed by such environments. The low growth rate found in the centre of the biofilm (Wentland *et al.* 1996), could for example lead to higher mutagenesis, due to the starvation of cells (Bjedov *et al.* 2003). Other physico-chemical factors, like oxygen gradients in colonies growing in structured environments could also cause differences in mutagenesis (Bjedov *et al.* 2003).

To test the influence of these potential consequences of spatial structure, we performed competition experiments between a mutator and a wild-type strain of *Escherichia coli* at varying ratio's in three environments with different heterogeneity and competitive constraints. We compared a well-mixed environment (shaken liquid culture) with a spatially structured heterogeneous environment (agar surface), with and without dispersal. The results show an invasion benefit for the mutator in the well-mixed environment. In contrast to our predictions, in the structured environments, the mutator gains no added benefits. The disadvantage in structured environments may be attributable to either the slower dynamics in such environments, or to the drift loss of rare beneficial mutations in the mutator lineage caused by our method of transfer.

Methods & Materials

Strains & media

The ancestral strain (*Escherichia coli* B*,* REL606) has been used in previous evolution experiments and is described elsewhere (Lenski *et al.* 1991). From this strain, a DNA-repair deficient strain (Ara^+ $mutS^-$) was derived that can be distinguished from the ancestral strain due to its ability to utilize arabinose (de Visser *et al.* 1999); on Tetrazolium Arabinose indicator agar (TA) Ara^- cells grow as red colonies, while Ara^+ cells produce white colonies. TA plates contain per liter: 10 g tryptone, 1 g yeast extract, 5 g NaCl, 16 g agar, 10 g arabinose, and 1 ml of a 5% stock of tetrazolium (2,3,4-triphenyltetrazolium chloride). Minimal Arabinose (MA) plates (Davis minimal medium in which glucose is replaced by arabinose) only allow the growth of Ara^+ strains. The mutation rate of the Ara^+mutS strain is approximately 33-fold higher than that of the ancestral strain (deVisser *et al.* 1999). The invasion experiments were done in 1/10 Luria Broth media (LB).

Invasion experiment

Individual colonies of the ancestral strain Ara^-mutS^+ and the Ara^+mutS^- were isolated from agar plates, grown overnight in LB, and used to inoculate three different 1/10 LB environments; a homogeneous environment (liquid culture in 20 ml shaken tubes; 225rpm) and two spatially structured environments (60mm agar plates), one where the population structure was kept intact (structured plate), and one where the population was mixed during each daily transfer to fresh medium (twist plate). In each treatment, the competitors were introduced at three different starting ratios of mutator to wild type strain: 1:100; 1:1,000 and 1:10,000. Every treatment was replicated fourfold. Culture volume for all treatments is 10 ml. Every day, 50µl (containing ~$2,5*10^7$ cells) of the stationary phase culture was transferred to fresh medium and incubated for 24 hours at 37°C. The plate populations were transferred using sterile velvet; the twist population was mixed by twisting the donor plate a few times while transferring the cells (Kerr *et al.* 2002). The populations grew approximately 200-fold each day until they reached a density of about $5*10^8$ cells per ml, which represents ~8 generations. Twice a week samples were plated on both TA and MA plates to estimate the ratio of the competitors. At generation 100 and 200, a sample of the population was stored in

15% glycerol at -80°C.

If mutators were no longer detectable at day 25, the mutator frequency was scored with the value corresponding to the lowest detectable limit. Therefore, the slope of the trajectories of these populations is a conservative estimate of their rate of extinction. In addition, one population was excluded because it had already fixed a beneficial mutation prior to the commencement of the experiment.

Fitness assay

Relative fitness of the evolved clones was measured for all populations except the 1:100 ratio treatments. To measure the fitness of both wild type and mutator subpopulations, we isolated 6 clones of each, and competed them together versus the reciprocally-marked ancestor. Competition assays were performed with threefold replication as described in Lenski *et al.* (1991). Since mutators are identified by the state of their arabinose marker and not their mutator phenotype, and wild-type cells revert at the Ara locus at very low frequencies, we tested whether cells carrying the mutator marker found in very low frequencies possessed an increased mutation rate by conducting fluctuation tests on rifampicin. When no cells with mutator phenotype were found in a population, we assumed a fitness of 1 for the mutator subpopulation, indicating that it declined because it failed to generate a beneficial mutation rather than that it declined owing to the accumulation of a deleterious one.

Fluctuation test

We estimated the mutation rate of ancestral strain REL606 in the unstructured (liquid culture in tube) and structured environment (agar surface) by fluctuation test analysis following the protocol of Sniegowski *et al.* (1997). Tests were replicated 15-fold for resistance to rifampicin and reversion to the ability to utilize arabinose. Analysis of the fluctuation test data was conducted with a local program (website P.D. Sniegowski: http://www.bio.upenn.edu/faculty/sniegowski/).

Results

Difference in invasion dynamics between treatments

We examined the invasion dynamics of mutator subpopulations introduced at various frequencies in environments with varying degrees of spatial structure. As anticipated, invasion rate, as measured by the slope of the change in frequency with time, was dependent upon the mutator's initial frequency (Fig 1; two-way ANOVA on all slopes: $F_{2,26} = 4.051$, P = 0.029). At low starting frequencies (1:10,000), the likelihood of the mutator to fix the first beneficial mutation and invade the wild-type cells is low. A mutator subpopulation requires a mutation rate which is increased by approximately the inverse of its numerical disadvantage to have a higher chance of fixing the next beneficial mutation (Chao & Cox 1983; de Visser & Rozen 2005). Consequently, at high initial frequencies, fixation of the mutator seems a likely future outcome for all treatments (Fig 1).

We also found a marginally significant difference between environments in the invasion rate of mutator populations (Fig 1; ANOVA on all slopes: $F_{2,26} = 3.233$, P = 0.056). This is mostly driven by the difference at the highest initial frequency (1:100). Although the mutator appears likely to fix in all treatments at this frequency, the rate at which this occurs differs (ANOVA on slopes of 1:100 frequency: $F_{2,9} = 5.167$, $P = 0.032$). This was anticipated, because we have previously found that the rate at which a beneficial mutation achieves fixation increases with the rate of dispersal (Habets *et al*. 2007), and this varied between the environments; the well-mixed treatment allowed the highest dispersal rate and the structured-plate treatment allowed almost no dispersal, while in the third treatment some dispersal was allowed by twisting the velvet during daily transfer in a structured environment

Due to the slower invasion dynamics in the structured and twist-plate treatment, we had anticipated that the mutator subpopulation would persist for a longer period of time in these environments when their initial frequency was low, whereas they might be driven to extinction in the well-mixed environment upon the fixation of a beneficial mutation in the wild-type population. Overall, we could not detect a significant difference in invasion rates between treatments at lower frequencies (1:1,000: $F_{2,8} = 3.633$, $P = 0.075$; 1:10,000: $F_{2,9} = 0.060$, $P = 0.942$). In the twist plate treatment at 1:10,000, (all but one) mutator populations are still present at roughly the same frequency as the initial frequency after 200 generations
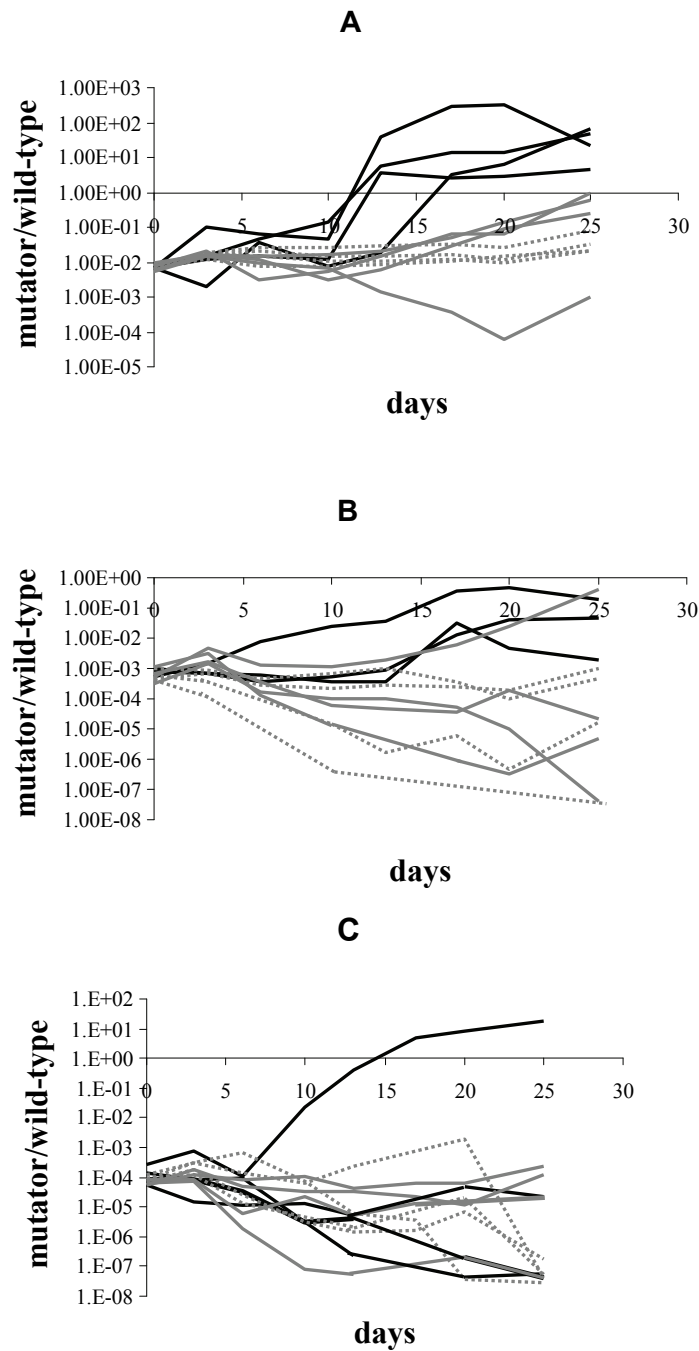
Figure 1:
Ratios of the mutator/wild-type population during 200 generations in either a well-mixed environment (tube: black lines), a structured environment with mixed population (gray lines), and a structured environment with intact population structure (dashed lines). A) represents populations where the initial ratio of the mutator wild-type was 1:100; B) represents populations with a 1:1,000 initial ratio; C) initial ratio of 1:10,000.

(one way ANOVA between beginning and ending frequency: $F_{1,7} = 0.288$, $P = 0.611$). In contrast and consistent with faster competitive dynamics, all but one of the mutators in the well-mixed environment have either been replaced or have invaded the population.

In the structured-plate environment, we failed to find any evidence for the persistence of the mutator population. We believe that genetic drift may have been a more powerful driver of this result than the fact that the competitive dynamics are slower in these populations. Serial transfer of each population was carried out using velvet, which may have caused sampling to be clumped, thereby sometimes missing small colonies. Because the subpopulation of the mutator is very low in the 1:10,000 treatment, this drift effect will hamper the mutator population more than the wild type, and may explain the decrease after 20 days in almost all mutator populations.

Fitness of mutator and wild-type subpopulation

Because no significant differences in the rate of invasion of the mutator could be found between treatments at lower initial frequencies, we examined the relative fitness of both the mutator and wild-type strains in order to extrapolate the outcome of competition. Significant differences between both strains were found (Fig 2; 3-way ANOVA using strains, initial mutator frequency and environment as factors: $F_{1,32} = 10.419$, $P = 0.003$), as well as significant differences between environments ($F_{2,32} = 6.982$, $P = 0.003$). In addition, the fitness difference between wild type and mutator varied marginally among environments (interaction strain x environment: $F_{2,32} = 3.270$, $P = 0.051$). In the structured environments with lowest initial frequency, the higher fitness of wild-type relative to mutator subpopulation is most apparent (one-way ANOVA: structured plate 1:10,000: $F_{1,7} = 7.991$, $P = 0.030$; twist plate 1:10,000: $F_{1,7} = 11.584$, $P = 0.014$). All other factors and interactions of the 3-way ANOVA are non-significant.

Two other interesting results were found. First, there is a difference in the final fitness of the wild-type between treatments, with lowest mean fitness in the well-mixed environment and

highest mean fitness in the twist-plate environment (Fig 2; $F_{2,16} = 11.566$, $P = 0.001$). This is in accordance with previous results of the same *E. coli* strain in the same environment after 900 generations of evolution (Habets *et al.* 2006). Mutator fitness did not conform to this pattern ($F_{2,16} = 1.404$, $P = 0.155$), possibly due to the small population size of mutator subpopulations. Second, we found that in the 1:10,000 twist-plate treatment, where the mutator subpopulations persisted, mutators have a significantly lower mean fitness ($F_{1,7} = 11.584$, $P = 0.014$) than the wild-type. Thus, even though the wild type has on average a $\approx$ 20% fitness advantage compared to the mutator (assuming that their fitness is transitive), it has not driven the mutator to extinction. This persistence indicates that spatial environments, owing to slower invasion dynamics, are somewhat protective of diversity.
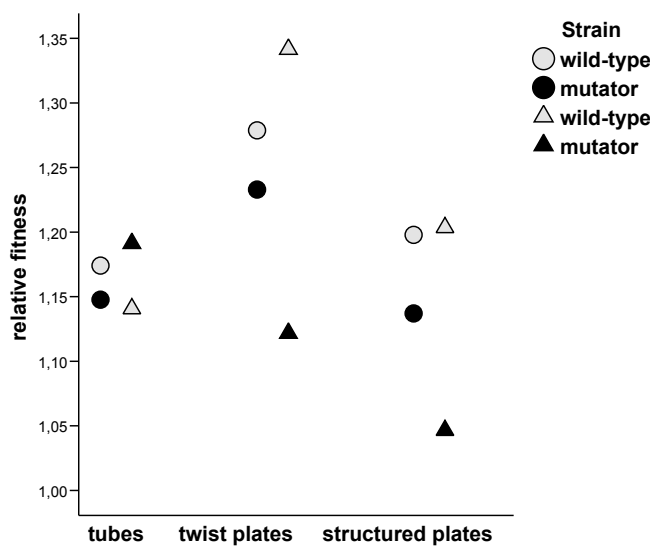

Figure 2:
Relative fitness of the wild-type and mutator subpopulations after 200 generations of evolution. Circles represent the values for the initial frequency of 1:1,000, triangles represent values for the initial frequency of 1:10,000.

Mutation rate in structured and unstructured environments

To test the possibility that mutator mutants arise more frequently in spatially-structured environments due to prevailing higher mutation rates in those environments, the mutation rate in the well-mixed and structured environment was investigated by fluctuation tests. For

rifampicin resistance, no difference in mutation rate was found between environments (Fig 3). However, for arabinose reversion, the mutation rate is 2-fold higher in tubes than in plates, indicating that mutators are unlikely to arise more frequently by mutation in spatial than in well-mixed environments.
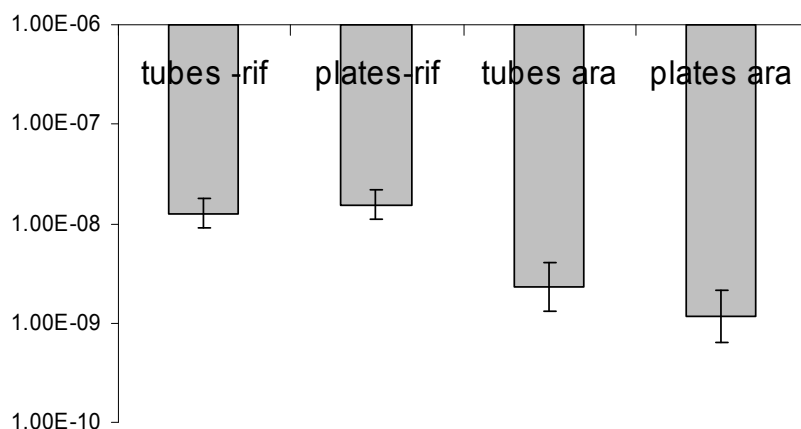

Figure 3:
Mutation rates for rifampicin resistance and arabinose reversion in both the unstructured (tube) and structured environment (plate). Error bars are 95% confidence limits.

Discussion

This study attempted to assess the competitive dynamics between mutator and wild-type subpopulations of *E. coli* in environments that differed in their degree of spatial structure. We had expected more frequent invasions of the mutator strain in spatially-structured environments, either because the prevailing heterogeneous conditions associated with such environments provide more opportunities for hitchhiking with the beneficial mutations they produce, or because mutators are given more time to substitute a beneficial mutation when at low frequency due to the slower competitive dynamics caused by local resource competition (Habets *et al.* 2007). Contrary to expectations, we found an invasion advantage for the mutator in the well-mixed environment, although this effect is only significant when the initial frequency of the mutator is relatively high (1:100). This result likely reflects the enhanced rate of mutator invasion due to faster competitive dynamics in a situation where the mutator is

anticipated to invade. However, we had not anticipated a benefit for the mutator when its initial frequency is low in well-mixed environments compared to structured environments.

Slower dynamics in structured environments

A possible explanation for the disadvantage in the structured-plate environment is the way we transferred population samples to fresh medium. Transfer by velvet produces clumped samples of the population and this may burden the smallest population most. Another possibility is that due to slower competitive dynamics, the mutator subpopulation does not increase in population size fast enough to fix additional beneficial mutations at a higher rate than the wild-type. On the other hand, its longer persistence in structured environments, what was especially surprising in light of the considerable fitness advantage of the wild-type over the mutator strains, may benefit the mutator population in the long term, perhaps particularly when the environment would change dramatically and selection is lethal. When the mutations fixed by the wild-type are neutral in the new environment, the competition would start over, with the renewed possibility that the mutator wins, whereas in an unstructured environment, due to a possible sweep of the wild-type beneficial mutation the mutant would have already gone extinct

Differences in dynamics due to heterogeneity

It has been shown, both theoretically and experimentally that mutators may benefit from novel environmental conditions such as encountered in fluctuating or heterogeneous environments (Mao *et al*. 1997; Taddei *et al*. 1997b; Tenaillon *et al*. 1999; Travis & Travis 2002; Tanaka *et al*. 2003; Oliver *et al*. 2003; Travis & Travis 2004). Even though our spatially structured environment provides more niches due to heterogeneity, we did not find an additional advantage for the mutator strain.

It is possible that the supply rate of beneficial mutations in our experimental system is very high. If so, having a higher mutation rate would only provide the mutator strain with a negligible advantage in finding new beneficial mutations, and this would fail to offset their numerical disadvantage versus the wild-type lineage. In a previous experiment using the same *E. coli* strain and environment, small and large structured populations adapted equally rapidly

to the complex environment used here (Rozen *et al.* unpublished) suggesting that beneficial mutations in this environment are abundant; for this reason, a higher mutation rate might not lead to faster adaptation. It could be argued in this case, that if the initial population was better adapted, the mutator might realize its hypothesized advantage, because then the population would be limited by the supply of beneficial mutations (de Visser *et al.* 1999). Accordingly, the likelihood that the mutator subpopulation will fix the first few mutations, necessary for invasion of the mutator lineage, may be higher when the population is beneficial mutation limited.

Differences in mutation rate

Our results show an invasion benefit for the mutator population in well-mixed environments, and a higher fitness for the wild-type populations, but no difference could be found in mutation rate. However, Taddei and colleagues (1997a) show that mutagenesis in structured environments increases with time spent in stationary phase, whereas mutagenesis remains equal for well-mixed populations. It is possible that gradients in physical and chemical factors increase with the amount of time a population has spent in a structured environment, and this, together with stress due to starvation, could lead to a higher mutation rate. Evidence for this is found by Bjedov *et al.* (2003); their results show that mutagenesis in aging colonies depends on starvation together with the diversity of selective pressures found in structured populations.

It remains questionable whether spatial structure can increase the likelihood of a mutator to invade a population from low frequencies either through the slower competitive dynamics or elevated heterogeneity associated with structured environments. Our results would suggest a benefit for the wild type populations when mutator subpopulations are rare, because we did find a higher fitness for the wild-type in the 1:10,000 treatment in structured – and twist plates. However, we believe that the method of transfer may have affected this outcome. Additionally, we believe that the increased persistence time of mutators, even in the face of sizable fitness deficits, does offer a potential clue to mutator invasion in spatially structured environments. Namely, in fluctuating environments that would allow the mutator the opportunity to gain secondary beneficial mutations. This two-step process, as outlined in

Tanaka *et al*. (2003), and suggested by our data, warrants testing and will represent a direction of our future work on this clinically important topic.

Acknowledgements

6 General Discussion

Spatial structure has the potential to provide solutions to the paradox of diversity – the problem of the coexistence of many species, all seemingly competing for few limiting resources. Population dynamics and species interactions can be strongly influenced by spatial structure, demonstrated by numerous mathematical models (Tilman & Kareiva 1997). Empirical evidence, supporting the models, is still scarce. We have attempted to investigate experimentally several consequences of spatial structure that can increase the adaptive radiation of populations.

Population fragmentation

In **Chapter 2,** the hypothesis was tested that small populations, representing population fragments in structured environments, are more likely to diverge than large populations. Twenty-four small and six large *E. coli* populations were serially transferred for 500 generations in either a simple or a complex environment. We found that small populations followed heterogeneous adaptive trajectories when adapting to a new environment, whereas large populations seem to adapt in a more deterministic way. We believe that large populations did not show divergence in adaptation because they tend to fix beneficial mutations conferring large benefits. Small populations, on the other hand, fix beneficial mutations with smaller benefits. Because the latter class of mutations is more abundant, more genetic diversity in adaptation arises between small populations than between large populations. Surprisingly, even though small populations are thought to be constrained due to the fixation of small mutations, this was not found when populations evolved in a complex nutrient environment. Due to a combination of epistasis among and greater diversity of selected mutations, the small populations that had fixed a smaller- effect beneficial mutation after 100 generations, reached higher fitness after 500 generations.

In a complex environment, the adaptive landscape consists of several adaptive peaks. Small populations explore the landscape more widely, with some populations finding low

peaks and others high peaks. Large populations, due to their wider sampling of the distribution of beneficial mutations, are more likely to fix rare large-effect beneficial mutations, and will be 'forced' to go up the steepest hills, which do not necessarily lead to the highest peak.

Bearing in mind many restrictions, our experiment can be viewed as relevant for the so-called SLOSS (Single Large Or Several Small) debate. In conservation biology, there is controversy as to whether effort and money should be put into reserving large natural reserves, or many small ones. Whereas some conservationists argue that large areas contain a higher diversity – based on the theory that species richness increases with habitat area (MacArthur and Wilson 1967) -, others challenge this view because it is based on the premise that large areas contain the same species as many small ones do. If small areas, however, contain unshared species, species richness may be higher in a collection of small areas.

Forty-eight small populations in our experiment have the same effective population size and resources as the single large population with which they were compared. Even though not all small populations were tested, it is clear from the fitness data that these show an enormous diversity when compared to one large population. When direct comparisons are made between many small islands and one large one, Quinn & Harrison (1988) found similar effects. For example, even though the island of Hawaii represents 63% of the land area of the Hawaii archipelago, only 30 of the 70 native land bird species are found here, whereas the small islands harbor 51 species

Obviously, the simple and controlled experimental conditions are very different from the complexity of interactions between many species in nature reserves. In addition, even though the population size of the small populations of *E. coli* is consistent with bottleneck sizes in microbes, $5*10^5$ is not a small population size for some higher organisms.

Competitive dynamics in spatially structured environments

In **chapter 4,** we examined how the rate of invasion and the realized fitness of a beneficial mutant are affected by dispersal in a structured environment. A beneficial mutant of a non-motile *E. coli* strain (isolated from the previous experiment) invaded populations of ancestral

cells under different dispersal regimes. In the treatment with lowest dispersal, cells of the beneficial mutant were introduced in one spot. In the second treatment, the mutant was introduced in a number of small spots. In the third and fourth treatment, they were completely mixed with the ancestral cells. The fourth treatment differed from the third in that cells were mixed before transfer to a fresh agar plate, making it the treatment with highest dispersal.

We found slower dynamics for an invading beneficial mutation when dispersal was limited. When dispersal was highest so was the rate of invasion. We also found that with increased invasion, the fitness of the mutant declines due to an increase in the ratio of intra-specific versus inter- specific competition.

Even though we have investigated the consequences of spatial structure on the adaptive radiation of populations in the framework of the niche differentiation hypothesis, both population fragmentation and slower competitive dynamics are capable of increasing diversity in populations in homogeneous structured environments: fragmentation most noticeably through allopatric divergence, slower dynamics through increased local competitive interactions, including those leading to stable coexistence.

Intra specific clustering due to limiting dispersal can allow coexistence if life history differences create spatial niche differences (Amarasekare 2003). For example, inter specific trade- offs between colonization ability and competitive ability may lead to coexistence because inferior competitors can persist by occupying sites the superior competitor has not occupied yet. In grasslands with nitrogen as the limiting resource, more than 100 species can coexist, even though one species is the best competitor (Tilman 1994). The superior competitor has, due to a greater allocation to roots, a lower allocation to reproduction. If by death and disturbance, open patches are always available, stable coexistence can arise by the competitive colonization trade-off.

Our results demonstrate transient coexistence of both strains; however, we could not test any of the coexistence mechanisms, since we used a non-motile *E. coli* strain in our experimental set up.

Intrinsic & extrinsic heterogeneity of the environment

Environmental heterogeneity is expected to be one of the main contributors to diversification of populations in structured environments. Microclimates arise due to gradients in light, oxygen, moisture, pH and many other abiotic or biotic factors. These gradients can be caused by extrinsic factors, but also by the organisms themselves (intrinsic factors, niche construction).

In **Chapter 3,** we have attempted to investigate the impact of environmental heterogeneity together with fragmentation and slower dynamics on the diversification of populations. To this end, thirty- six *E. coli* populations were serially transferred for 900 generations in three different environments; an unstructured environment with a well-mixed population, a structured environment with a well-mixed population, and a structured environment where the population structure was left intact with each transfer.

Extrinsic heterogeneity by itself did not lead to the diversification of populations in our model system. In the presence of fragmentation and slower dynamics, however, stable polymorphisms arose. These can be explained either by intrinsic heterogeneity – organisms have provided additional niches, allowing the population to diverge (Laland 1999) or by the higher likelihood of fragments of the population to diverge into the niches present in the environment.

Few studies have experimentally tested the influence of spatial heterogeneity on adaptive radiation (Korona *et al.* 1994; Rainey and Travisano 1998; Losos 1997) although many studies have looked at correlations between environmental gradients and communities (for a review see Sollins 1998), or have examined trade-offs of species in laboratory settings (Ter Steege 1994) or both. Other studies focus on source- sink dynamics as a mechanism for coexistence of species; locally inferior competitors are transported from source habitats to sink habitats, preventing exclusion (Codeco & Grover 2001). Abiotic heterogeneity of the environment increases the likelihood of source-sink dynamics.

In some organisms, maintenance of polymorphisms due to environmental heterogeneity can also arise due to genotype specific habitat selection. For example, for the

African Finch Pyrenestes, billsize and feeding preference are correlated; billsize is related to the time it takes to crack seeds; large morphs feed on hard seeds, small morphs on soft seeds (Smith 1993).

Consequences of spatial structure on the invasion of mutators.

In **Chapter 5,** we investigated whether mutator populations benefit from the heterogeneity and slower dynamics found in spatially structured environments. Thirty-six competition experiments between a wild-type and a mutator *E. coli* strain were performed for 200 generations in three environments with different heterogeneity and competitive constraints. A well-mixed environment was compared to two structured environments, one where the population was dispersed, and one without dispersal. Contrary to expectations, the results showed an advantage for the mutator lineage in well-mixed environments. A possible explanation for this is that faster competitive dynamics allow for a faster increase in population size, and hence of the supply of mutations for subsequent adaptation in situations where the mutator fixes the first beneficial mutation. However, these results only apply to constant environmental conditions. If fluctuations would occur, the delay in mutator extinction at low frequencies caused by slower dynamics, could give them an advantage.

Investigating conditions favoring the emergence of mutators is interesting scientifically, but more importantly, critical to human health. Because mutators are found in high frequencies in pathogenic bacteria, and because they have a higher mutation rate, they can cause antibiotic treatment failure.

Future studies

Several theoretical studies have shown the importance of environmental heterogeneity for the adaptive radiation of populations, although most focus on fluctuations in time, rather than space. Moreover, few empirical studies have detailed information on adaptive radiations (Hedrick 2006). We too have not yet been able to unravel the specific details of the interactions between our coexisting strains found in the experiment in **chapter 3**. Further research will focus on examining the nature of this stable coexistence. It is possible that cross-feeding takes place, especially since this has been observed before in experimental evolution

studies using *E. coli* (Helling *et al.* 1987).

More generally, a possible direction for further research in population fragmentation, slower competitive dynamics and environmental heterogeneity would be to study biofilms in batch culture. This would mimic natural conditions better than growth on agar plates. In addition, several species could be studied simultaneously, looking at the effects of their mutually, evolving interactions on each other. Also, interactions between factors increasing diversity should be studied. Due to the many advantages of working with microbes, studying complex systems will be easier than it is for higher organisms.

Another line of research could be to study natural isolates to confirm predictions made in the laboratory. For the influence of environmental heterogeneity on diversity, investigations could focus on strains living in biofilms compared to "free living" micro-organisms, in the water column. The amount of heterogeneity in the environment may be related to the amount of diversity found.

Further study of the slower competitive dynamics, associated with limited dispersal in structured habitats, should focus on the colonization-competition trade-off. Like plants, it may be possible for micro-organisms to coexist due to a trade-off between colonization and competition. For example, studies could examine commensal bacteria coexisting in populations. It is possible that some species spread well, while others are better at competing for e.g. iron, or other limiting factors in human bodies. Strains could be isolated and tested for dispersal ability and competitive ability in laboratory settings.

In addition, isolating strains from humans living in densely populated areas (major cities) and comparing those with strains from island inhabitants in more remote parts of the world would be a way to test if fragmentation has led to a higher amount of diversity among commensal bacteria.

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Nederlandse samenvatting

Er is een groeiende bezorgdheid over de biodiversiteit aangezien door ontbossing, versnippering van leefgebieden en vele andere humane oorzaken, soorten bedreigd worden. Dit geldt niet alleen voor tijgers, pandaberen, de das en de otter; soorten in alle rijkdommen worden bedreigd. Van de amfibieën zijn zelfs eenderde van de soorten wereldwijd bedreigd. Om effectieve strategieën te ontwikkelen om deze afname tegen te gaan is een goed begrip nodig van mechanismen die een rol spelen bij het ontstaan en behoud van diversiteit. Dit proefschrift onderzoekt één van de factoren die een rol spelen bij het ontstaan en behoud van diversiteit, namelijk spatiële structuur.

De enorme biodiversiteit in de wereld heeft vele onderzoekers verwonderd; immers, de verwachting is dat competitie voor nutriënten, zonlicht of andere noodzakelijke bronnen, zal leiden tot de overwinning van de beste concurrent. Dit blijkt echter niet het geval en een aantal hypothesen is opgesteld om de coëxistentie van concurrenten te verklaren, waarvan de niche-differentiatie hypothese de belangrijkste is. Deze stelt dat wanneer er meerdere niches aanwezig zijn in de omgeving, en er competitie voor resources is, de populatie zal differentiëren in verschillende specialisten, elk aangepast aan een andere niche. Een voorbeeld hiervan zijn stekelbaarsjes in kleine meren in Canada. Wanneer voedsel schaars is, kan competitie leiden tot de differentiatie in twee soorten: een grote soort die leeft van vertebraten die op de bodem leven, en een kleine soort die plankton eet in de waterkolom – op deze manier is er minder concurrentie tussen de nieuwe soorten. In meren waar geen schaarste is, komt een intermediaire soort voor, die zowel plankton als vertebraten consumeert.

Dit voorbeeld is een van de weinige bewijzen voor de niche-differentiatie hypothese. Het zoeken naar bewijzen voor deze hypothese is onder andere moeizaam, omdat men niet terug kan gaan in de tijd om te onderzoeken waarom diversificatie heeft plaatsgevonden. Micro-organismen daarentegen, geven deze mogelijkheid wel, en zijn daarom uitermate geschikt voor onderzoek naar evolutionaire processen.

Het werken met micro-organismen heeft meerdere voordelen. Zo hebben micro-organismen een zeer korte generatie tijd, waardoor binnen enkele maanden 1000-en generaties evolutie bereikt kunnen worden in het laboratorium. Micro-organismen kunnen worden ingevroren bij -80°C. Dit betekent dat de voorouder van een evolutie experiment direct in competitie kan worden gebracht met de geëvolueerde lijnen. Bovendien, nemen ze weinig plaats in het laboratorium, waardoor vele replica's tegelijkertijd kunnen worden gekweekt.

Resultaten

Wij hebben gekeken naar de invloed van spatiële structuur op de diversificatie van *E. coli* populaties. Ons onderzoek heeft uitgewezen dat wanneer populaties in een gestructureerd milieu (agar plaat) voorkomen, in tegenstelling tot een gemengd milieu (geschudde buis), er een grotere diversiteit optreedt, mits de populatie-structuur intact wordt gelaten. Dit wordt door een aantal factoren veroorzaakt.

Ten eerste zullen populaties, wanneer ze zich enkel beperkt kunnen verspreiden in een gestructureerde omgeving, gefragmenteerd worden in meerdere kleinere subpopulaties. Kleine populaties vertonen een hogere variatie dan grote populaties (hoofdstuk 2), aangezien kleine populaties meestal mutaties fixeren met een relatief klein fitness-voordeel. Omdat er meer mutaties met een klein dan met een groot fitness-voordeel zijn, zullen kleine populaties vaker verschillende mutaties fixeren. Aangezien grote populaties mutaties fixeren met een groot voordeel, en deze schaars zijn, adapteren zij vaker met behulp van dezelfde mutaties. Een opmerkelijke vondst was dat het fixeren van grotere mutaties op de lange termijn niet hoeft te leiden tot een hogere fitness. Kleine populaties die adapteren in een fitness landschap met meerdere pieken (zeg meerdere adaptieve oplossingen) kunnen een hogere fitness bereiken vanwege hun groter vermogen dit landschap te exploreren.

Spatiële structuur zorgt niet alleen voor de fragmentatie van de populatie, het kan ook zorgen voor heterogeniteit van de omgeving. In de bodem kan er bijvoorbeeld een gradiënt zijn van zuurstof, waardoor coëxistentie mogelijk is van twee soorten, waarbij de ene soort beter concurreert bij een lage en de ander bij een hoge zuurstofconcentratie. Heterogeniteit zorgt dus voor het voorkomen van meerdere niches. Het kan ook zijn dat heterogeniteit in een

gestructureerd milieu wordt veroorzaakt door de organismen zelf. Organismen kunnen het milieu veranderen op vele manieren: planten veroorzaken bijvoorbeeld een schaduwrijke omgeving, met een hogere humiditeit en temperatuur. Hierdoor wordt een nieuwe niche gecreëerd, die evolutie kan vullen met nieuwe specialisten. Hoofdstuk 3 beschrijft de positieve rol van de heterogeniteit van het milieu – veroorzaakt door ruimtelijke structuur - op de diversiteit van evolueerde *E. coli* populaties. Voorwaarde voor een hogere diversiteit is echter wel dat de populatie structuur behouden wordt.

De resultaten van hoofdstuk 4 tonen aan dat de verspreiding van een voordelige mutatie sneller gaat in een gemengd milieu dan in een gestructureerd milieu. Doordat in een gestructureerd milieu een mutant met een hoger competitief vermogen dan de voorouder, voornamelijk met zichzelf in competitie is, zal de toename niet zo snel gaan als in een gemengd milieu - de mutant zal immers alleen aan de rand van de kolonie met de voorouder in competitie zijn, en kan dus enkel hier zijn voordeel realiseren. Doordat de mutant langzaam toeneemt in aantal, zal de voorouder enige tijd coëxisteren met de mutant. Dit verhoogt de kans op langdurige coexistentie van sommige mutanten via de evolutie van specifieke frequentie-afhankelijke interacties.

In het laatste hoofdstuk wordt de invloed van fragmentatie en een langzamere invasie van mutanten in een gestructureerde omgeving onderzocht op de toename van mutators. Mutators zijn mutanten met een beschadigd DNA-repair mechanisme, waardoor zij meer mutaties produceren dan het wildtype. Mutators zijn medisch gezien belangrijk aangezien ze vaker resistent zijn tegen antibiotica en gevonden worden in patiënten, zoals in het longweefsel van cystische fibrose patiënten, waar ze enorme problemen opleveren. Aangezien het interne milieu van een gastheer (bijvoorbeeld een patiënt) wordt gezien als sterk gestructureerd, hebben we onderzocht of door deze ruimtelijke structuur mutators een voordeel hebben. Echter, wij konden geen duidelijk voordeel vinden. Wel blijven mutators, zoals verwacht, langer aanwezig in een gestructureerd milieu in vergelijking met een gemengde milieu, wanneer ze in lage frequentie geïntroduceerd worden. Het is daarom mogelijk dat de mutator een voordeel krijgt wanneer de omgeving verandert, bijvoorbeeld door het toedienen van antibiotica, en daardoor alsnog in frequentie toeneemt. De tragere

invasie van mutanten in gestructureerde milieu's zou in dit geval zorgen voor een voordeel voor mutators.

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