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The Distribution of Fitness Effects of Spontaneous Mutations in Vibrio fischeri

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Abstract

Mutations are the ultimate source of the biological diversity on which natural selection acts, but the vast majority of these mutations are harmful. As such, mutations lead to disease states like cancer, extinction of small populations, and can drive pathogen evolution. Unfortunately, because mutations are rare and past studies have been subject to detection biases, very little is known about the distribution of fitness effects from naturally occurring mutations. In this study, we used mutation accumulation and full genome sequencing to capture naturally occurring mutations before they were exposed to the sieve of natural selection in *Vibrio fischeri*. We then measured the effects of these mutations on the fitness of the individuals harboring these mutations. We hypothesized that most mutations would be deleterious, and that deletions and insertions would be more detrimental to fitness than base substitutions, particularly in coding regions. Additionally, we expected to show that mutations on primary chromosomes, which are more highly expressed and evolutionarily conserved, would have more harmful effects than mutations on accessory secondary chromosomes. Using a subset of eleven mutation accumulation isolates, each harboring between two and nine mutations, we show that the majority of mutations have minor deleterious effects, with a subset of those errors resulting in more drastic fitness declines. However, extending this study to more genotypes will be required to examine the relationship between particular mutation types and fitness. Ultimately, an enhanced understanding of the relationship between genotype and fitness will broaden our understanding of the distribution of mutational effects and elucidate the susceptibility of different genome regions to deleterious variation.

Introduction:

Genetic mutations provide the raw material for evolution, as they result in the phenotypic diversity on which natural selection acts. Mutations have led to the extensive biological diversity that can be seen today. However, the majority of genetic mutations are damaging, or deleterious. In humans, deleterious mutations are responsible for the origin of nearly all diseases, including cancer. It is important to study the effects of these mutations, as it could aid in the understanding of such diseases. Diseases, such as cancer, are the result of mutations that accumulate throughout a person's lifetime in somatic cells. In an average lifetime, a person can accumulate between 4,000-40,000 mutations (Lynch, 2009). It is also something that pertains to everyone, as it is estimated that every person receives more than 100 new mutations in the germline, which are mutations that are passed from one generation the next (Crow et al., 1993).

There are a number of problems associated with studying mutations in humans directly, the first and most important of which is that the effects of these mutations often only appear in a population after a number of generations. To study evolution in humans, or any other large organism for that matter, would take an impossibly long time. Therefore, most studies of genetic mutation are conducted on bacteria or unicellular eukaryotes, which can experience up to 30 generations a day. The results of such studies have extremely broad implications, the most applicable of which is the development of a null hypothesis for the molecular evolution of organisms. By examining organisms with such short generation times, we can study evolution directly, and genetic sequencing allows us to observe the changes that are occurring at the molecular level. Eventually, this hypothesis may be applied to human evolution, as all organisms share the same basic genetic code, and mutations play similar roles in human evolution and microbial evolution.

It has been suggested that the percent of genetic mutations that are beneficial is substantially influenced by the initial fitness of the organism in its environment. This is based on how close the organism is to reaching its fitness plateau, or the point at which fitness is maximized for that environment, and beneficial mutations are either occurring much more rarely, or simply do not have an observable effect. Organisms that are approaching a fitness plateau will experience a greater percent of deleterious mutations than those that are poorly adapted to their environment (Silander et al, 2007).

Based on studies in *E. coli,* it is estimated that microbes undergo around 0.3 deleterious mutations per haploid genotype per generation (Kibota and Lynch, 1996). The percent of genetic mutations that are beneficial versus harmful may vary between species. In an environment free of selection, about 25% of mutations in yeast appeared to be beneficial (Dickinson, 2008). However, a similar study of *Arabidopsis* concluded that as high as 50% of mutations were beneficial (Shaw et al., 2002), although the results of this experiment were highly scrutinized. In response, an alternative model concluded that most of the mutations were, in fact, deleterious (Keightley and Lynch, 2003).

Although deleterious mutations are widely studied, studying the natural mutation spectrum has proven difficult. One problem that researchers are faced with is that these mutations can have an array of fitness effects on an organism, ranging from mild to lethal. Oftentimes, mildly deleterious mutations are overlooked because researchers pick and study a mutant only after sufficient fitness divergence from a wild type ancestor (Eyre-Walker and Keightley, 2007). This results in a detection bias of only mutations that are deleterious enough to significantly change the fitness of an organism relative to the ancestor.

With regards to genome conservation, previous studies have found that mutations in the non-coding region underwent weaker selection than those in the coding region (Eyre-Walker and Keightley, 2007). Moreover, in humans, it was found that certain parts of the genome are more susceptible to mutations that lead to disease (Kowarsch et al., 2010).

This study aims to address and overcome the problems in studying the natural mutation spectrum by examining mutations in *Vibrio fischeri* that accumulated without the sieve of natural selection. The studied strains were not grown in congruence with the ancestor, and were bottlenecked to one cell daily to avoid natural selection. Mutants were chosen after a certain number of elapsed generations, rather than any visible fitness divergence from the ancestor, which allows for an essentially unbiased screen for all naturally occurring mutations.

Once genetic sequencing was performed on these lines, our focus turned to whether or not certain types of mutations occur more commonly, and whether certain parts of the genome are more subject to deleterious mutations than others. We hypothesized that deletions and insertions would be more deleterious than base substitutions due to the resulting frameshifts. This is something that is already well documented in many other systems (Eyre-Walker and Keightley, 2007).

We also used the same genetically sequenced *V. fischeri* mutants, in order to determine the effects of locations of mutations on the fitness of the organism, relative to the ancestor. We expected to find that mutations located in coding regions would result in larger declines in fitness than those in non-coding regions, as these coding regions are transcribed into functional proteins. Further, we expected that mutations on the primary chromosome would be more deleterious than those on the secondary chromosome. Our results would either support or dispute the hypotheses that genes on the secondary chromosome are less significant to cellular function than those on

the primary chromosome, and that gene location has an influence on whether or not a specific, perhaps less important site, in the genome is more susceptible to variation than others that are more central to the functionality of the organism (Cooper et al., 2010).

We found that there was no relationship between the locations of the mutations, whether it is on chromosome one or two or in the coding or non-coding region, and its effect on the change in relative fitness. We also found no relationship between the different types of mutations and their effects on change in relative fitness. We did, however, find that the majority of mutations have relatively negligible effects on relative fitness, and can conclude that large changes in fitness are most likely due to "driver" mutations.

Methods:

Mutation Accumulation:

The strains of *V. fischeri* used in this study were obtained from a previous mutation accumulation experiment. In this MA experiment, a fully sequenced *V. fischeri* strain, which is a symbiont of the Hawaiian bobtail squid, was used as the ancestor. This strain was streaked from frozen onto 75 T-Soy NaCl plates and incubated at 28C overnight. Each day for a period of eight months, a single colony was picked from each plate, re-streaked onto a new plate, and incubated at 28C for another 24 hours. Individual colonies were chosen randomly to limit the influence of natural selection. After 5187 generations, mutants were frozen, and the complete genomes of 48 isolates were sequenced in order to determine the genetic identity of each evolved strain. Sequencing was conducted by using Illumina paired-end reads, which were each 100bp in length. All 100bp fragments were then aligned to a reference genome using two independent reference alignment algorithms: bwa and novoalign. Mutations were identified using mutation

calls with pindel and in house perl scripts. Following the completion of genetic sequencing, mutation accumulation lines *Vf1-3* and *Vf5-12* were used for this study.

***Fluorescent Marking of VfES114 strains using E. coli pVSV102gfp and pVSV108Red plasmids*:**

Strains of *V. fischeri* ES114 were fluorescently marked with either a green or a red plasmid using conjugation. An *E. coli* helper was also used to facilitate the process of conjugation, in which the fluorescently marked *E. coli* donor plasmid is transferred into the *V. fischeri* cell. The following *V. fischeri* strains were fluorescently marked both red and green using *E. coli* plasmid donors: VfES114-WT, VfES114-1, VfES114-2, VfES114-3, VfES114-5, VfES114-6, VfES114-7, VfES114-8, VfES114-9, VfES114-10, VfES114-11, and VfES114-12. The *E. coli* donor strain containing the green plasmid was *E. coli* pVSV102gfp, which harbored resistance to the antibiotic, kanamycin. The helper strain, *E. coli* pEVS104 Helper, was also resistant to kanamycin. The *E. coli* donor strain containing the red plasmid was *E. coli* pVSV208dsRed, which harbored resistance to the antibiotic, chloramphenicol.

All *V. fischeri* strains were streaked from frozen onto LBS plates and incubated at 28C for 24 hours. *E. coli* pVSV102gfp and *E. coli* pEVS104 Helper were streaked from frozen onto LB plates with a kanamycin concentration of 40 ug/ml. *E. coli* pVSV208dsRed was streaked from frozen onto an LB plate with a chloramphenicol concentration of 20ug/ml. All *E. coli* strains were incubated at 37C for 24 hours.

Following 24 hours of growth, a single colony of each *V. fischeri* strain was inoculated into liquid LBS broth and grown overnight (>8 hours) at 28C. Each *E. coli* strain was also inoculated into the same media as before, but as a liquid culture, and grown overnight at 37C.

The next day, each *V. fischeri* and *E. coli* strain were inoculated into 5mL of the same media as the previous day via a 1/50 dilution (100uL into 5mL). These cultures were grown to mid-log phase (OD600~0.5). 750uL of each *E. coli* donor strain was added to three separate 1.5mL centrifuge tubes, along with 750uL of the *E. coli* helper strain. Each centrifuge was spun down, then re-suspended in 1mL of LBS in order to wash the cells of the antibiotics from the previous media. After re-suspension, each tube was spun down again and re-suspended in 100uL of LBS. We then spread-plated 100uL from each centrifuge tube onto LBS plates using glass beads. Each recipient *V. fischeri* strain was spotted as 50uL onto one of four locations on the plates, which were then incubated right side up at 28C overnight.

The resulting individual spots were streaked onto separate LBS with antibiotic plates in order to capture the plasmid bearing *V. fischeri* cells. Strains that had been plated with *E. coli* pVSV102gfp were streaked on LBS with a kanamycin concentration of 100ug/ml, and strains plated with *E. coli* pVSV208Red were streaked on LBS with a chloramphenicol concentration of 2ug/ml. These plates were incubated at room temperature for one to two days. *V. fischeri* colonies were then picked and re-streaked onto the same LBS antibiotic plates, and incubated overnight at 28C. The following day, each strain was inoculated in 5mL of the same media as the night before, with antibiotic selection, and incubated at 28C overnight. Fluorescence was verified the next day using flow cytometry. Once verified, each strain was frozen down in 8% DMSO. Each of the twelve *V. fischeri* strains was successfully marked with both the red and green plasmids, and these marked strains were used in the subsequent reciprocal fitness assays.

Fitness Assays:

Cultures of the previously marked strains were inoculated from frozen into 5mL of LBS with a pipette tip, and incubated in a roller drum at 28C for 24 hours. The following day, we

transferred 50uL of each culture into 5mL of T-soy NaCl and incubated in a roller drum at 28C for 24 hours.

On the third day, competitions were created by combining 25uL of the green test strain to 25uL of the red ancestor into 5mL of T-Soy NaCl. This was repeated with a reciprocal competition containing 25uL of the red test strain and 25uL of the green ancestor. This was done for each of the 11 total competitor strains. The following controls were present at the beginning of each assay: blank culture, WT-R Alone (50uL), WT-G Along (50uL), and a WT-R versus WT-G competition. Each competition was incubated in a roller drum at 28C for 24 hours. For each of the Day 0 competitions created, the same competition was created in filter sterilized PBS by combining 15uL of each competitor into 270uL of filter sterilized PBS in a 96 well plate. Individual controls were recreated by pipetting 30uL of each into 270uL of filter sterilized PBS in the 96 well plate. Competitions were diluted to $10^{-4\ 1/5}$ by transferring 30uL of the cultures into 270uL filter sterilized PBS a subsequent three times, and 60uL into 240uL filter sterilized PBS once. The $10^{-4\ 1/5}$ dilution was read in the flow cytometer using the following settings: forward scatter 30, side scatter 2, threshold 7, green 200, yellow 200, five decade on, blue laser only, low flow rate, and 10,000 events.

The next day, 50uL of each culture was transferred into 5mL of fresh T-Soy NaCl and incubated in a roller drum at 28C for 24 hours. This process was repeated again the following day. On Day 3 of the assay, competitions were again diluted to $10^{-4\ 1/5}$ and read in the flow cytometer using the same settings as Day 0. This process was repeated three additional times, for a total of four replicates.

In order to correct for the marker overlap and determine the true concentrations of each competition, the following equations were applied to the results:

$GFP_{Actual} = GFP_{Count} - ((RFP_{count}-(GFP_{count} \bullet X)) \bullet Y) + ((GFP_{count} - (RFP_{count} \bullet Y)) \bullet X)$

$RFP_{Actual} = RFP_{Count} - ((GFP_{count}-(RFP_{count} \bullet Y)) \bullet X) + ((RFP_{count} - (GFP_{count} \bullet X)) \bullet Y)$

Where X equal the number of red counted per green in the GFP only control, and Y equals the number of green counted per red in the RFP only control.

The WT-R versus WT-G competitions were used to calculate the correction value in order to correct for any marker effect. The correction value was used to correct each competition by either adding or subtracting the benefit or harm of harboring the plasmid that is harbored in that strain. The average of the two corrected reciprocal competitions was taken to calculate final fitness.

The equation used to calculate fitness was:

$$W = \frac{\ln(\frac{M_F \times 10^6}{M_I})}{\ln(\frac{a_F \times 10^6}{a_I})}$$

Results:

After 5187 generations in the near-absence of natural selection, the twelve lines, on average, had an overall fitness of 0.977, relative to the ancestor. Nine of the eleven mutant lines experienced a significant decrease in relative fitness, one line was neutral (falling within the error of the ancestral fitness), and one line increased to a relative fitness of 1.016 (Fig.1).

The absolute fitness effects of individual mutations were estimated by dividing the total fitness decline of each genotype by the number of mutations in each line. The effects of these single mutations ranged from 0-3% but the majority of mutations resulted in a less than a 1% decline in relative fitness (Fig. 2). An increased number of mutations in an organism resulted in no change in relative fitness. Surprisingly, the organism with the fewest mutations (1) had a

change in fitness of 0.34 and the organism with the most mutations (9) had relative change in fitness of 0.008 (Fig. 3).

We also found no relationship between the change in relative fitness and the number of insertions and deletions in the coding region of the genome (Fig. 4). There was likewise no relationship between the change in relative fitness and increased number of genic non-synonymous substitutions (Fig. 5), genic synonymous substitutions (Fig. 6), substitutions in the non-coding region of the genome (Fig. 7), and increased number of insertions and deletions in the non-coding region of the genome (Fig. 8).

The results for the relationship between a mutation's location on either chromosome and change in relative fitness also proved negligible. Both chromosome one and chromosome two had no correlation between number of mutations and change in relative fitness, represented by insignificant r^2 values, 0.001 and 0.016 respectively (Fig. 9, Fig. 10). After sorting the individual mutations by exactly where they occur in the replication timing of the organism, we, again, observed no relationship (Fig. 11).

Discussion:

A large misconception in both the general and scientific communities regarding mutation is that the majority of mutations are beneficial. However, here, we corroborate previous findings that the vast majority of mutations have nearly neutral or slightly deleterious effects (Keightley and Lynch, 2003; Kibota and Lynch, 1996). This is evident, first, in the overall decline in relative fitness observed after 5187 generations in the near-absence of natural selection. Previous studies have found a similar pattern of decline in average relative fitness in a variety of organisms, from yeast (Dickinson, 2007) to *Daphnia* (Schaacketal et al., 2013) to *E. coli* (Kibota

and Lynch, 1996). The 2.3% average relative fitness decline that we observe here supports this idea that most mutations are deleterious, rather than beneficial.

Observing the effects of individual mutations on an organism's relative fitness contributes further support that most mutations are neutral or slightly deleterious. We observed a less than 1% change in relative fitness for the large majority of individual mutations. Nearly 75% of the mutations harbored within the eleven tested lines had less than 1% effect on the change in relative fitness of the organism. We can thus conclude from this data that the majority of mutations are slightly deleterious or neutral, as has been hypothesized using theoretical modeling and quantitative-genetics (Keightley and Lynch, 2003).

The data that we have collected regarding relative fitness and specific types and locations of mutations is strikingly insignificant. We would expect to see a steep decline in relative fitness of strains containing more genic insertions/deletions than those with more substitutions, as previous work has shown that insertions and deletions tend to be more detrimental to the organism than other types of mutations (Eyre-Walker and Keightley, 2007). This is because frameshifts can cause changes to a number of proteins that are coded by that region, whereas synonymous mutations cause no changes, and non-synonymous mutations cause a single protein to be coded differently. However, here we observe no relationship between the change in relative fitness and increased number of insertions/deletions, non-synonymous mutations, or synonymous mutations. The data between mutations of the coding region and those of the non-coding region is also insignificant, and no concrete conclusion may be drawn from it.

One explanation for the lack of relationship observed between relative fitness and an increased number of mutations could be due to "driver" mutations. A "driver" mutation is a single mutation that causes a major change in relative fitness (McFarland et al., 2013). Combined

with the fact that most mutations have extremely minor effects, it is possible that the mutant with a single mutation has a "driver" mutation, while the mutant with nine mutations simply has nine nearly neutral mutations.

The idea of a "driver" mutation is a possibility, but even so, the skew between the relative fitness of the line with a single mutation and the line with nine mutations is extremely unexpected. It has previously been shown that random mutations acquired during these mutation accumulation experiments only slightly combine fitness effects, and that organisms may have a buffering mechanism that protects against additive effects of multiple mutations (Maisnier-Patin et al., 2005). However, a number of other studies have found that there is a near-linear relationship between number of mutations and relative fitness (Elena and Lenski, 1997; Kibota and Lynch, 1996) and that epistasis between deleterious mutations is minimal (Peters and Keightley, 2000). Others have concluded that fitness declines exponentially with the number of accumulated mutations (Dickinson, 2007).

It was also surprising that we observed no relationship between the location of the mutation, in terms of replication timing, and change in relative fitness. It has been shown that *V. fischeri* exhibits a higher evolutionary rate on the second chromosome than the first. This suggests that the second chromosome, which has less essential functions and is used less (Cooper et al., 2010), could have a higher rate of mutation. We would therefore predict to see a higher change in relative fitness for mutations in early replication on the more conserved and essential primary chromosome than those later in replication on the secondary chromosome. The fact that we do not see this is most likely due to insufficient data. Future studies should include a greater number of mutant strains in order to improve the significance of these results.

Our findings demonstrate that the majority of mutations are neutral or slightly deleterious, with a less than 1% decline in relative fitness in the *V. fischeri* system. Large changes in relative fitness may be due to "driver" mutations, which cause significant deviations in fitness compared to the near neutrality of the majority of mutations. All of the results regarding types and locations of mutations are not significant enough for us to make meaningful conclusions at this stage. Analysis of a greater number of mutation accumulation strains of *V. fischeri,* and in other systems such as *Vibrio cholerae* and *Burkholderia cenocepacia,* should result in an improved capacity to examine the relationship between mutational effects, type of mutation, and replication timing.

Mutation accumulation experiments, along with genetic sequencing and fitness measurements, significantly contribute to our understanding of the distribution of naturally occurring mutations. Such methods allow us to comprehend the overall fitness effects of mutations for a species in a particular environment (Eyre-Walker and Keightley, 2007). Ultimately, the results of this study contribute to our understanding of the natural mutation spectrum, as we have confirmed that the majority of naturally occurring mutations are neutral or slightly deleterious.

Here, no significant relationship between mutation location and change in relative fitness was observed. However, in congruence with a larger number of replicates, the results of this study should help to either strengthen or diminish the idea that regions of the genome that are more centrally important to the organisms survival are conserved (Cooper et al., 2010). This could potentially change how we view naturally occurring mutations, and therefore the processes of natural selection and evolution. It can illustrate that evolution is not actually accomplished via "random" mutations, thus putting another common misconception, regarding mutations, to rest.

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Figures:

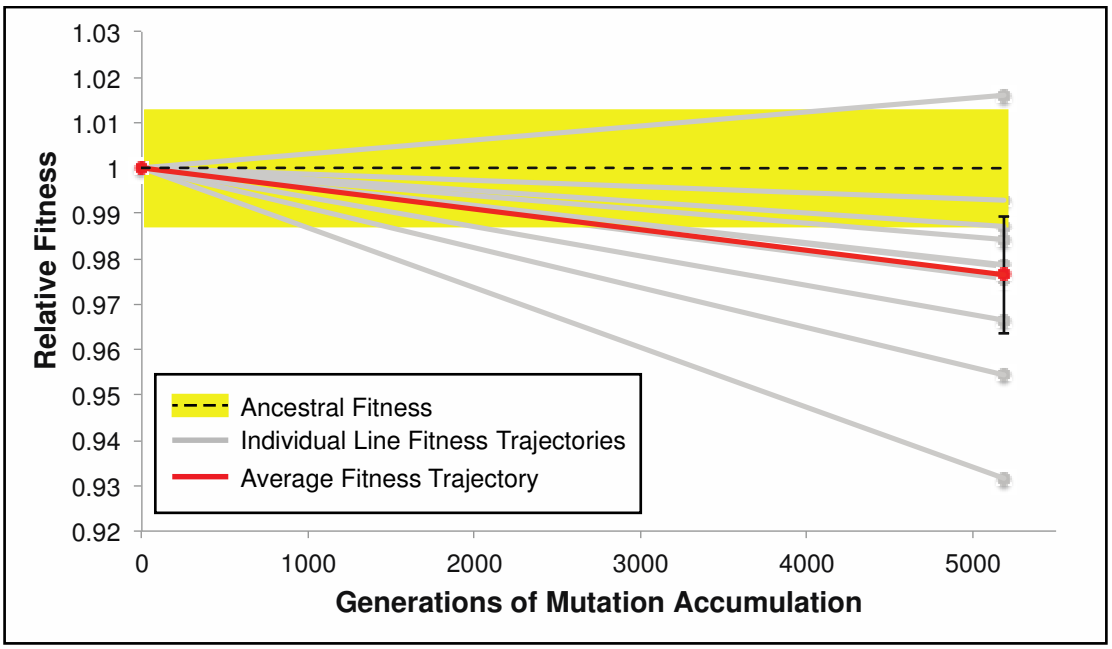

Figure 1: Fitness of each of the twelve *V. fischeri* mutant lines after 5187 generations. The yellow block symbolizes the ancestral fitness, and the red line represents the average decline in fitness observed.

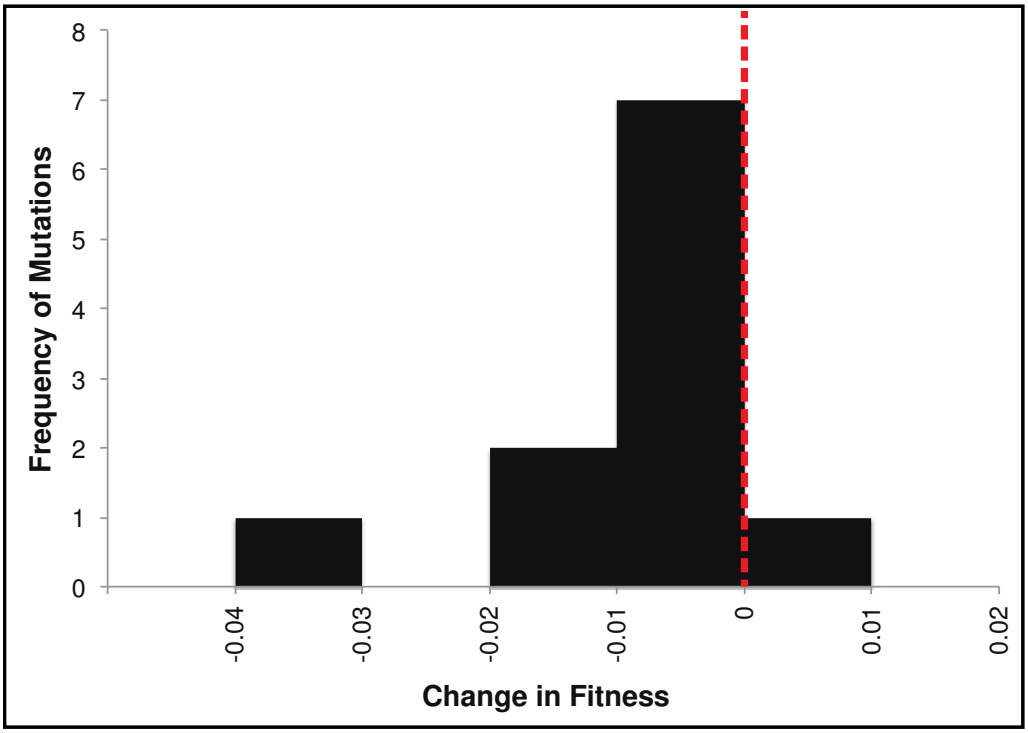

Figure 2: The frequency of mutations and their effects on the change in relative fitness of the organism.

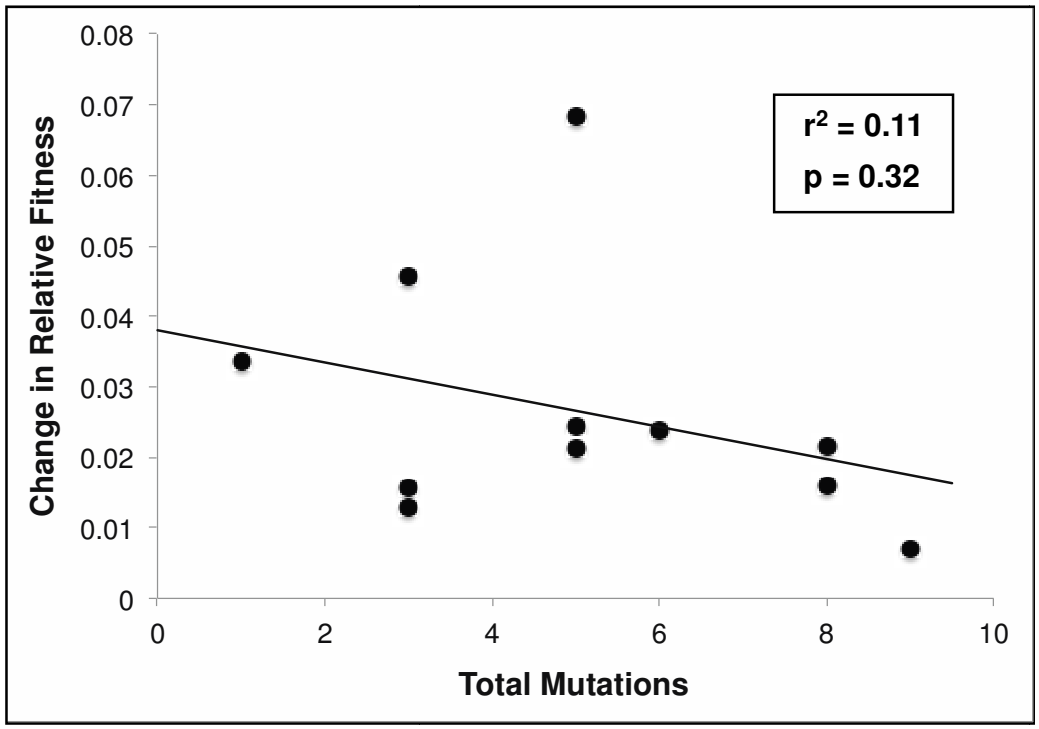

Figure 3: Effect of total number of mutations on change in relative fitness in *V. fischeri*.

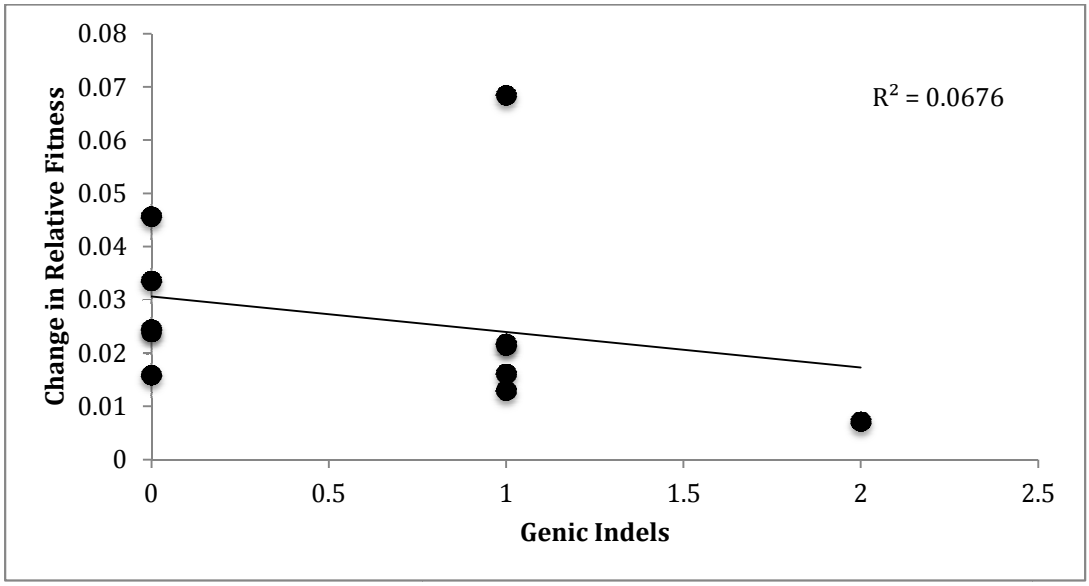

Figure 4: Impact of number of insertions/deletions in the coding region of the genome on change in relative fitness of the organism.

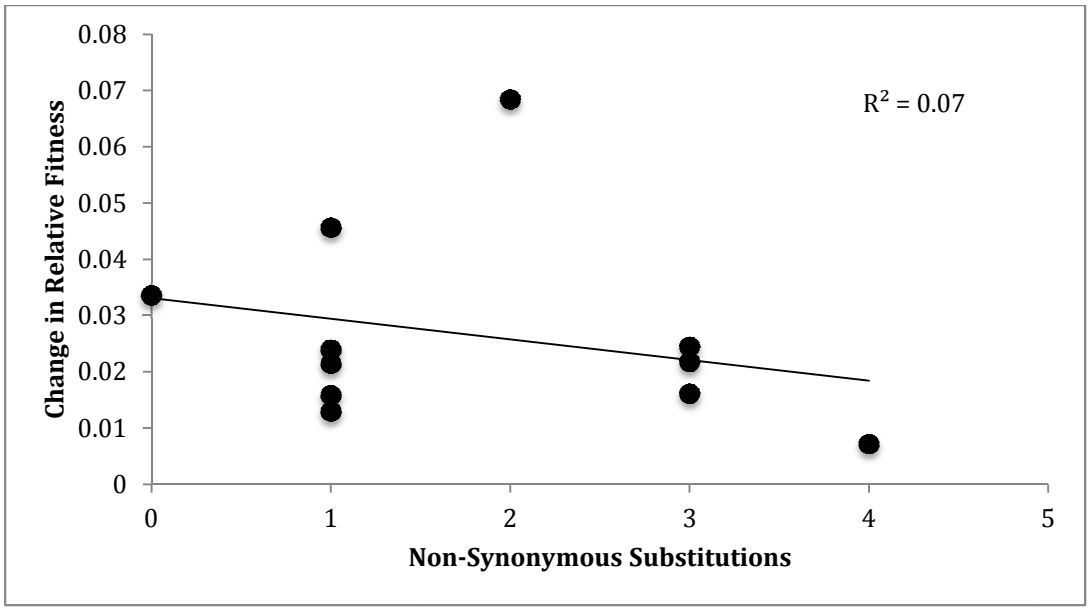

Figure 5: The number of non-synonymous substitutions in the coding region of the genome and the resulting change in relative fitness.

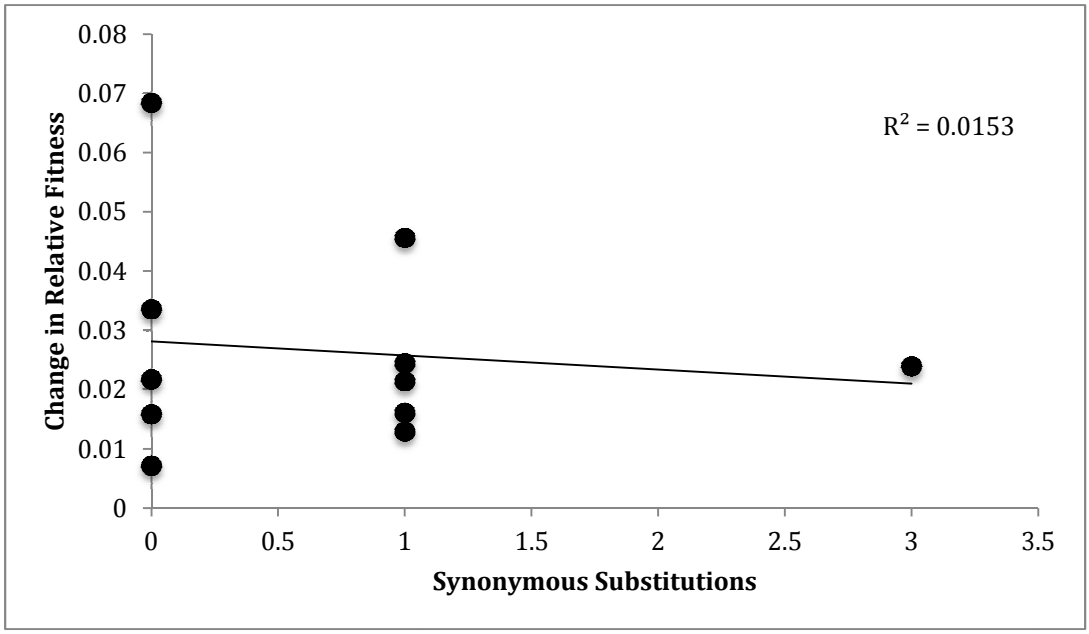

Figure 6: The number of synonymous mutations in the coding region of the genome and their impacts on change in relative fitness in the organism.

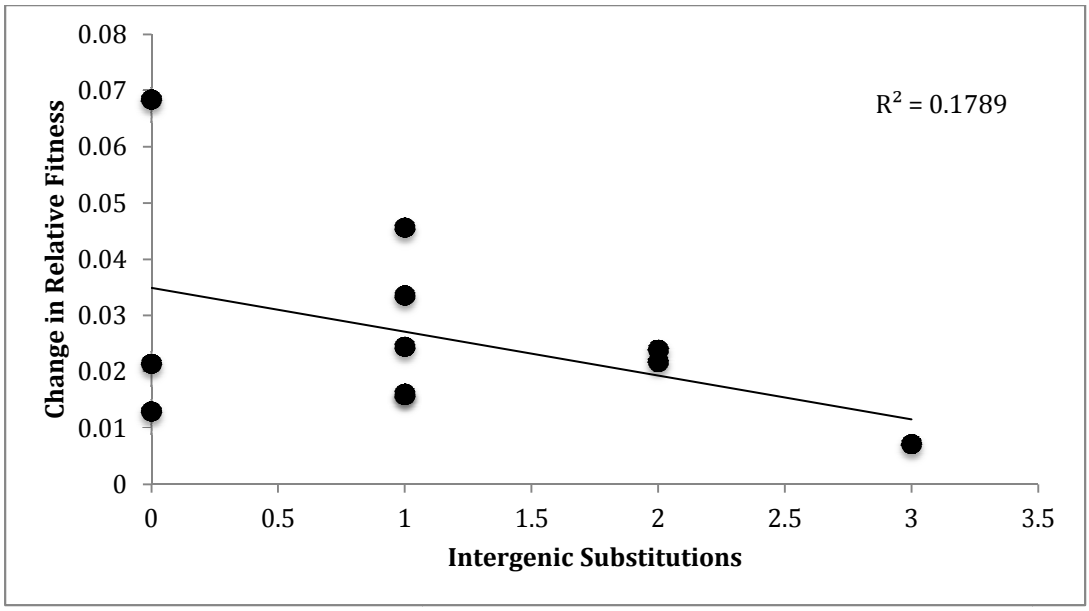

Figure 7: Effect of the number of substitutions in the non-coding region of the genome on the change in relative fitness of the organism.

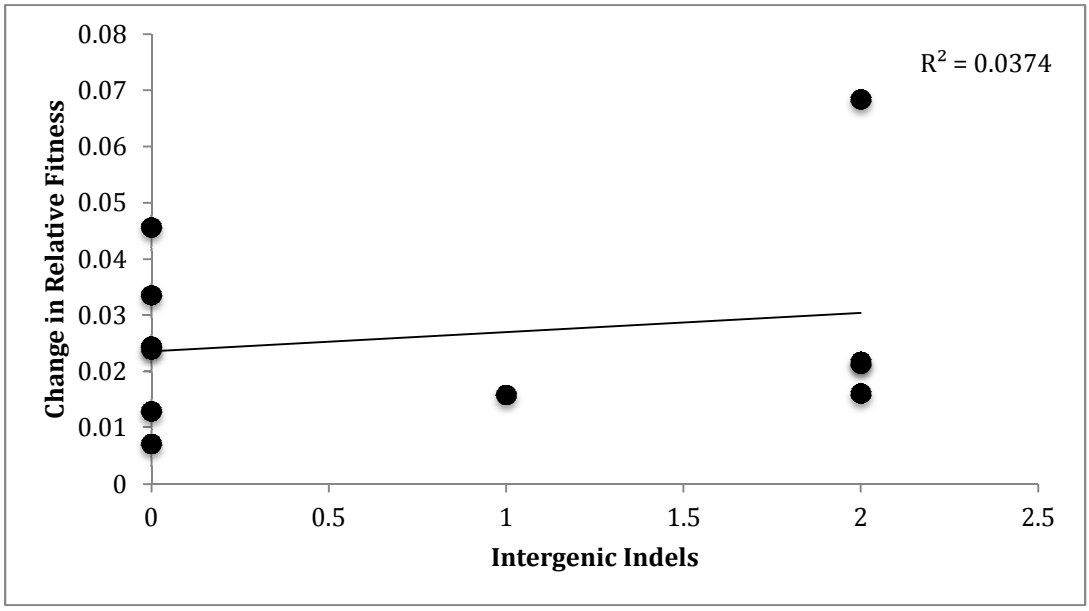

Figure 8: Effect of the number of insertions/deletions in the non-coding region of the genome on the change in relative fitness of the organism.

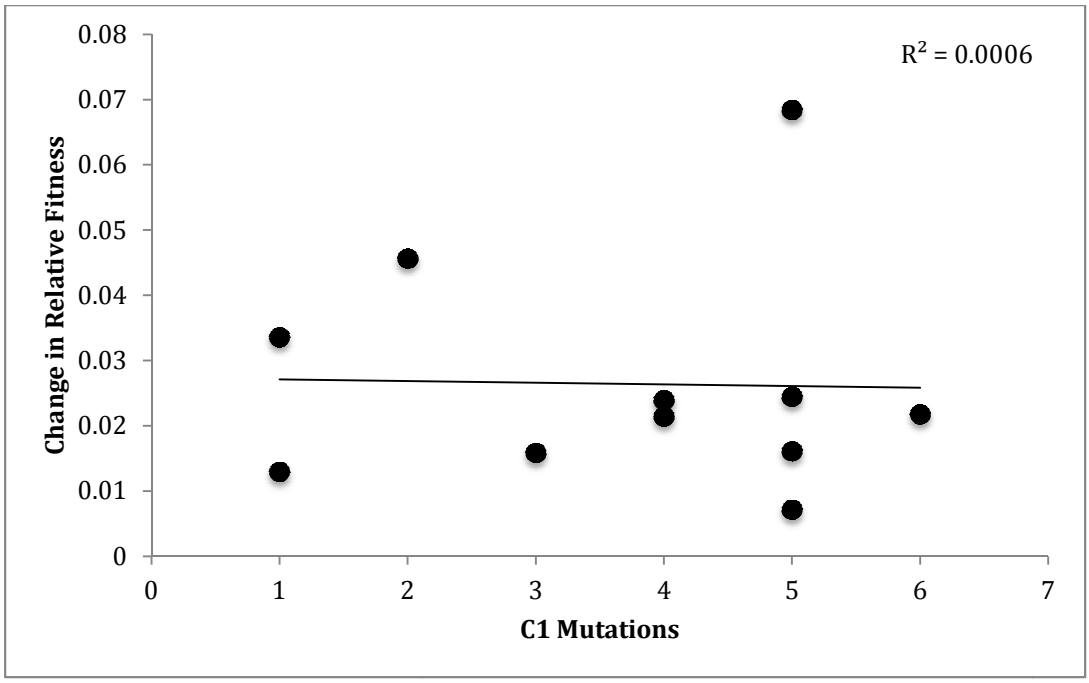

Figure 9: Effect of the number of mutations on chromosome one on the change in relative fitness.

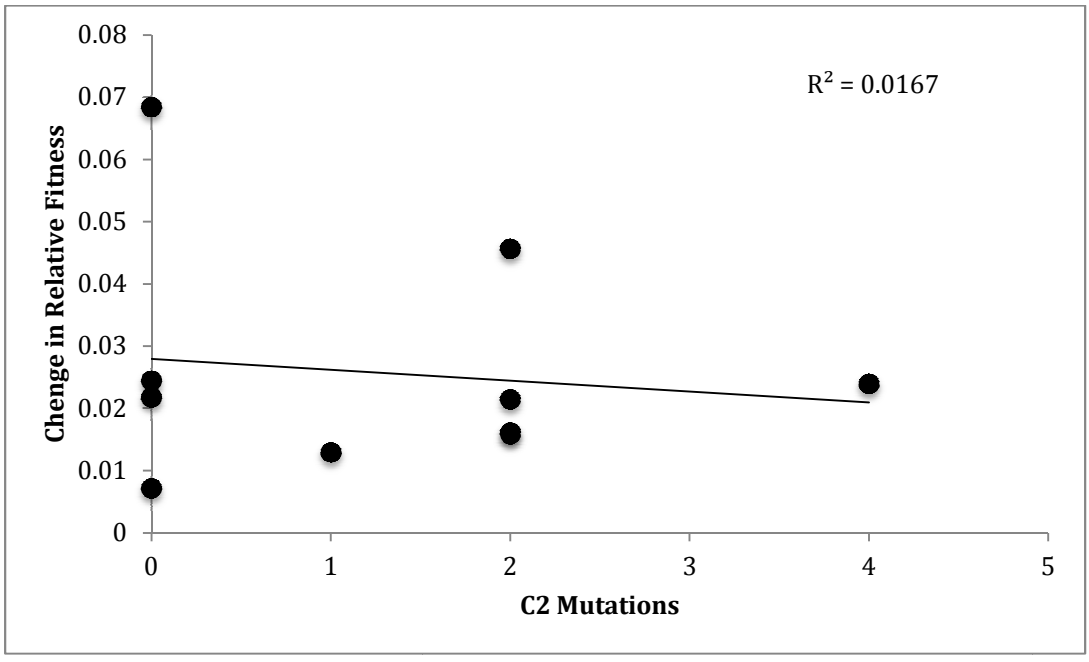

Figure 10: Effect of the number of mutations on chromosome two on the change in relative fitness.

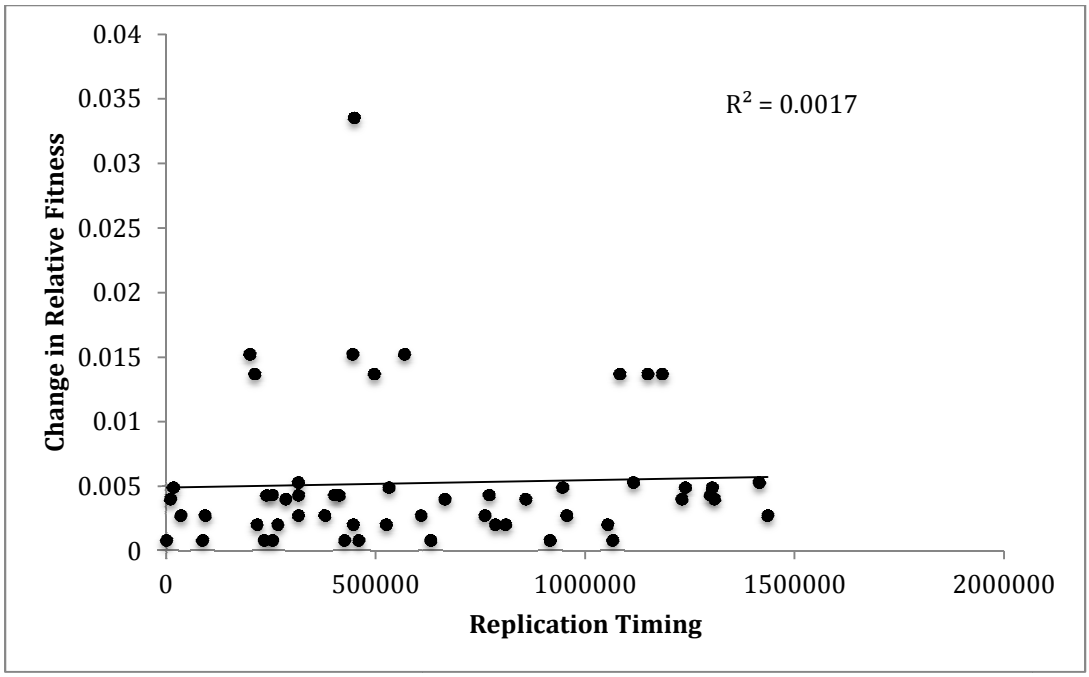

Figure 11: Location of mutations, in terms of replication timing of the organism, and the resulting change in relative fitness.