Extracting Characteristic Properties of Fitness Landscape from *In Vitro* Molecular Evolution: A Case Study on Infectivity of fd Phage to *E.coli*

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Notation

ν: Number of amino acid residues in a variable region to be targeted in the random mutagenesis

k: Mean number of sites that affect a certain site in a sequence

ε: Mean of site-fitness over λ letters, where $\lambda = 20$ in this article

σ: Standard deviation of site-fitness over λ letters, where $\lambda = 20$ in this article

W: Fitness of an amino acid sequence for an arbitrary individual

W_m: Fitness of the m-th fittest individual among N offsprings

$\mathscr{E}$: Mean of the fitness over all possible sequences, where $|\mathscr{E}|$ corresponds to the height of the fitness landscape

$\mathscr{V}$: Variance of the fitness over all possible sequences

O: Absolute fitness at the global peak.

N: Number of offsprings (=mutants) or library size of mutants to be screened in a single generation

d: Number of mutated sites, Hamming distance between a parent sequence and each of its offspring (=mutant) sequences

ζ: Expected value of the greatest among N random numbers from the standard Gaussian. A measure of selection pressure.

$\mathscr{N}(x|a,b)$: normal distribution of a variable x with the mean a and variance b

$E[x|a\ ]$: Conditional expectation of a variable x with a given a

$V[x|a\ ]$: Conditional variance of a variable x with a given a

$\mathscr{W}$: Fitness of a parent or fitness of an adaptive walker

$\mathscr{W}_t$**:** Fitness of the adaptive walker at the t-th generation, where "adaptive walker" at the t-th generation is identified with the fittest individual among N mutants at the t-th generation and becomes the parent at the $t+1$-th generation

$\Delta\mathscr{W}$**:** Change of $\mathscr{W}$ after a single generation, climbing rate of the adaptive walker

$\mathscr{W}^*$**:** Stationary value of $\mathscr{W}$ in the stationary state

ΔW**:** Fitness change from the fitness of a parent ($\mathscr{W}$) to the fitness of its arbitrary mutant (W): $\Delta W \equiv W - \mathscr{W}$

$\overline{d_{\rm eff}}$**:** Mean number of sites changing their site-fitnesses as a result of d-fold point mutations: $\overline{d_{\rm eff}} \approx d(1+k)$.

1 Abstract

We have developed a methodology for extracting characteristic properties of a fitness landscape of interest by analyzing fitness data on an *in vitro* molecular evolution. The *in vitro* evolution is required to be conducted as the following "adaptive walk": a single parent sequence generates N mutant sequences as its offsprings, and the fittest individual among the N offsprings will become a new parent in the next generation. N is the library size of mutants to be screened in a single generation. Our theory of the adaptive walk on the "NK landscape" suggests the following: The adaptive walker starting from a random sequence climbs the landscape easily in an early stage, and then reaches a stationary phase in which the mutation-selection-random drift balance sets in. The stationary fitness value is nearly proportional to $\sqrt{\ln N}$. Our analysis is performed from the following points: (1) stationary fitness values, (2) time series of fitness in the transitional state, (3) mutant's fitness distribution, and (4) the strength of selection pressure. Applying our methodology, we analyzed experimental data on the *in vitro* evolution of a random polypeptide (139 amino acids) toward acquiring infectivity (= ability to infect) of fd phage. As a result, we estimated that k is about 27 in this system, indicating that an arbitrary residue in a sequence is affected from other 23 % residues. In this article, we demonstrated that the experimental data is consistent with our theoretical equations quantitatively, and that our methodology for extracting characteristic properties of a fitness landscape may be effective.

2 Keywords

Fitness landscape; Sequence space; Adaptive walk; NK model; *In vitro* evolution

3 Introduction

In vitro molecular evolution can be considered as a hill-climbing or an adaptive walk on a fitness landscape in a sequence space (Wright,1931). "Fitness" is defined as a quantitative

measure of a certain physicochemical property of a biopolymer (i.e., thermostability or enzymatic activity) (Maynard-Smith, 1970; Eigen, 1985; Kauffman, 1993). The statistical properties of fitness landscapes are regarded as the "evolutionary attribute" of biopolymers. Therefore, exploring fitness landscapes and comprehending the statistical properties of the global and local landscapes is an important issue. A number of theoretical studies on the fitness landscape and adaptive walk on the landscape have been reported. Voigt *et al.* summarized these studies in their review (Voigt *et al.*, 2000). We have theoretically analyzed the statistical properties of the fitness landscape and adaptive walk, based on simple mathematical models of the Mt. Fuji-type landscape, and Kauffman's NK landscape. As a result of this study, we succeeded in deriving analytical solutions on the evolutionary dynamics on the fitness landscape (Aita & Husimi, 2000;Aita & Husimi, 2003; Aita *et al.*, 2004; Aita *et al.*, 2005).

In this article, we proposed a methodology for extracting characteristic properties of a fitness landscape of interest by analyzing the time series of fitness in an *in vitro* molecular evolution according to the $(1, N)$-evolution strategy (Rechenberg, 1984), which obeys the following rule: A single parent sequence generates N mutant sequences as its offsprings, and the fittest individual among the N offsprings will become the parent in the next generation. The evolution process according to this protocol is called the "adaptive walk" on the landscape. Our theory of the adaptive walk on the NK landscape expects the following: The adaptive walker starting from a random sequence climbs the landscape easily in an early stage, and then reaches a stationary phase in which the mutation-selection-random drift balance sets in. The stationary fitness value is nearly proportional to $\sqrt{\ln N}$. Our analysis is performed from the following points: (1) stationary fitness values, (2) time series of fitness in an ascending phase, (3) mutant's fitness distribution, and (4) the strength of selection pressure.

The NK landscape is a rugged mountainous landscape constructed by the NK model (Kauffman & Weinberger, 1989; Weinberger, 1991; Kauffman, 1993). The NK model is defined as a mathematical model for a complex system in which an arbitrary element is affected from other k elements. For a protein, an amino acid site corresponds to the

element in the NK model. The landscape with $k = 0$ has a single global peak and is called the "Mt. Fuji-type", whereas the landscape becomes more rugged with k increasing. The k-value is inherent in the physicochemical property of individual biopolymers. Kauffman and Weinberger applied the NK model to affinity maturation of the V region in immunoglobulin and estimated that k is about 40, from the number of steps of adaptive walk, up to the local optima (Kauffman & Weinberger, 1989; Kauffman, 1993). Kauffman & Macready analyzed the stepwise evolution of short peptides of 6 amino acid residues towards acquiring affinity to a receptor molecule, and estimated that k is about 3 (Kauffman & Macready,1995).

In the interim, we have been engaged in an *in vitro* evolution experiment to explore the possibility that arbitrary sequences can evolve towards acquiring a functional role (Yamauchi, *et al.*, 2002; Hayashi, *et al.*, 2003; Ito , *et al.*, 2004). One of our interests was the evolvability of infectivity (= ability to infect) of fd phage to *E.coli* (Hayashi, *et al.*, 2003). By replacing the D2 domain of the g3p minor coat protein of the fd-tet phage genome with the soluble random polypeptide RP3-42 of 139 amino acids, we made a defective phage, fd-RP, which yielded a seven-order magnitude lower infectivity than the wild-type fd-tet phage. A variable region of 119 amino acids in the RP3-42 was targeted by a random mutagenesis. Then, over 20 generations through iterative mutation and selection, we improved the infectivity up to 1.7×10^4-fold as compared with the original fd-RP (Hayashi, *et al.*, 2006).

The iterative process was performed according to the following: A single polypeptide as a parent sequence was subjected to a random mutagenesis by error-prone PCR, generating a population of mutant polypeptides (=offspring sequences). Subsequently, N mutant clones were arbitrarily chosen from the population and the clone with the highest infectivity was selected from among the N mutants. The selected clone became the parent in the next generation. We found that the infectivity increased rapidly in early generations but plateaued in later generations. Apparently, the stationary infectivity at the plateau was dependent on the library size of the mutants to be screened (N). We considered that this phenomenon was caused by the "mutation-selection-random drift balance". This is

where a convergence force by a selection event and a divergence force by a mutation event are balanced. In the latter half of this article, we estimated characteristic parameters of the infectivity landscape. As a result, we estimated that k is about 27 and found that the NK landscape model with $k = 27$ could explain all the experimental data quantitatively.

In our previous paper (Hayashi, *et al.*, 2006), we summarized our methodology of the landscape analysis and applied simplified equations to analyze the same experimental data as those shown in this paper, and determined $k = 21$. Although there is a slight discrepancy between the two estimated k-values ($k = 27$ and $k = 21$), the semi-quantitative description of the landscape structure presented in Hayashi *et al.* is guaranteed. In this article, describing the details of the theory and methodology and employing more sophisticated equations, we demonstrated that the experimental data was consistent with the equations quantitatively, and that our methodology for extracting characteristic properties of a fitness landscape was effective.

4 Theory of the adaptive walk on a fitness landscape

4.1 Model of the NK fitness landscape and adaptive walk

We consider all conceivable amino acid sequences with the chain length of ν, where λ ($= 20$) naturally occurring amino acids are available letters at every site. Then, each sequence is mapped into the corresponding point in the λ-valued ν-dimensional sequence space. The fitness W for a given sequence "$\alpha_1\alpha_2\cdots\alpha_\nu$" is defined by

$$W = \sum_{j=1}^{\nu} w_j(\alpha_j|\alpha_{j_1}, \alpha_{j_2}, \cdots, \alpha_{j_k}), \tag{1}$$

where $w_j(\alpha_j|\alpha_{j_1}, \alpha_{j_2}, \cdots, \alpha_{j_k})$ is the "site-fitness", *i.e.*, a fitness contribution from a particular letter α_j at the jth site when the k sites $\{j_1, j_2, \cdots, j_k\}$ are occupied by the particular letters $\{\alpha_{j_1}, \alpha_{j_2}, \cdots, \alpha_{j_k}\}$. The k sites $\{j_1, j_2, \cdots, j_k\}$ are randomly chosen from all $\nu - 1$ sites except the j-th site. The assignment of site-fitness values is modeled as follows: With a set of letters $\{\alpha_{j_1}, \alpha_{j_2}, \cdots, \alpha_{j_k}\}$ given, the site-fitness for an arbitrary letter a (e.g., a =Ala,Cys,$\cdots$,Tyr) at each site is randomly assigned from the following set of λ

values, but the degeneracy of assignment is not allowed:

$$w_j(a|\alpha_{j_1},\alpha_{j_2},\cdots,\alpha_{j_k}) \in \left\{ \frac{2\varepsilon}{\lambda-1}\, h \middle| h = 0,1,2,\cdots,\lambda-1 \right\} \tag{2}$$

under the condition that $w_j(a|\cdots) \neq w_j(a'|\cdots)$ for $a \neq a'$. ε (≤ 0) is a negative constant equivalent to the mean of the site-fitness over all available letters. We note that there is no significant effect on the theoretical conclusion when the ε-value is different by sites (Aita & Husimi, 2004). Since the site-fitness distribution is according to the comb-type function, then the variance of the site-fitness, denoted by σ^2, is related with ε by

$$\sigma^2 \equiv \frac{1}{\lambda}\sum_{i=0}^{\lambda-1}(w_j(a_i|\alpha_{j_1},\alpha_{j_2},\cdots,\alpha_{j_k})-\varepsilon)^2 \approx \frac{\varepsilon^2}{3}. \tag{3}$$

The fitness landscape resulting from this model is called the "NK landscape", although the original NK landscape was defined in the binary sequence space (Kauffman, 1993). In the case of $k = 0$, the fitness landscape is called the "Mt. Fuji-type" with a single global peak. As the k-value increases, the surface on the fitness landscape becomes more rugged.

The probability density of fitness W over all possible sequences is approximately given by the following normal distribution with the mean $\mathscr{E}$ and variance $\mathscr{V}$: $\mathscr{N}(W|\mathscr{E},\mathscr{V})$, where

$$\mathscr{E} = \varepsilon\,\nu, \tag{4}$$

$$\mathscr{V} = \sigma^2\,\nu \approx \frac{\varepsilon^2\nu}{3}. \tag{5}$$

$\mathscr{E}$ is equivalent to the expectation of the fitness of a randomly generated sequence, and then corresponds to the fitness at the foot of the landscape (while, region where $W < \mathscr{E}$ corresponds to an undersea and is negligible for the adaptive walk). Since the fitness at the global peak takes about zero (this is not necessarily guaranteed), then $|\mathscr{E}|$ corresponds to the height of the landscape.

We adopted the cloning-screening type evolution protocol as a rule of the adaptive walk: a single parent generates N offsprings (=mutants) in the t-th generation, and subsequently, the fittest individual among the N offsprings will become a new parent

in the $t+1$-th generation. That is, N is the "library size" of mutants to be screened in a single generation. The number of mutated sites is a constant d, *i.e.*, the Hamming distance between a parent and each of its offsprings is d. An "adaptive walker" is a general term for these parents over all the generations.

In the NK model mentioned above, an arbitrary single-point mutation causes the changes in site-fitness at about $1+k$ sites, that include both of the mutated site and other k sites affected by the mutation. That is, the values of w_j's at about $1+k$ sites are randomly re-assigned from eqn. (2) as a result of the mutation. Random d-fold point mutations cause the changes in site-fitness at about $d(1+k)$ sites. Then, we introduced the "effective number of mutations", $d_{\rm eff}$, as the number of sites changing their site-fitnesses. Each mutant takes a stochastic value of $d_{\rm eff}$, but we focus on the expectation of $d_{\rm eff}$, which is given by

$$\overline{d_{\rm eff}} \approx \nu - (\nu - d)\left(1 - \frac{k}{\nu - 1}\right)^d \tag{6}$$

$$\approx d(1+k) \qquad \text{for } d \ll \nu. \tag{7}$$

The derivation of eqns (6) and (7) is shown in Appendix A. Fig.1 shows the expectation and standard deviation of $d_{\rm eff}$ as a function of d. The expectation, $\overline{d_{\rm eff}}$, is one of the important parameters in the theory described below.

4.2 Evolutionary dynamics on the NK fitness landscape

We consider a "typical" adaptive walk with fixed d and N values on various NK landscapes realized with fixed ν and k values, instead of a particular realization of the landscape, and we describe the movement of a walker on the one-dimensional fitness coordinate. One of our remarkable conclusions is that the movement is mainly governed by the walker's fitness as a single variable, and is not explicitly dependent on the details of its sequence. That is, walkers that have different sequences but have the same fitness can be treated as the same walker in our theory. The walker starting from the foot of the landscape climbs rapidly and then reduces its climbing rate, and finally reaches a stationary state which occurs in the specific region of the "mutation-selection-random drift balance".

First, we will begin by considering the set of all sequences at distance d from a parent sequence in the sequence space. That is, the set consists of all conceivable d-fold point mutants generated from the parent sequence. According to Fontana and Schuster (1998), we call the set "d-boundary" of the parent sequence. The size of the d-boundary is $\binom{\nu}{d}(\lambda-1)^d$. Let $\mathscr{W}$ be the fitness of the parent, and ΔW be the change between $\mathscr{W}$ and a fitness W of its arbitrary mutant in the d-boundary ($\Delta W = W - \mathscr{W}$). Let $\psi_d(\Delta W|\mathscr{W})$ be the probability density of ΔW over the d-boundary, when $\mathscr{W}$ is fixed. $\psi_d(\Delta W|\mathscr{W})$ is approximately given by the following normal distribution:

$$\psi_d(\Delta W|\mathscr{W}) \approx \begin{cases} \mathcal{N}\left(\Delta W|E[\Delta W|\mathscr{W}], V[\Delta W|\mathscr{W}]\right) & \text{for } (\Delta W)_{\mathrm{l}} \leq \Delta W \leq (\Delta W)_{\mathrm{u}}, \\ 0 & \text{otherwise,} \end{cases} , \quad (8)$$

where $E[\Delta W|\mathscr{W}]$ and $V[\Delta W|\mathscr{W}]$ are the conditional expectation and variance of ΔW over the d-boundary. These are respectively given as follows:

$$E[\Delta W|\mathscr{W}] = -(\mathscr{W} - \mathscr{E})\frac{\overline{d_{\mathrm{eff}}}}{\nu}, \quad (9)$$

$$V[\Delta W|\mathscr{W}] \approx \left(\mathscr{V} + \frac{\mathscr{W}^2}{3\nu}\right)\frac{\overline{d_{\mathrm{eff}}}}{\nu} \qquad \text{for } \overline{d_{\mathrm{eff}}} \ll \nu, \quad (10)$$

where $\mathscr{E}$ and $\mathscr{V}$ are the mean and variance of fitness over all possible sequences in the sequence space, respectively (eqn.(4) and (5)). $(\Delta W)_{\mathrm{l}}$ and $(\Delta W)_{\mathrm{u}}$ are the lower bound and upper bound, respectively. In almost cases,

$$\frac{(\Delta W)_{\mathrm{u}} - E[\Delta W|\mathscr{W}]}{\sqrt{V[\Delta W|\mathscr{W}]}} \gg 2, \frac{E[\Delta W|\mathscr{W}] - (\Delta W)_{\mathrm{l}}}{\sqrt{V[\Delta W|\mathscr{W}]}} \gg 2,$$

then the truncation is small and then negligible, actually. The full derivation of $E[\Delta W|\mathscr{W}]$ and $V[\Delta W|\mathscr{W}]$ is described in Appendix A in Aita & Husimi,2003.

Consider that N mutants are randomly chosen from the d-boundary and the fittest mutant with the highest fitness among the N mutants becomes the new parent. In terms of statistics, the d-boundary and randomly chosen N mutants correspond to the "population" and "sample", respectively. Let $\Delta\mathscr{W}$ be the change in fitness from the parent to a new parent after a single generation, that is the "climbing rate" of the walker. Let $\varphi(\Delta\mathscr{W}|\mathscr{W})$ be the probability density of $\Delta\mathscr{W}$ when the (old) parent sequence has the fitness $\mathscr{W}$. By using eqn.(8), $\varphi(\Delta\mathscr{W}|\mathscr{W})$ is given by

$$\varphi(\Delta\mathscr{W}|\mathscr{W}) = N\,\psi_d(\Delta\mathscr{W}|\mathscr{W})\left(\int_{-\infty}^{\Delta\mathscr{W}} \psi_d(\Delta W|\mathscr{W})\,\mathrm{d}\Delta W\right)^{N-1}. \quad (11)$$

The expectation and variance of $\Delta\mathscr{W}$ based on eqn.(11) are respectively given by

$$E[\Delta\mathscr{W}|\mathscr{W}] = E[\Delta W|\mathscr{W}] + \sqrt{V[\Delta W|\mathscr{W}]} \times \zeta \qquad (0 \leq \zeta \leq \zeta_{\max}), \tag{12}$$

$$V[\Delta\mathscr{W}|\mathscr{W}] \approx \frac{V[\Delta W|\mathscr{W}]}{\zeta^2 + 1}, \tag{13}$$

where ζ is defined as follows. Consider that N random numbers are chosen according to the standard Gaussian probability density $\mathscr{N}(x|0,1)$, and let x_1 be the greatest number among the N numbers. We denote the expectation of x_1 by $\zeta\,(\equiv E[x_1])$ or $\zeta(N)$ as a function of N. ζ is approximately given by transforming N via:

$$\zeta \, \exp(\zeta^2/2) \int_{-\infty}^{\zeta} \exp(-s^2/2)\mathrm{d}s = N - 1. \tag{14}$$

Numerical values of ζ calculated by eqn.(14) are plotted as a function of N in Fig.2. Since the number of all the conceivable d-fold point mutants is $\binom{\nu}{d}(\lambda-1)^d$, then ζ has an upper limit:

$$\zeta_{\max} = \zeta\left(\binom{\nu}{d}(\lambda-1)^d\right). \tag{15}$$

In eqn. (12), ζ governs the climbing rate, then we designate ζ as a measure of "selection pressure". When $N = 1$, $\zeta = 0$ and then $E[\Delta\mathscr{W}|\mathscr{W}] = E[\Delta W|\mathscr{W}]$. This does not cause adaptive walk, but random walk under no selection pressure. When N takes a large value, ζ is approximately given by the following explicit form:

$$\zeta \approx \sqrt{2\ln\frac{N}{\sqrt{2\pi}}}. \tag{16}$$

The derivation of equations (12)-(14) is described in Appendix B.

Substituting eqn.(9) and (10) into eqn.(12), we can see that eqn.(12) is the linerly decreasing function of $\mathscr{W}$. As the walker climbs the landscape, the climbing rate decreases gradually and finally the walker reaches the stationary state at $\mathscr{W} = \mathscr{W}^*$, where $\mathscr{W}^*$ is defined as a specific $\mathscr{W}$-value satisfying $E[\Delta\mathscr{W}|\mathscr{W}^*] = 0$. By substituting $E[\Delta\mathscr{W}|\mathscr{W}] = 0$ into eqn.(12) and solving the resulting equation, the stationary value $\mathscr{W}^*$ is approximately determined as follows:

$$\mathscr{W}^* = \mathscr{E} + \kappa^{-1}\zeta\sqrt{\frac{2\mathscr{V}\,\nu}{d_{\mathrm{eff}}}} \tag{17}$$

$$\approx \mathscr{E} + \zeta\sqrt{\frac{2\mathscr{V}\,\nu}{d_{\mathrm{eff}}}} \qquad \text{for } \sqrt{d_{\mathrm{eff}}}/\zeta \geq 1. \tag{18}$$

κ in eqn.(17) is a correction factor defined by $\kappa \equiv (1+\sqrt{6\tau^2-1})/\sqrt{6}\tau$ with $\tau \equiv \sqrt{\overline{d_{\rm eff}}}/\zeta$. κ takes values in the following range: $1 < \kappa \leq \sqrt{2}$. In this article, we postulate the condition of $\sqrt{\overline{d_{\rm eff}}}/\zeta \geq 1$ and adopt eqn.(18). Using the stationary fitness $\mathscr{W}^*$, the expected climbing rate (eqn.(12)) is approximately rewritten as follows:

$$E[\Delta\mathscr{W}|\mathscr{W}] \approx -(\mathscr{W}-\mathscr{W}^*)\frac{\overline{d_{\rm eff}}}{\nu}. \tag{19}$$

We consider an adaptive walk from an initial fitness $\mathscr{W} = \mathscr{W}_0$ up to the stationary state $\mathscr{W} = \mathscr{W}^*$. Let $\mathscr{W}_t$ be the fitness of a parent sequence at each generation t. The probability density of $\mathscr{W}_t$ is described by the following the path integral:

$$P(\mathscr{W}_t) = \int_{-\infty}^{0}\int_{-\infty}^{0}\cdots\int_{-\infty}^{0}\prod_{t'=1}^{t}\varphi(\mathscr{W}_{t'}-\mathscr{W}_{t'-1}|\mathscr{W}_{t'-1})\,{\rm d}\mathscr{W}_1{\rm d}\mathscr{W}_2\cdots{\rm d}\mathscr{W}_{t-1}. \tag{20}$$

By approximating eqn.(11) as a normal distribution with the mean and variance given by eqn.(12) and eqn.(13), respectively, the path integral in eqn.(20) can be performed by the mathematical induction. The expectation and variance of $\mathscr{W}_t$ is respectively given by

$$E[\mathscr{W}_t] \approx \mathscr{W}_0 + \left(1-\left(1-\frac{\overline{d_{\rm eff}}}{\nu}\right)^t\right)(\mathscr{W}^*-\mathscr{W}_0), \tag{21}$$

$$V[\mathscr{W}_t] \approx \frac{1-(1-\overline{d_{\rm eff}}/\nu)^{2t}}{1-(1-\overline{d_{\rm eff}}/\nu)^2}\times\frac{2\sigma^2\overline{d_{\rm eff}}}{\zeta^2}. \tag{22}$$

Eqn.(21) tells that the ascending time is dependent only on $\overline{d_{\rm eff}}/\nu$. Thus, when $\overline{d_{\rm eff}}$ takes a large value (this is caused when d or k takes large values), the ascending time is short and then the walker reaches the stationary state in early stage.

In *in vitro* evolution experiments, the mutagenesis is frequently carried out by error-prone PCR. Consider that, in the mutagenesis process, a residue substitution occurs with the probability of μ for each site. μ is called the "mutation rate". Then, the mole fraction of the d-fold point mutants generated from a parent sequence obeys binomial distribution:

$$\binom{\nu}{d}\mu^d(1-\mu)^{\nu-d}. \tag{23}$$

The mean of d is given by $\mu\nu$ and is denoted by $\overline{d}$: $\overline{d} = \mu\nu$. Let ΔW be the change in fitness from a parent with the fitness $\mathscr{W}$ to its arbitrary mutant. By using eqn.(8), the probability density of ΔW is described by

$$\widetilde{\psi}(\Delta W|\mathscr{W}) = \sum_{d=0}^{\nu}\binom{\nu}{d}\mu^d(1-\mu)^{\nu-d}\ \psi_d(\Delta W|\mathscr{W}). \tag{24}$$

The expectation of ΔW based on eqn.(24) is equal to eqn.(9) with $d = \overline{d}$, while the variance of ΔW is given by

$$V[\Delta W|\mathscr{W}] \approx \left(\mathscr{V} + \frac{\mathscr{W}^2}{3\nu} + \frac{(\mathscr{W} - \mathscr{E})^2}{\nu} \right) \frac{\overline{d_{\rm eff}}}{\nu} \qquad \text{for } \mu \ll 1 \tag{25}$$

with $d = \overline{d}$. The probability density of the climbing rate, $\Delta\mathscr{W}$, is given by replacing $\psi_d(\Delta W|\mathscr{W})$ in eqn.(11) by $\widetilde{\psi}(\Delta W|\mathscr{W})$ shown in eqn.(24). As a result, equations (18), (19) and (21) can be applied to this case by substituting d with $\overline{d} = \mu\nu$, as an approximation.

If the noise in the fitness measurements is not negligible, the following modification should be added. Consider that a random noise obeying Gaussian density with standard deviation ϱ is added to the fitness values. The fitness measurements with large noise is likely to select the second- or third- or m-th fittest mutant as the "pseudo-fittest" instead of the fittest mutant. In this case, with $R \equiv \varrho/\sqrt{V[\Delta W|\mathscr{W}]}$, the effective quantity of ζ,

$$\zeta_{\rm eff} \equiv \zeta \times \sqrt{(1 + R^2)^{-1}}, \tag{26}$$

should be applied, or the effective quantity of N,

$$N_{\rm eff} \equiv \sqrt{2\pi} \left(\frac{N}{\sqrt{2\pi}} \right)^{\sqrt{(1+R^2)^{-1}}}, \tag{27}$$

should be applied. The effect shown in eqn.(26) is easily derived from eqn.(33) in Aita and Husimi, (2000), and eqn.(27) is derived from eqn.(26) with eqn.(16).

4.3 Estimating characteristic parameters for a real fitness landscape through adaptive walk process

For handling of the realistic data in our fitness model, we add a constant term O to the fitness model (eqn.(1)) as follows:

$$W = \sum_{j=1}^{\nu} w_j(\alpha_j|\alpha_{j_1}, \alpha_{j_2}, \cdots, \alpha_{j_k}) + O. \tag{28}$$

Since the first term in the right-hand side in eqn.(28) takes about zero for the globally optimal sequence as the global peak (this is not necessarily guaranteed), the second term O is determined as the absolute fitness for the global peak. Known parameters are ν, d (or $\overline{d}$) and N, while unknown parameters yet to be estimated are ε, σ, O, $\mathscr{E}$, $\mathscr{V}$ and k.

Suppose that we have experimental data on several time series of fitness, $\{\mathscr{W}_{\mathrm{obs},t}|t = 0, 1, 2, \cdots\}$ and data on the stationary fitness observed, $\mathscr{W}^*_{\mathrm{obs}}$, through the adaptive walks with different N-values (e.g. $N_1 = 10, N_2 = 10^2, N_3 = 10^3, \cdots$) (Fig.3a). Note that N should take values less than $\binom{\nu}{d}(\lambda - 1)^d$. If the initial sequence as a starting point is a randomly generated one, the initial fitness $\mathscr{W}_{\mathrm{obs},0}$ is regarded as $\mathscr{W}^*_{\mathrm{obs}}$ for $N = 1$. The reason is that an initial fitness $\mathscr{W}_0$ is expected to take a value close to $\mathscr{E}$, that corresponds to the stationary fitness $\mathscr{W}^*$ for $N = 1$ ($\zeta = 0$).

First, plot the $\mathscr{W}^*_{\mathrm{obs}}$-values against their respective ζ-values. That is, $\{(\zeta(N), \mathscr{W}^*_{\mathrm{obs}}(N))|N = 1, N_1, N_2, N_3, \cdots\}$ is plotted into the X-Y plane (Fig.3b). Then, get the regression line for this plots:

$$\mathscr{W}^* = a + b \times \zeta. \tag{29}$$

The $\mathscr{W}^*$-value obtained by interpolation from the regression line is regarded as an estimate of the true value for the stationary fitness. Comparing eqn.(29) with eqn.(18) (a constant O is added to the right-hand side), we can obtain the following condition:

$$O + \mathscr{E} = a \tag{30}$$

$$\frac{\mathscr{V}}{\overline{d_{\mathrm{eff}}}/\nu} = \frac{b^2}{2}. \tag{31}$$

Second, the time series of fitness, $\{\mathscr{W}_{\mathrm{obs},t}|t = 0, 1, 2, \cdots\}$, is fitted to the following regression curve:

$$\mathscr{W}_t = e + \left(1 - c^t\right)(\mathscr{W}^* - e), \tag{32}$$

where $\mathscr{W}^*$ is not the observed value but the estimated value via eqn.(29), on the reason that the observed value is likely to contain a considerable fluctuation or measurement error. The parameter c governs the ascending time of the regression curve. Identifying eqn.(32) with eqn.(21), we can obtain the following condition:

$$\frac{\overline{d_{\mathrm{eff}}}}{\nu} = 1 - c. \tag{33}$$

Solving simultaneous equations (4), (5), (6), (30), (31) and (33), we can estimate characteristic parameters for the fitness landscape as follows.

$$\overline{d_{\mathrm{eff}}} = (1 - c)\nu, \tag{34}$$

$$k = (\nu - 1)\left(1 - \left(\frac{\nu - \overline{d_{\rm eff}}}{\nu - d}\right)^{1/d}\right), \tag{35}$$

$$\mathscr{V} = \frac{b^2(1-c)}{2}, \tag{36}$$

$$\varepsilon = -\sqrt{\frac{3\mathscr{V}}{\nu}}, \tag{37}$$

$$\sigma = \sqrt{\frac{\mathscr{V}}{\nu}}, \tag{38}$$

$$\mathscr{E} = -\sqrt{3\mathscr{V}\nu}, \tag{39}$$

$$O = a + \sqrt{3\mathscr{V}\nu}. \tag{40}$$

We conducted verification of the effectiveness of our methodlogy as follows: initially, a NK landscape according to eqn.(1) with a given k-value (other parameters were fixed: $\nu = 120$, $\lambda = 20$, $\varepsilon = -1$) was realized in silico, and adaptive walk simulation that started from a randomly generated sequence was performed for each N-value of $N_1 = 10$, $N_2 = 10^2$, $N_3 = 10^3$ and $N_4 = 10^4$, with $d = 2$; next, regarding a period from the 20-th to the 40-th generation as the stationary phase, we estimated values of k and ε through the procedure mentioned above. Table 1 shows a comparison between the estimated values and true values for k and ε. We can see that the estimated values almost agree with the true values and confirm that our methodlogy is effective. The discrepancy observed for $k = 40$ suggests that the estimated values are more discrepant as $(k+1)d$ is closer to the chain length ν.

5 Analysis of the experimental data on the *in vitro* evolution for infectivity of fd phage

5.1 Correspondence between the *in vitro* evolution system and our theoretical model

To begin analysis of the experimental data of the *in vitro* evolution conducted by Hayashi *et al.* (Hayashi *et al.*, 2006), we take a correspondence of variables and parameters between the *in vitro* evolution system and our theoretical model. The important variables and

parameters are as follows: the number of amino acid residues in the variable region (ν), definition of fitness (W), average number of sites mutated in a single mutagenesis process ($\overline{d}$), and library size of mutants to be screened (N).

The polypeptide to be targeted for *in vitro* evolution was a random polypeptide RP3-42, which has 139 amino acid residues and was an artificial substituent for the D2 domain of the g3p minor coat protein of the fd-tet phage. The evolving polypeptide includes 119 amino acid residues as a variable region (the 12th-130th sites in 139 sites), which were subjected to random mutagenesis through error-prone PCR. Thus, $\nu = 119$ in this case.

The polypeptide as a substituent for the D2 domain, contributes to the infectivity of the corresponding mutant phage (that possesses the polypeptide) into *E.coli* host cells. The *in vitro* evolution of the targeted polypeptide was driven toward improving the infectivity through evaluating the CFU values, where CFU (=Colony Forming Unit) indicates the number of infected *E.coli* host cells per unit titer of phage suspension. That is, the CFU values reflect on the rate constant of phage propagation. Some experimental studies showed exponential increase of RNA virus in a given environment (Novella,*et al.*,1995;Novella,*et al.*,1999). Under the assumption that CFU is proportional to $\exp(-\Delta G/k_{\rm B}T)$, where ΔG is the apparent free energy in the infection process, we defined the fitness W of the evolving polypeptide with a given amino acid sequence as follows:

$$W \equiv \ln(\mathrm{CFU}), \tag{41}$$

where CFU represents the CFU-value for *E.coli* JM109 strain. Therefore, the fitness W is handled in the energy level. We assumed that the D2 domain and other domains contribute to the phage infectivity independently, and considered the intradomain interaction. Fig.4 shows the time series of fitness in the adaptive walk experiment through the 0th-20th generations.

In each of the 1st-7th and 8'th generations, we have arbitrarily chosen about ten mutant phage clones that possess respective mutant polypeptides, and subsequently evaluated the infectivity for each of them and selected a single clone with the highest infectivity from among them. This obeys the cloning-screening type evolution protocol mentioned in

the previous section. In each of the 8th-20th generations, the mutant phage clones with the highest infectivity were selected from among $10^2 \sim 10^6$ mutants through iteration of infection and production of phage particles (Fig.5). This iteration process is called the "enrichment process". This obeys the natural selection-type evolution protocol. We assumed the enrichment of the best clone had been sufficiently done. The N-value, which is the library size of mutants to be screened, was as follows: $N \approx 10$ (for the 1st-7th and 8'th generations), 10^2 (for the 8th generation), 10^3 (for the 9th-13th generations), 10^4 (for the 14th-15th generations), 10^5 (for the 16th-18th generations) and 10^6 (for the 19th-20th generations). The mutation analysis in Hayashi *et al.* (2006) showed that the mutation rate μ was 0.02 in all generations, and thus we adopted $\overline{d} = 2.4$ in this case.

5.2 Estimating parameters: analysis of the stationary state and transitional state

In this subsection, we fitted the theoretical equations (described in the previous section) to the experimental data obtained by Hayashi *et al.* (2006). Known parameters are $\nu = 119$, $\overline{d} = 2.4$ and N values given at each generation.

From the time series of fitness in the adaptive walk shown in Fig.4, we considered that the adaptive walker at the 7th-8'th, 12th-13th, 17th-18th and 19th-20th generations reached the stationary state for $N = 10$, 10^3, 10^5 and 10^6, respectively. Assuming that the stationary fitness values, $\mathscr{W}^*_{\rm obs}$, is given as the mean fitness of the last two generations for each N-value, we plotted the $\mathscr{W}^*_{\rm obs}$-values in Fig.6 against their respective ζ-values calculated by eqn.(14). The resulting regression line has a slope of $b = 1.85$ and Y-segment of $a = 6.11$ with correlation coefficient of 0.94 ($p = 0.016$). This observed linearity between $\mathscr{W}^*_{\rm obs}$ and ζ was compatible with eqn.(18).

The theoretical curve shown in eqn.(32) was fitted onto the series of fitness through the 1st-8'th generations (the fitness of the initial generation was not included in the fitting because the Hamming distance between the initial and 1st generation is 11). As a result of the parameter fitting, we obtained $c = 0.47$ (Fig.4). Then, using eqn.(34) and eqn.(35), we obtained $\overline{d_{\rm eff}} = 63$ and $k = 31$. In addition, we fitted eqn.(32) onto the series of

fitness through the 8th-13th generations using the same method and obtained $c = 0.59$ (Fig.4). At which point, we obtained $\overline{d_{\rm eff}} = 49$ and $k = 22$. We adopted $k = 27$ as the intermediate value between the two k-values. Using eqn.(36) $\sim$ eqn.(40), we determined other parameters. The estimated parameters as the landscape properties are listed in Table.1.

In the meantime, the fitness of the wild-type fd-tet phage that possesses the native D2 domain was observed as $W = 22.2 \pm 0.2$, and the difference between the fitness of the starting sequence (random polypeptide RP3-42) at the 0th generation and the wild-type fitness is $22.2 - 4.9 = 17.3$. The values, 22.2 and 17.3, are similar to $O = 23.1$ and $|\mathscr{E}| = 17.0$, respectively. If the starting sequence and the wild-type sequence correspond to the foot of the landscape and the global peak, respectively, it is suggested that our theoretical analysis succeeded in predicting the height of the fitness landscape.

5.3 Fitness distribution over the d-boundary

For each of the 2nd-8'th generations ($N \approx 10$), we have data on the fitness for each individual of about ten mutant clones and their parent clone. Note that these clones were randomly chosen from the d-boundary. The fitness distribution among the sample clones for each generation is plotted in Fig.7(a). In this figure, we show the change in fitness from the parent to each of its mutants, $\Delta W = W - \mathscr{W}$, where $\mathscr{W}$ is the parent's fitness. We can see in Fig.7(a) that there are some outlier clones which have salient values of fitness change. Discrimination of outlier clones was performed according to the description in Appendix C. About 10 percent of the sample clones were identified as the outlier for each generation. From the fitness data of the sample clones excluding the outlier clones, we estimated the average $E[\Delta W|\mathscr{W}]$ and variance $V[\Delta W|\mathscr{W}]$ over the d-boundary (Fig.7(a)).

For each of the 8th-20th generations ($N \approx 10^2 \sim 10^6$), we do not have data on the fitness of individual mutant clones, then we estimated values of $E[\Delta W|\mathscr{W}]$ and $V[\Delta W|\mathscr{W}]$ in the following. Fig.5 shows the mean CFU-value of the mutant population at each round in the enrichment process. Here, we note that the term "generation t" is used as the step number in the evolution process and "round r" is used as the step number in the

enrichment process for each generation. The mean CFU-value at an arbitrary round is denoted by $\langle \mathrm{CFU} \rangle$. We assumed that the fitness distribution over the d-boundary is given by the Gaussian with the mean $E[W|\mathscr{W}]$ and variance $V[W|\mathscr{W}]$, and that the enrichment process obeys the typical scheme of natural selection. On the assumption, the $\langle \mathrm{CFU} \rangle$ at the r-th round in the ascending phase is given by

$$\ln\langle \mathrm{CFU} \rangle \approx E[W|\mathscr{W}] + \left(r + \frac{1}{2}\right) V[W|\mathscr{W}]. \tag{42}$$

Derivation is described in Appendix D. We estimated values of $E[W|\mathscr{W}]$ and $V[W|\mathscr{W}]$ for each generation from the slope and Y-segment of the regression line shown in Fig.5. The estimated values of $E[\Delta W|\mathscr{W}]$ $(= E[W|\mathscr{W}] - \mathscr{W})$ and $V[\Delta W|\mathscr{W}]$ $(= V[W|\mathscr{W}])$ for each generation are shown in Fig.7(a).

On the other hand, we calculated the theoretically predicted values of $E[\Delta W|\mathscr{W}]$ and $V[\Delta W|\mathscr{W}]$, by substituting the determined parameters into eqn.(9) and eqn.(25), respectively. These theoretical values are shown in Fig.7(a). Fig.7(b) shows a comparison of the experimental values for $E[\Delta W|\mathscr{W}]$ with theoretical values, for the 2nd-20th generations. As a result, we can see that the experimental values for $E[\Delta W|\mathscr{W}]$ and $V[\Delta W|\mathscr{W}]$ are almost in agreement with the theoretical values of them. Student's t-tests at a significance level of 5 % did not indicate that there are differences between the experimental values for $E[\Delta W|\mathscr{W}]$ and the theoretical values. Therefore, eqn.(9) and eqn.(25) were supported.

5.4 The strength of selection pressure

We have data on the change in fitness, $\Delta\mathscr{W}$, from the parent to the fittest individual among its N mutants, for the 2nd-20th generations. Then, we calculated the following quantity:

$$Z = \frac{\Delta\mathscr{W} - E[\Delta W|\mathscr{W}]}{\sqrt{V[\Delta W|\mathscr{W}]}}, \tag{43}$$

where $E[\Delta W|\mathscr{W}]$ and $V[\Delta W|\mathscr{W}]$ are the experimental values indicated by symbols and error-bars, respectively, shown in Fig.7(a).

On the other hand, we calculated the theoretically predicted values of selection pressure, ζ, described in eqn.(14) with given N-values. Fig.8 shows a comparison of the

experimental values (Z) with the predicted values (ζ), for the 2nd-20th generations. As a result, we can see that Z-values are in good agreement with ζ-values, indicating that eqn.(12) with eqn.(14) was almost exemplified and then the selection process was theoretically carried out. Student's t-tests at a significance level of 5 % did not indicate that there are differences between the Z-values and the ζ-values. Therefore, eqn.(12) with eqn.(14) was supported.

6 Discussions

We proposed a methodology for extracting characteristic properties of a fitness landscape by analyzing time series of fitness through the adaptive walk on the landscape, and applied to analyze data on the *in vitro* evolution of a random polypeptide toward acquiring infectivity of fd phage (Hayashi *et al.*, 2006). It is interesting that, in spite of the theoretical model of the fitness landscape being a simple mathematical model called the NK model, the experimental data analyzed was almost quantitatively consistent with the theoretical prediction for the NK fitness landscape. Our methodology is mainly based on the analysis of the stationary phase. In practice, it is difficult to judge the stationary phase in a time series of fitness with small number of steps of the adaptive walk. If an analyst judges a set of certain values observed, $\{\mathscr{W}^*_{\rm obs}(N)|N = N_1, N_2, N_3, \cdots\}$, as the set of stationary fitnesses and the parameters determined after the analysis of the set is compatible with other additional data, then it is probably safe to conclude that the set of values as being that of the true stationary fitness values.

We estimated $k = 27$ in this case. This result suggests that an arbitrary residue is affected from about 23 percent over all residues ($27/119 = 0.23$). Our theoretical study presumed that if k is small, the walker would climb the smooth landscape gradually toward a high stationary point, whereas as k increases, the walker climbs the rugged landscape rapidly and reaches a low stationary point in the early stages (see eqn.(18) and (19)). It seems that this large number of interaction caused the ruggedness on the fitness surface, and thus the ruggedness caused the rapid ascending rate of the adaptive walk. In

the meanwhile, Kauffman and Weinberger applied the NK model to affinity maturation of the V region ($\nu = 110 \sim 120$) in immunoglobulin and estimated that k is about 40 (Kauffman & Weinberger,1989; Kauffman,1993). Although they estimated the k-value from the number of steps of the adaptive walk up to the local optima, our estimate of k is similar to their estimate. These values ($k = 27 \sim 40$) suggest that the landscape from the foot through the middle is considerablely rugged.

In our previous paper (Hayashi, *et al.*, 2006), we summarized our methodology of the landscape analysis and applied simplified equations to analyze the same experimental data as those shown in this paper, and determined $k = 21$. Although there is a slight discrepancy between the two estimated k-values ($k = 27$ and $k = 21$), the semi-quantitative description of the landscape structure presented in Hayashi *et al.* is guaranteed by our additional mathematical analysis. We may leave the details of the landscape structure to Hayashi *et al.* (2006).

It is noticeble that there are some outlier mutant clones that show a drastic decrease in fitness. We found about 10 percent of the sample clones were identified as the outlier. This effect can not be explained by the non-additivity handled in the NK model, but may be explained by a cooperative molecular conformational change. The emergence of the defective phage clones suggests that there are "hollows" on the fitness landscape. This seems to be related to a study on the RNA genotype-phenotype mapping (Fontana & Schuster,1998).

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Appendix A: Derivation of eqn.(6)

Consider that random d-fold point mutations occur in a parent sequence. We will determine the expected number of sites changing their site-fitness as a result of the mutations. We approximate that a single mutation affects on other k sites. Fig.9 shows three kinds of sites: unchanged sites, mutated sites and nonmutated sites that changed their site-fitness by the other mutations, which are indicated with $\odot$, $\oplus$ and $\otimes$, respectively. First, fix d symbols of $\oplus$. Next, choose k sites randomly from among $\nu - 1$ sites except a site of $\oplus$, and repeat this procedure by d times. Let $p_t(n)$ be the probability that the number of sites that have changed their site-fitness ($\oplus$ and $\otimes$) is n for t times ($t = 0, 1, 2, \cdots, d$). $p_t(n)$ obeys the following master equation:

$$p_t(n) = \sum_{\Delta n=0}^{k} p_{t-1}(n - \Delta n) \times \frac{\binom{n-\Delta n-1}{k-\Delta n}\binom{\nu-(n-\Delta n)}{\Delta n}}{\binom{\nu-1}{k}}, \qquad 0 \le n \le \nu, \tag{44}$$

where the initial condition is $p_0(n) = \delta_{n,d}$ (Kronecker's delta). The probability that the number of sites that have changed their site-fitness as the result of the d-fold point mutations is $d_{\rm eff}$, is given by $p_d(d_{\rm eff})$. Let $E_t[n]$ be the expectation of n: $E_t[n] = \sum_{n=0}^{\nu} n\, p_t(n)$.

$E_t[n]$ approximately obeys the following master equation:

$$E_t[n] = E_{t-1}[n] + \left(\frac{\nu - E_{t-1}[n]}{\nu - 1}\right) k.$$

Solving the above equation, we obtain the solution

$$E_t[n] = \nu - (\nu - d)\left(1 - \frac{k}{\nu - 1}\right)^t.$$

Then, substituting $t = d$ into the above equation, we obtain

$$\overline{d_{\rm eff}} = \nu - (\nu - d)\left(1 - \frac{k}{\nu - 1}\right)^d.$$

Using

$$\left(1 - \frac{k}{\nu - 1}\right)^d \approx 1 - \frac{dk}{\nu - 1},$$

we obtain the following approximated form:

$$\overline{d_{\rm eff}} \approx d(1 + k) \qquad \text{for } d \ll \nu.$$

Appendix B: Derivation of equations (12)-(14)

We neglect the truncation of the normal distribution shown in eqn.(8). Substituting eqn.(8) into eqn.(11) and transforming the resulting equation by using

$$x \equiv \frac{\Delta\mathscr{W} - E[\Delta W|\mathscr{W}]}{\sqrt{V[\Delta W|\mathscr{W}]}}, \tag{45}$$

we obtain the probability density function of x as follows:

$$\widetilde{\varphi}(x) = N\mathscr{N}(x|0,1)\left(\int_{-\infty}^{x}\mathscr{N}(y|0,1)\mathrm{d}y\right)^{N-1}. \tag{46}$$

$\widetilde{\varphi}(x)$ represents the probability density function of the greatest value among N random numbers from the standard Gaussian probability density. Then, the expectation of $\Delta\mathscr{W}$ is given by

$$\begin{aligned} E[\Delta\mathscr{W}|\mathscr{W}] &= \int_{-\infty}^{\infty}\Delta\mathscr{W}\,\varphi(\Delta\mathscr{W}|\mathscr{W})\,\mathrm{d}\Delta\mathscr{W} \\ &= \int_{-\infty}^{\infty}\left(E[\Delta W|\mathscr{W}] + \sqrt{V[\Delta W|\mathscr{W}]}\,x\right)\widetilde{\varphi}(x)\,\mathrm{d}x \\ &= E[\Delta W|\mathscr{W}] + \sqrt{V[\Delta W|\mathscr{W}]}\;\zeta, \end{aligned} \tag{47}$$

where ζ is defined by

$$\zeta \equiv \int_{-\infty}^{\infty} x\,\widetilde{\varphi}(x)\,\mathrm{d}x. \tag{48}$$

Eqn.(47) corresponds to eqn.(12).

$\widetilde{\varphi}(x)$ in eqn. (46) is rewritten as follows:

$$\widetilde{\varphi}(x) = \frac{N}{\sqrt{2\pi}}e^{-A(x)}, \tag{49}$$

$$A(x) \equiv \frac{x^2}{2} - (N-1)\ln\int_{-\infty}^{x}\mathscr{N}(y|0,1)\mathrm{d}y. \tag{50}$$

$\widetilde{\varphi}(x)$ has a maximum when $A(x)$ has a minimum. We approximate $A(x)$ by a truncation of its Taylor series, expanded about $\widehat{x}$, that is defined as

$$\widehat{x} \equiv \arg\max\{\widetilde{\varphi}(x)\} = \arg\min\{A(x)\}. \tag{51}$$

A first differentiation of $A(x)$ is

$$\frac{dA(x)}{dx} = x - (N-1)\frac{\mathscr{N}(x|0,1)}{\int_{-\infty}^{x}\mathscr{N}(y|0,1)\mathrm{d}y}.$$

Since the condition that $\widehat{x}$ must satisfy is

$$\left.\frac{dA(x)}{dx}\right|_{x=\widehat{x}} = 0,$$

then, $\widehat{x}$ satisfies

$$\widehat{x}\, e^{\widehat{x}^2/2} \int_{-\infty}^{\widehat{x}} e^{-y^2/2} \mathrm{d}y = N - 1. \tag{52}$$

It is guaranteed that eqn. (52) has a unique solution. A second differentiation is

$$\frac{d^2A(x)}{dx^2} = 1 + (N-1)\left(\frac{x\, e^{-x^2/2}}{\int_{-\infty}^{x} e^{-y^2/2}dy} + \left(\frac{e^{-x^2/2}}{\int_{-\infty}^{x} e^{-y^2/2}dy}\right)^2\right).$$

Using eqn. (52), we obtain

$$\begin{aligned}\left.\frac{d^2A(x)}{dx^2}\right|_{x=\widehat{x}} &= 1 + \frac{N}{N-1}\,\widehat{x}^2 \\ &\approx 1 + \widehat{x}^2. \end{aligned} \tag{53}$$

$A(x)$ is approximated by

$$A(x) \approx A(\widehat{x}) + \frac{1}{2}\left.\frac{d^2A(x)}{dx^2}\right|_{x=\widehat{x}} (x-\widehat{x})^2. \tag{54}$$

Then, using eqn. (54) and eqn. (53), $\widetilde{\varphi}(x)$ in eqn. (49) is rewritten as follows:

$$\begin{aligned}\widetilde{\varphi}(x) &\approx \widetilde{\varphi}(\widehat{x})\, e^{-(x-\widehat{x})^2/2(1+\widehat{x}^2)^{-1}}, \\ &\approx \mathcal{N}(x|\widehat{x}, (1+\widehat{x}^2)^{-1}). \end{aligned} \tag{55}$$

From the relation $\Delta\mathscr{W} = E[\Delta W|\mathscr{W}] + \sqrt{V[\Delta W|\mathscr{W}]} \times x$ (eqn.(45)), the variance of $\Delta\mathscr{W}$ is given by

$$V[\Delta\mathscr{W}|\mathscr{W}] \approx \frac{V[\Delta W|\mathscr{W}]}{1+\widehat{x}^2}. \tag{56}$$

Here, ζ defined in eqn.(48) is approximated to $\widehat{x}$ defined in eqn.(51):

$$\zeta \approx \widehat{x}. \tag{57}$$

Substituting eqn.(57) into eqn.(52) and eqn.(56), we obtain eqn.(14) and eqn.(13), respectively.

Appendix C: Discrimination of outlier clones

Let MED and MAD be "median" and "median absolute deviation" of the fitness for the N mutant clones ($\{W_m | m = 1, 2, \cdots, N\}$), respectively, where median absolute deviation, MAD, is defined as the median of absolute deviation, $|W_m - \mathrm{MED}|$ ($m = 1, 2, \cdots, N$), except 0. When the fitness W_m of the m-th clone satisfied

$$|W_m - \mathrm{MED}| > 5 \times \mathrm{MAD},$$

the clone was identified as the outlier.

Appendix D: Derivation of eqn.(42)

Let $k(W)$ be the propagation rate-constant of a phage clone with the fitness W and let $x(r, W)$ be the mole fraction of the phage clone with the fitness W at the r-th round in the natural selection process. We assume that $x(r, W)$ obeys the following equation:

$$x(r+1, W) = \frac{1 + k(W)}{D(r+1)} \times x(r, W), \tag{58}$$

where $D(r)$ is the dilution parameter to keep the equation $\int_{-\infty}^{\infty} x(r, W)\mathrm{d}W = 1$ for any round r. $D(r)$ is determined as $D(r) = 1 + \int_{-\infty}^{\infty} k(W)\, x(r, W)\mathrm{d}W$. Solving eqn.(58), we obtain the following solution:

$$x(r, W) = \frac{x(0, W)(1 + k(W))^r}{\int_{-\infty}^{\infty} x(0, W)(1 + k(W))^r \mathrm{d}W}. \tag{59}$$

Thus, the population mean of $k(W)$ at the r-th round is given by

$$\langle k \rangle_r = \int_{-\infty}^{\infty} k(W)\, x(r, W)\mathrm{d}W \tag{60}$$

$$= \frac{\sum_{i=0}^{r} \binom{r}{i} \int_{-\infty}^{\infty} x(0, W) k(W)^{i+1} \mathrm{d}W}{\sum_{i=0}^{r} \binom{r}{i} \int_{-\infty}^{\infty} x(0, W) k(W)^{i} \mathrm{d}W}. \tag{61}$$

In our scheme, $k(W)$ corresponds to CFU, and then $k(W) = e^W$. $x(0, W)$ corresponds to the density function of fitness for the N sample mutants, which are randomly chosen from the d-boundary. We assume $x(0, W)$ is approximately given by the following truncated

Gaussian:

$$x(0,W) \approx \begin{cases} \mathcal{N}(W|E[W],V[W]) & \text{for } W \leq E[W] + \sqrt{V[W]}\,\zeta, \\ 0 & \text{otherwise,} \end{cases} \tag{62}$$

where ζ is described in eqn.(14). For not so large r satisfying $r \ll (\zeta - 2)/\sqrt{V[W]}$, using the equation

$$\frac{1}{\sqrt{2\pi V[W]}} \int_{-\infty}^{\infty} e^{-\frac{(W-E[W])^2}{2V[W]} + Wi} \mathrm{d}W = e^{iE[W] + i^2 V[W]/2},$$

we obtain

$$\begin{aligned} \langle k \rangle_r &\approx \frac{\sum_{i=0}^{r} \binom{r}{i} e^{(i+1)E[W] + (i+1)^2 V[W]/2}}{\sum_{i=0}^{r} \binom{r}{i} e^{iE[W] + i^2 V[W]/2}} && (63) \\ &\approx \frac{e^{(r+1)E[W] + (r+1)^2 V[W]/2}}{e^{rE[W] + r^2 V[W]/2}} && (64) \\ &= e^{E[W] + (r+1/2)V[W]}. && (65) \end{aligned}$$

$\langle k \rangle_r$ corresponds to $\langle \mathrm{CFU} \rangle$ in eqn.(42).

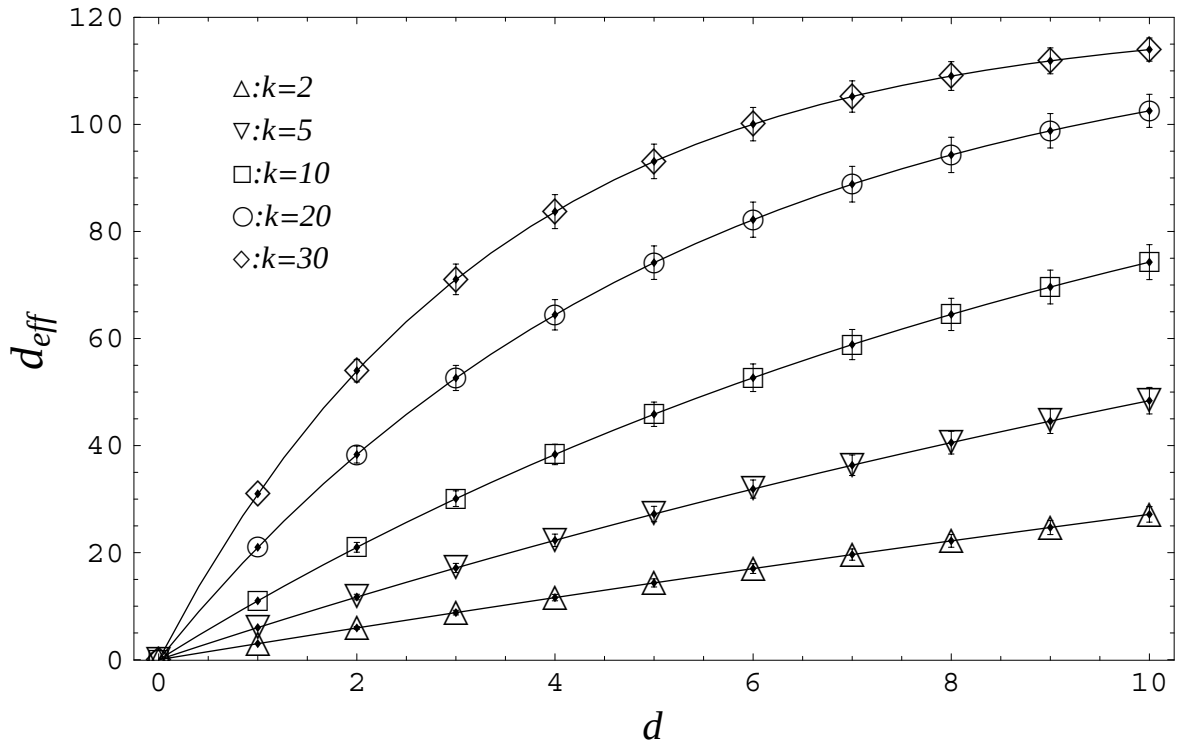


Figure 1: **The effective number of mutations, $d_{\rm eff}$, against the number of mutations, d.** The symbols and error-bars represent the expectation and standard deviation of $d_{\rm eff}$. These values were obtained by solving eqn.(44). The solid lines represent approximated expectations of $d_{\rm eff}$. These values were obtained from eqn.(6). $\nu = 120$.

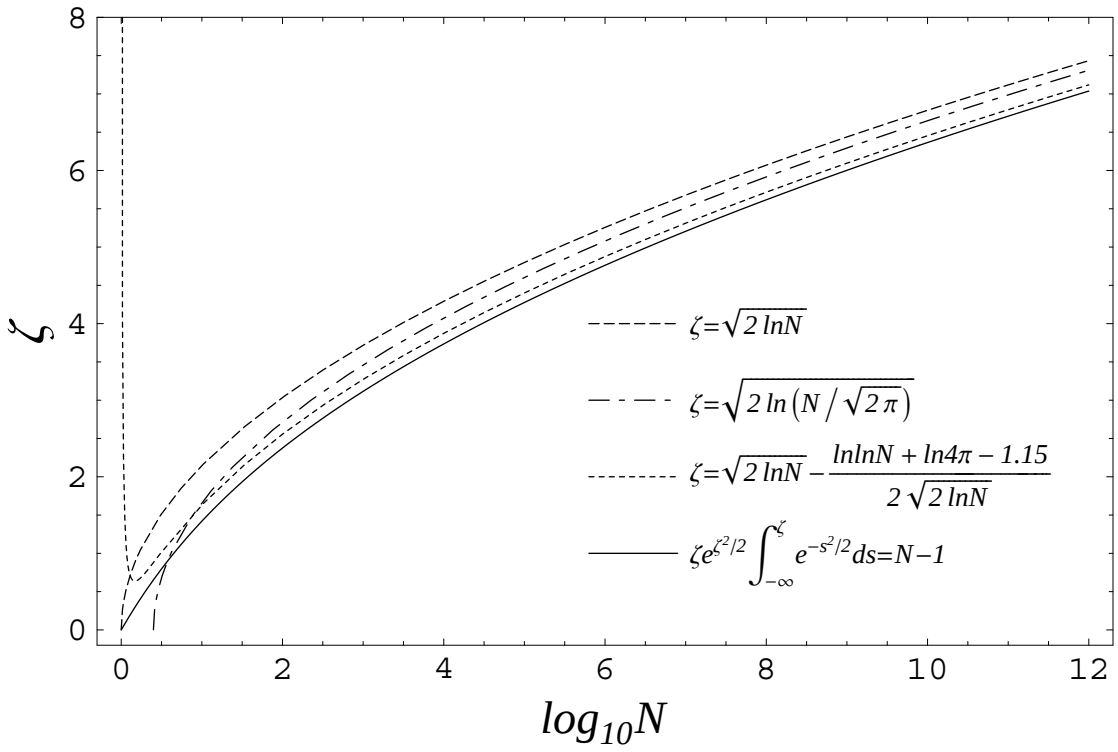
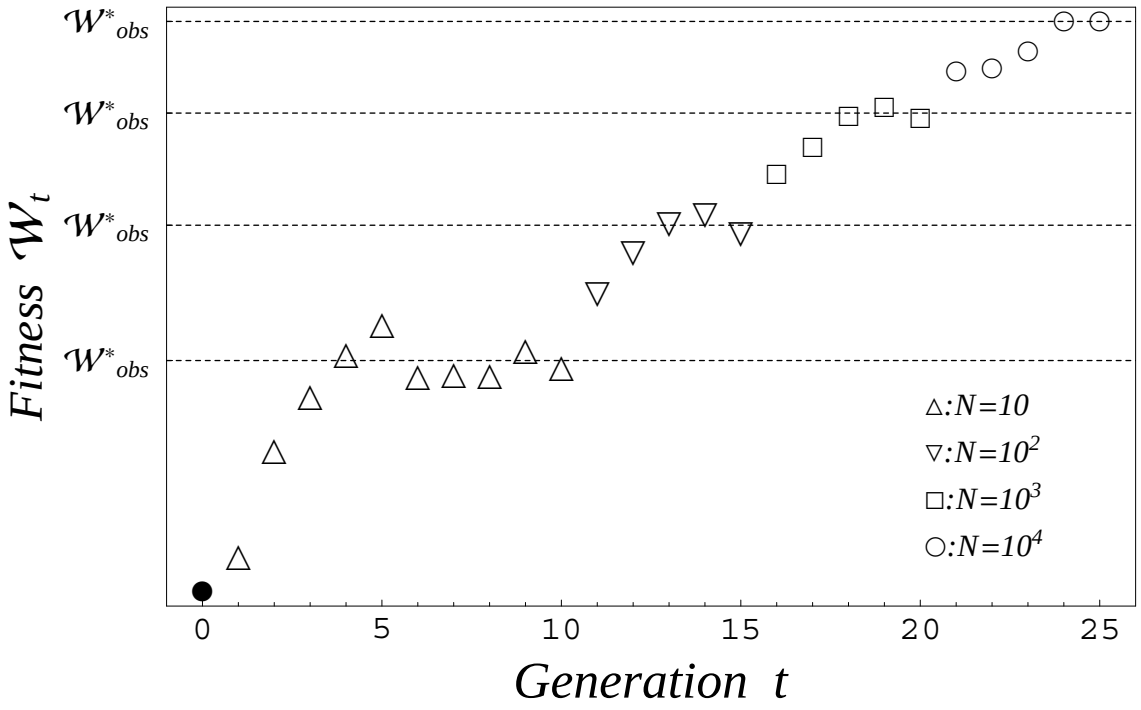


Figure 2: **A measure of selection pressure, ζ, against the library size N.** ζ is the function of N. Four different curves represent four types of approximation. The function shown with the dotted curve is cited from Nov & Wein,(2005).

Parameter	k	k	k	k
True value	10	20	30	40
Estimated value	11.6 ± 1.5	20.1 ± 4.2	29.1 ± 5.0	36.2 ± 8.1
Parameter	ε	ε	ε	ε
True value	-1.0	-1.0	-1.0	-1.0
Estimated value	-0.94 ± 0.06	-0.91 ± 0.10	-0.90 ± 0.10	-0.85 ± 0.10

Table 1: **Comparison between estimated values and true ones for landscape parameters k and ε.** The estimated values are indicated with the average and standard deviation over 100 different realizations of NK landscapes for each parameter set. $\nu = 120$, $\lambda = 20$ and $\varepsilon = -1.0$. $N_1 = 10$, $N_2 = 10^2$, $N_3 = 10^3$, $N_4 = 10^4$ and $d = 2$.



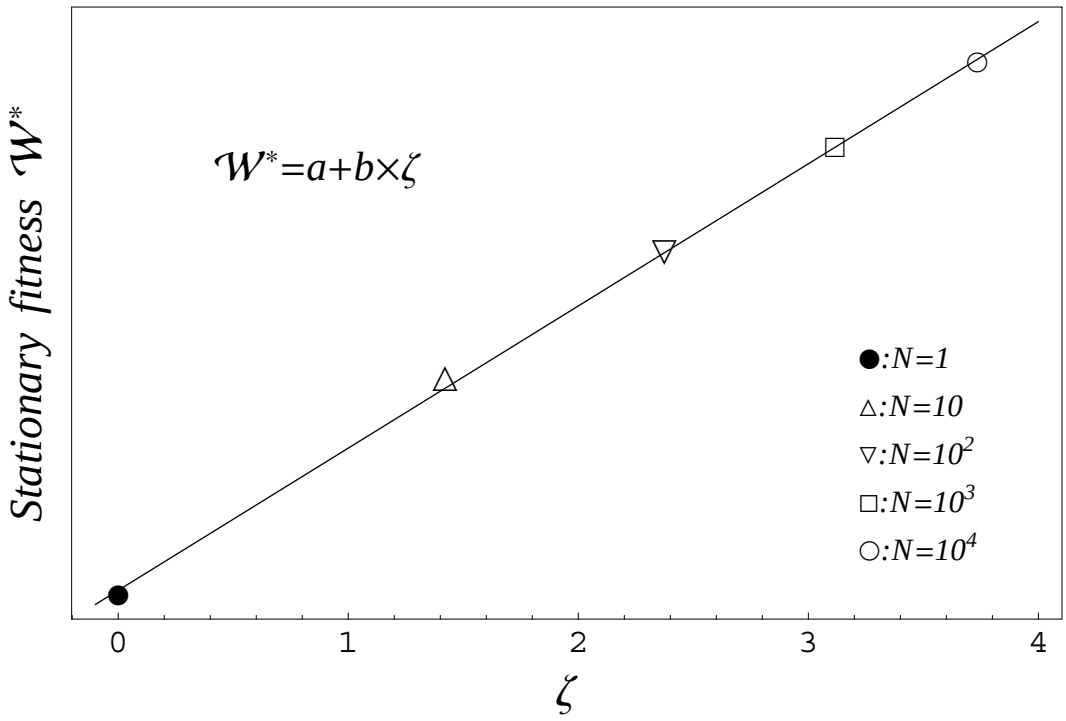


Figure 3: **(a) An example of time series of fitness in the adaptive walk simulation on the NK landscape.** $\lambda = 20$. $\nu = 120$. $\varepsilon = -1$. $O = 0$. $k = 0$. $d = 40$. The library size of mutants to be screened, N, was changed as shown. The stationary fitness observed, $\mathscr{W}^*_{\rm obs}$, for each N-value was obtained as the mean fitness of the last two generations for each N. **(b) Stationary fitness against ζ.** The stationary fitness observed, $\mathscr{W}^*_{\rm obs}$, for each N-value is plotted against ζ, which is calculated from eqn.(14).

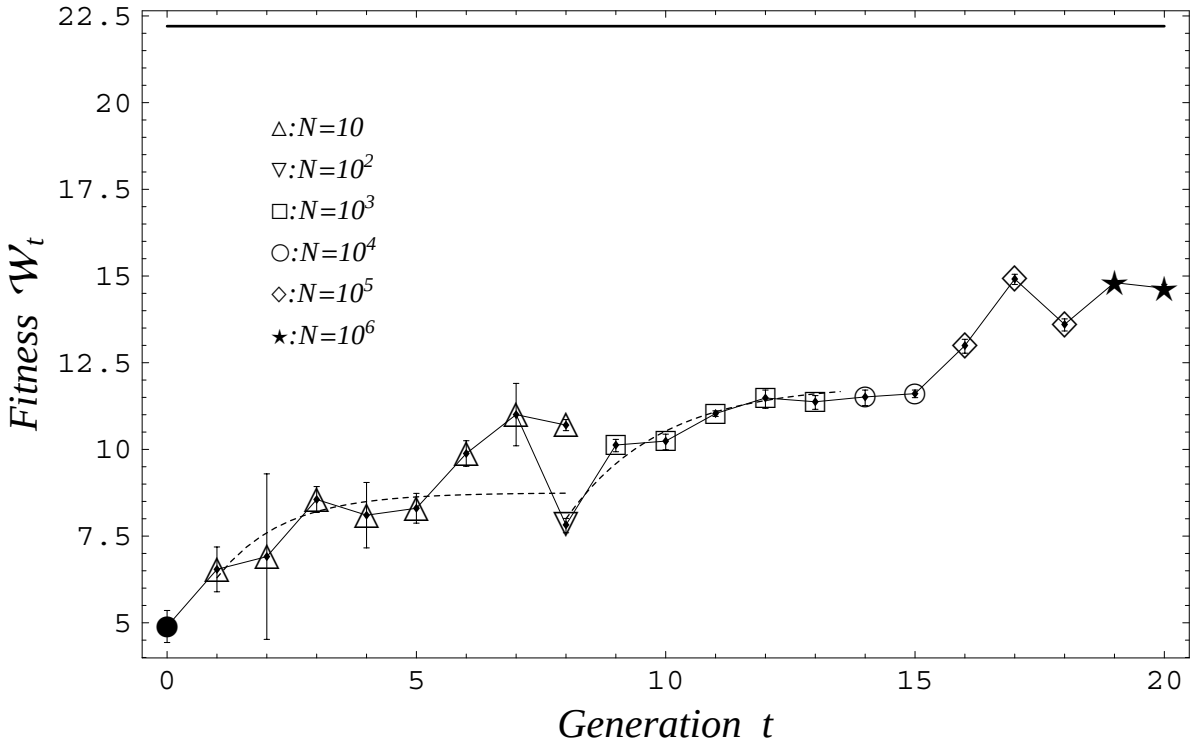


Figure 4: **Time series of fitness in the adaptive walk experiment through the 0th-20th generations.** The lineage of the adaptive walk is shown by the series of symbols joined by lines. The library size of mutants to be screened, N, is represented by different symbols. The upper triangle at the 8th generation is designated the "8'th" walker. The horizontal line at the top of the figure indicates the fitness of the wild-type fd-tet phage that possesses the native D2 domain. The dotted lines for $N = 10$ and $N = 10^3$ are the fitted curves described in eqn.(32).

parameter	λ	ν	ε	σ	$\mathscr{E}$	$\mathscr{V}$	k	O
estimated value	-	-	-0.14	0.082	-17.0	0.81	27	23.1
observed value	20	119	-	-	-17.3	-	-	22.2

Table 2: **Landscape properties for the infectivity landscape.** 22.2 is the fitness of the wild-type fd-tet phage that possesses the native D2 domain. 17.3 is the difference between the fitness of the starting sequence (random polypeptide RP3-42) at the 0th generation and the wild-type fitness.

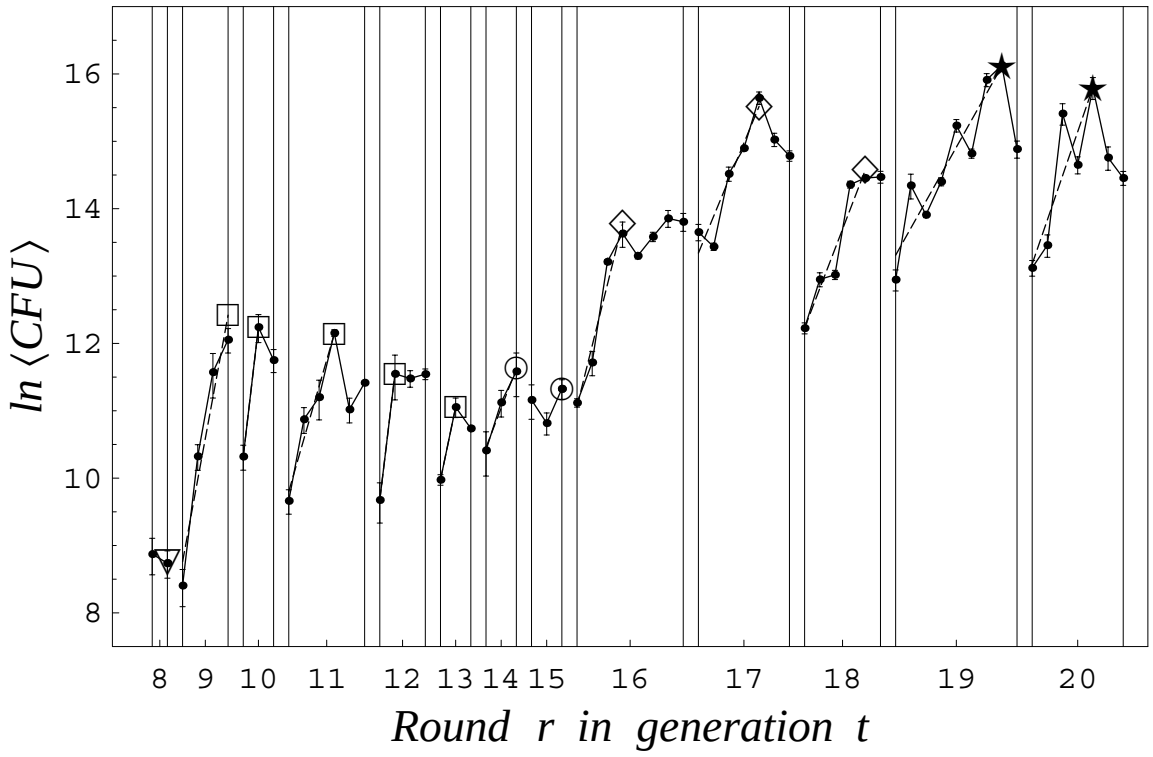


Figure 5: **Time series of the mean CFU-value, ⟨CFU⟩, of a mutant population in the natural selection process for the 8th-20th generations**. For each generation, a mutant library was prepared by subjecting the parent population to the random mutagenesis by the error-prone PCR, and then a cycle of infection and production of mutant phage particles was iterated starting from the initial population. The mutagenesis was not introduced to the subsequent rounds. The numbers indicated along the abscissa represent generations t. In each generation, the time series of ⟨CFU⟩ is shown against round r with the series of dots joined by lines. The dashed line is the regression line for each ascending phase. The symbols represent the estimated fitness of the selected population as the parents for the next generation. The same symbols as those in Fig.4 are shown. Note that these values show slight discrepancies from those in Fig.4 due to the difference in the experimental details used for evaluation.

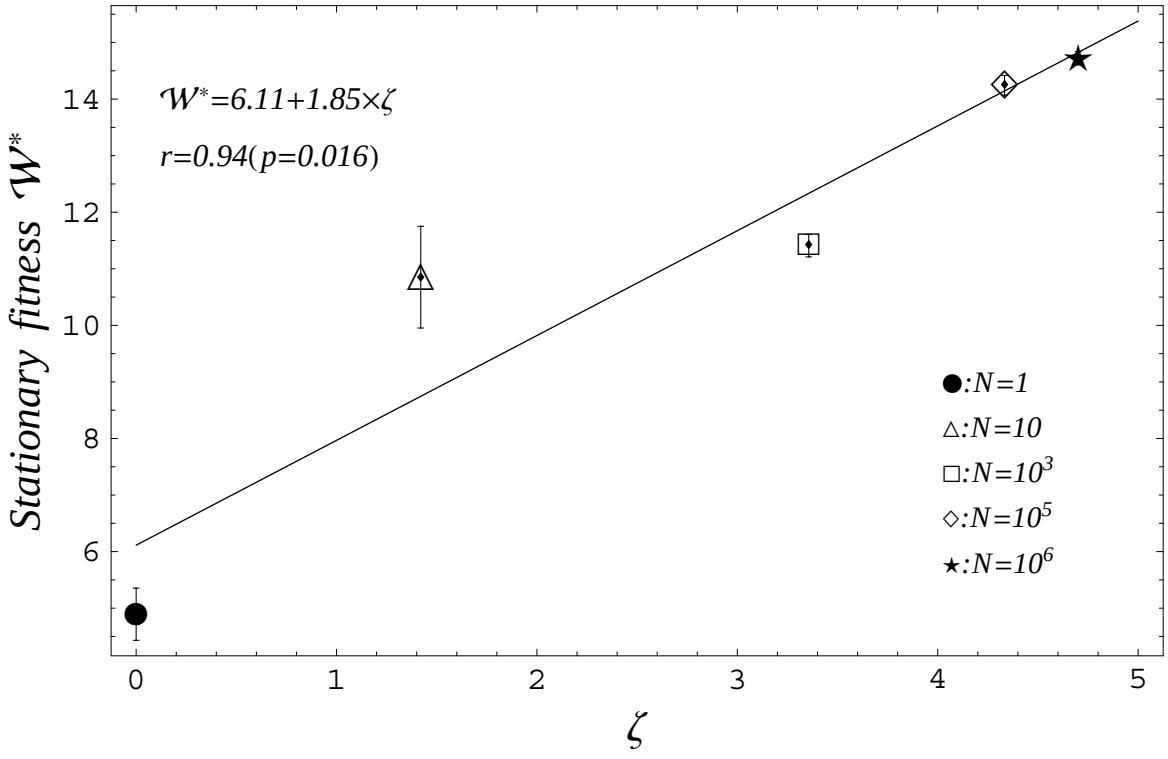


Figure 6: **Stationary fitness against ζ.** The stationary fitness observed, $\mathscr{W}^*_{\rm obs}$, for each N-value is plotted against ζ, which is calculated from eqn.(14). The filled circle indicates the walker's fitness at the initial generation. The triangle, square, diamond and star indicate the mean fitness of the 7th and 8'th, that of the 12th and 13th, that of the 17th and 18th, and that of the 19th and 20th generations, respectively.

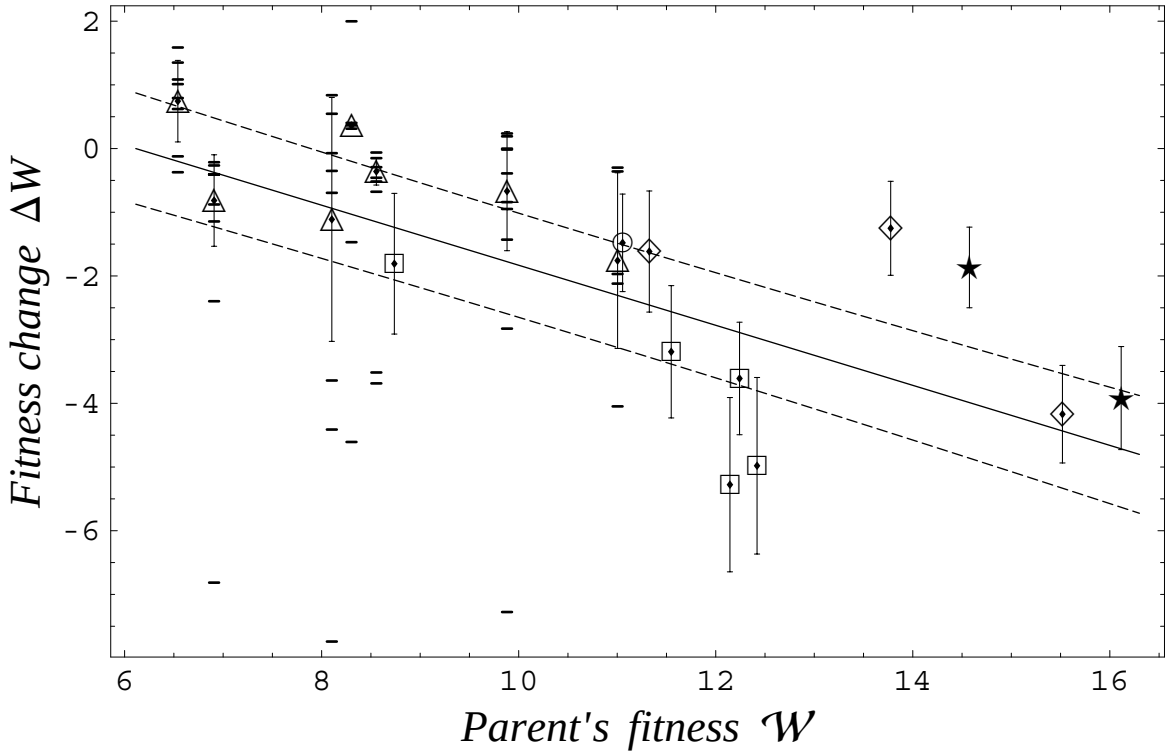


Figure 7: (a) Fitness distribution over the d-boundary. Ordinate is the fitness-change ΔW from a parent's fitness to an arbitrary mutant's fitness in the d-boundary. The average $E[\Delta W|\mathscr{W}]$ and standard deviation $\sqrt{V[\Delta W|\mathscr{W}]}$ for each generation are indicated with symbols and error-bars, respectively. For the 2nd-8'th generations, the fitness-change ΔW for individual mutant clone is indicated with a short horizontal bar. According to the Student's distribution with $N = 5 \sim 10$, the true value of $E[\Delta W|\mathscr{W}]$ takes a value within $E[\Delta W|\mathscr{W}] \pm (0.8 \sim 1.4) \times \sqrt{V[\Delta W|\mathscr{W}]}$ with a probability of 95 %. For the 8th-20th generations, values of $E[\Delta W|\mathscr{W}]$ and $V[\Delta W|\mathscr{W}]$ were estimated by applying eqn.(42) to the regression line shown in Fig.5. The parent's fitness $\mathscr{W}$ is also taken from the value indicated with the symbol in Fig.5. The descending straight solid-line represents the theoretically predicted value for $E[\Delta W|\mathscr{W}]$ and deviation from the solid line to each broken line represents $\sqrt{V[\Delta W|\mathscr{W}]}$. These were obtained by substituting $\overline{d_{\rm eff}} = 56, \nu = 119, \mathscr{E} = -17.0, O = 23.1, \mathscr{V} = 0.81$ into eqn.(9) and (25).

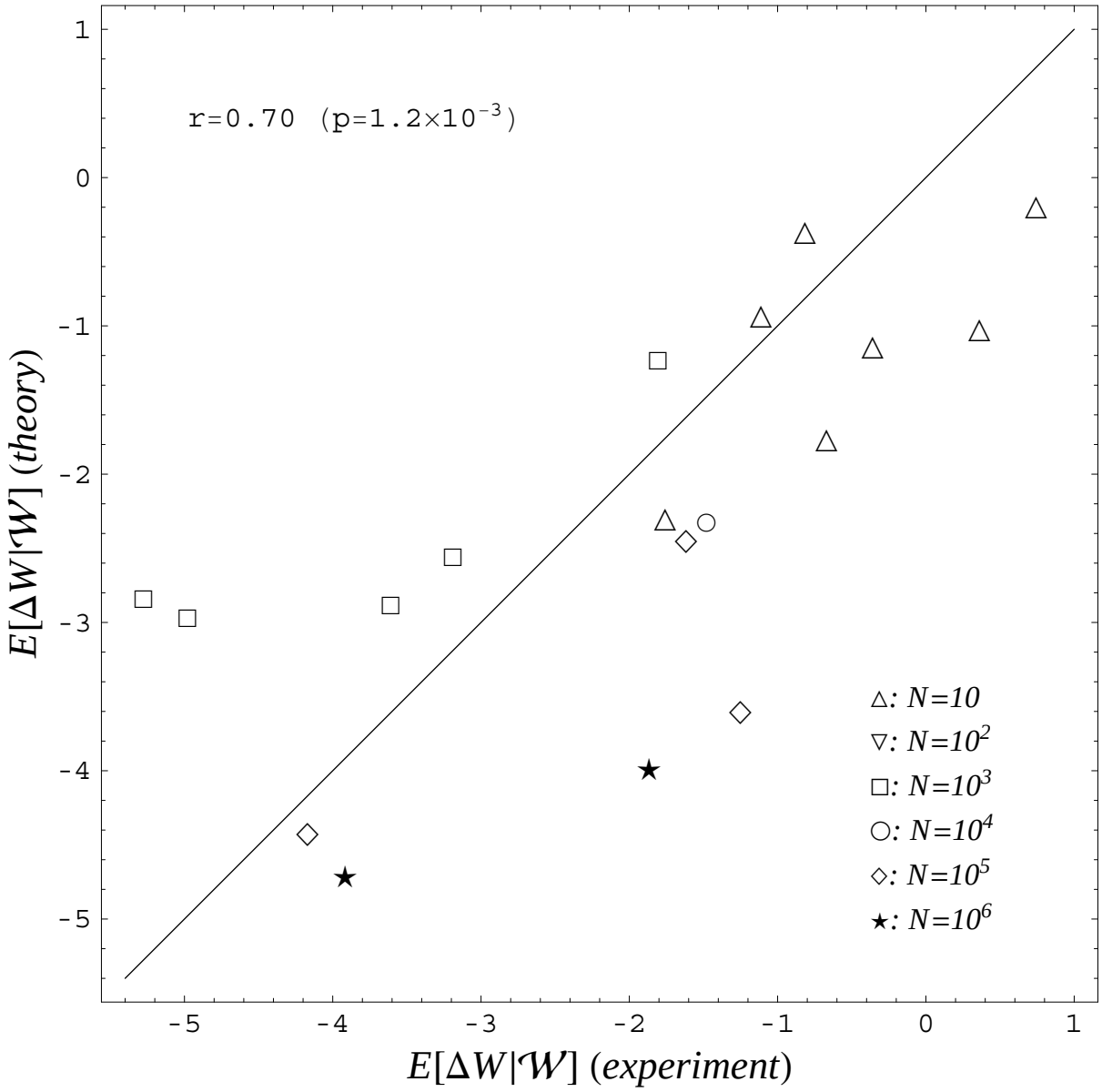


Figure 8: **Figure.7(b) The experimentally estimated values for $E[\Delta W|\mathscr{W}]$ vs the theoretically predicted values.** The symbols representing the N-values are the same as those in Fig.4. Correlation coefficient is 0.70 with $p = 1.2 \times 10^{-3}$.

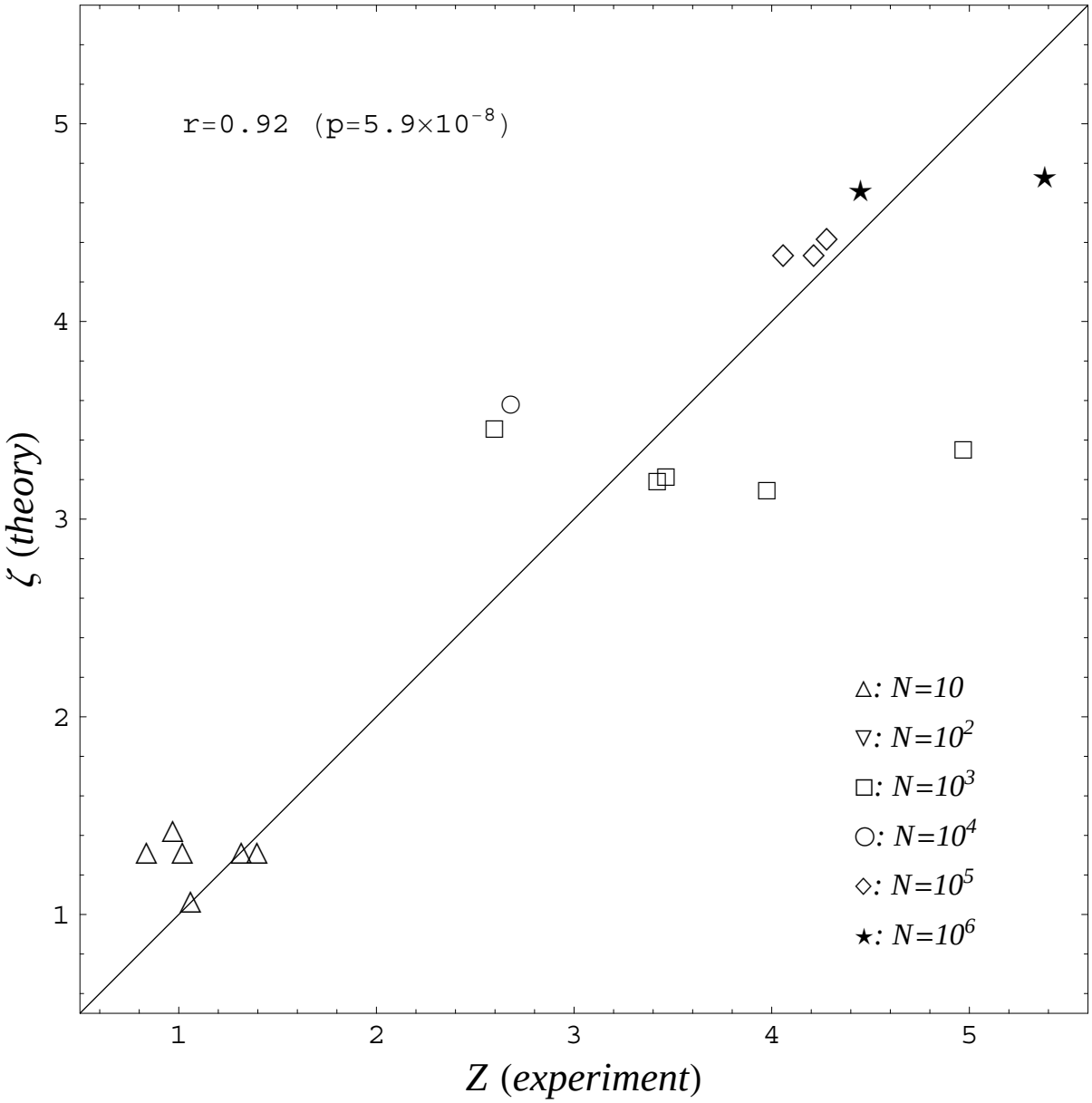


Figure 9: **Figure.8 Experimentally observed selection pressure Z vs theoretically predicted selection pressure ζ.** The Z and ζ values were calculated by eqn.(43) and eqn.(14), respectively. The symbols representing the N-values are the same as those in Fig.4. Note that the Z and ζ values for the 6-th generation were 43.9 and 1.16, respectively. Then, identifying it as the outlier, we removed this point. Correlation coefficient is 0.92 with $p = 5.9 \times 10^{-8}$.

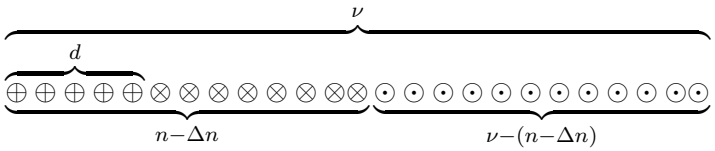


Figure 10: Figure.9 $\odot$ represents unchanged sites. $\oplus$ represents mutated sites. $\otimes$ represents non-mutated sites that change their site-fitness by the other mutations. Thus, the sites represented by $\oplus$ and $\otimes$ change their site-fitness.