
Relationship among phenotypic plasticity, phenotypic fluctuations, robustness, and evolvability; Waddington's legacy revisited under the spirit of Einstein

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Questions on possible relationship between phenotypic plasticity and evolvability, and that between robustness and evolution have been addressed over decades in the field of evolution-development. Based on laboratory evolution experiments and numerical simulations of gene expression dynamics model with an evolving transcription network, we propose quantitative relationships on plasticity, phenotypic fluctuations, and evolvability. By introducing an evolutionary stability assumption on the distribution of phenotype and genotype, the proportionality among phenotypic plasticity against environmental change, variances of phenotype fluctuations of genetic and developmental origins, and evolution speed is obtained. The correlation between developmental robustness to noise and evolutionary robustness to mutation is analysed by simulations of the gene network model. These results provide quantitative formulation on canalization and genetic assimilation, in terms of fluctuations of gene expression levels.

[Kaneko K 2009 Relationship among phenotypic plasticity, phenotypic fluctuations, robustness, and evolvability; Waddington's legacy revisited under the spirit of Einstein; *J. Biosci.* **34** 529–542]

1. Introduction

1.1 *Questions on evolution*

(i) How is evolvability characterized? Is it related with developmental process? Naively speaking, one may expect that if developmental process is rigid, phenotype is not easily changed, so that evolution does not progress so fast. Then one may expect that evolvability is tightly correlated with phenotypic plasticity. Here the plasticity refers to the changeability of phenotype as a result of developmental dynamics (Callahan *et al.* 1997; West-Eberhard 2003; Kirschner and Gerhart 2005; Pigliucci *et al.* 2006). If there is correlation between plasticity and evolvability (Ancel and Fontana 2002), one may formulate relationship between evolvability and development quantitatively.

(ii) Besides plasticity, an important issue in evolution is robustness. Robustness is defined as the ability to function against possible changes in the system (Barkai and Leibler 1997; Wagner *et al.* 1997; Alon *et al.* 2000; Wagner 2000,

2005; Ciliberti *et al.* 2007; Kaneko 2007). In any biological system, these changes have two distinct origins: genetic and epigenetic. The former concerns structural robustness of the phenotype, i.e. rigidity of phenotype against genetic changes produced by mutations. On the other hand, the latter concerns robustness against the stochasticity that can arise in environment or during developmental process, which includes fluctuation in initial states and stochasticity occurring during developmental dynamics or in the external environment.

Now, there are several questions associated with robustness and evolution. Is robustness increased or decreased through evolution? In fact, Schmalhausen (1949) proposed stabilizing evolution and Waddington (1942, 1957) proposed canalization. Both of them discussed the possibility that robustness increases through evolution. On the other hand, since the robustness increases rigidity in phenotype, the plasticity as well as the evolvability may be decreased accordingly through evolution.

Besides these robustness-evolution questions, another issue to be addressed is the relationship between the two

Keywords. Evolvability; genetic assimilation; phenotypic fluctuation; phenotypic plasticity

types of robustness, genetic and epigenetic. Do the two types of robustness increase or decrease in correlation through evolution? In fact, the epigenetic robustness concerns with developmental stability. Higher epigenetic robustness is, more stable the phenotype in concern shaped by development is. On the other hand, genetic robustness concerns with the stability against mutational change, i.e. evolutionary stability. Hence the question on a possible relationship between genetic and epigenetic robustness concerns with the issue on evolution-development relationship, that is an important topic in biology.

1.2 Formulation of the problem in terms of stochastic dynamical systems

Here we study the questions raised above, in terms of fluctuations. Let us first discuss epigenetic robustness, i.e. the degree of phenotype change as a result of developmental process. The *developmental dynamics* to shape a phenotype generally involves stochasticity. As has been recognized extensively, gene expression dynamics are noisy due to molecular fluctuation in chemical reactions. Accordingly, the phenotype, as well as the fitness, of isogenic individuals is distributed (Oosawa 1975; Spudich and Koshland 1976). Still, too large variability in the phenotype relevant to fitness should be harmful to the survival of the organisms. Phenotype that is concerned with fitness is expected to keep some robustness against such stochasticity in gene expression, i.e. robustness in ‘developmental’ dynamics to noise.

When the phenotype is less robust to noise, this distribution is broader. Hence, the variance of isogenic phenotypic distribution, denoted as V_{ip} here, gives an index for robustness to noise in developmental dynamics (Kaneko 2006, 2007). For example, distributions of protein abundances over isogenic individual cells have been measured, by using fluorescent proteins. The fluorescence level which gives an estimate of the protein concentration is measured either by flow cytometry or at a single cell microscopy (McAdams and Arkin 1997; Hasty *et al.* 2000; Elowitz *et al.* 2002; Bar-Even *et al.* 2004; Furusawa *et al.* 2005; Kaern *et al.* 2005; Krishna *et al.* 2005).

Genetic robustness is also measured in terms of fluctuation. Due to mutation to genes, the phenotype (fitness) is distributed. Since even phenotype of isogenic individuals is distributed, the variance of phenotype distribution of heterogenic population includes both the contribution from phenotypic fluctuation in isogenic individuals and that due to genetic variation. To distinguish the two, we first obtain the average phenotype over isogenic individuals, and then compute the variance of this average phenotype over heterogenic population. Then this variance

is due only to genetic heterogeneity. This variance is denoted as V_g here. Then, the robustness to mutation is estimated by this variance. If V_g is smaller, genetic change gives little influence on the phenotype, and larger genetic (or mutational) robustness is implied.

Phenotypic plasticity, on the other hand, represents the degree of change in phenotype against variation in environment. Quantitatively, one can introduce the following “response ratio” as a measure of plasticity. It is defined by the ratio of change in phenotype to environmental change. For example, measure the (average) concentration (x) of a specific protein. We measure this concentration by (slightly) changing the concentration (s) of an external signal molecule. Then, response ratio R is computed as the change in the protein concentration divided by the change in the signal concentration ($R = dx/ds$)¹. This gives a quantitative measure for the phenotypic plasticity. In general, there can be a variety of ways to change the environmental condition. In this case, one may also use the variance of such response ratios over a variety of environmental changes, as a measure of phenotypic plasticity.

By using these variances V_{ip} , V_g and plasticity the questions we address are represented as follows (Gibson and Wagner 2000; de Visser *et al.* 2000; Weinig 2000; Ancel and Fontana 2002; West-Eberhard 2003; Kirschner and Gerhart 2005):

- (i) Does the evolution speed increase or decrease with robustness?— *relationship between evolution speed with V_g or V_{ip} .*
- (ii) Are epigenetic and genetic robustness related? — *relationship between V_g and V_{ip} .*
- (iii) Does robustness in-(de-)crease through evolution? — *change of V_g or V_{ip} through evolution.*
- (iv) How is phenotypic plasticity related with robustness and evolution speed? — *relationship between R and V_g or V_{ip} .*

To answer these questions, we have carried out evolution experiments by using bacteria (Sato *et al.* 2003) and numerical simulations on catalytic reaction (Kaneko and Furusawa 2006) and gene networks (Kaneko 2007, 2008), while we have also developed a phenomenological theory on the phenotype distribution. Here we briefly review these results and discuss their relevance to evolution-development.

¹As will be discussed in Section 2.3, it is often relevant to define the phenotype variable after taking a logarithm. In this case a phenotype variable is defined as $x = \log(X)$ with X as the original variable, say, the protein concentration. Likewise, external signal often works in a logarithmic scale, so that the external parameter s is also defined as $\log(S)$, with S as a signal concentration. In this case $R = d\log(X)/d\log(S)$, which, indeed, is often adopted in cell biology.

2. Macroscopic approach I: Fluctuation-response relationship

One might suspect that isogenic phenotype fluctuations V_{ip} are not related to evolution, since phenotypic change without genetic change is not transferred to the next generation. However, the variance, a degree of fluctuation itself, can be determined by the gene, and accordingly it is heritable (Ito *et al.* 2009). Hence, there may be a relationship between isogenic phenotypic fluctuation and evolution. In fact, we have found evolutionary fluctuation-response relationship in bacterial evolution in the laboratory, where the evolution speed is positively correlated with the variance of the isogenic phenotypic fluctuation (Sato *et al.* 2003). This correlation or proportionality was confirmed in a simulation of the reaction network model (Kaneko and Furusawa 2006). The origin of proportionality between the isogenic phenotypic fluctuation and genetic evolution has been discussed in light of the fluctuation-response relationship in statistical physics (Einstein 1926; Kubo *et al.* 1985). In the present section we briefly summarize these recent studies.

2.1 Selection experiment by bacteria

We carried out a selection experiment to increase the fluorescence in bacteria and checked a possible relationship between evolution speed and isogenic phenotypic fluctuation. First, by attaching a random sequence to the N terminus of a wild-type Green Fluorescent Protein (GFP) gene, protein with low fluorescence was generated. The gene for this protein was introduced into *Escherichia coli*, as the initial generation for the evolution. By applying random mutagenesis only to the attached fragment in the gene, a mutant pool with a diversity of cells was prepared. Then, cells with highest fluorescence intensity are selected for the next generation. With this procedure, the (average) fluorescence level of selected cells increases by generations. The evolution speed at each generation is computed as the difference of the fluorescence levels between the two generations. Then, to observe the isogenic phenotypic fluctuation, we use a distribution of clone cells of the selected bacteria. The distribution of the fluorescence intensity of the clones was measured with the help of flow cytometry, from which the variance of fluorescence was measured.

From these data we plotted the evolution speed divided by the synonymous mutation rate versus the fluorescence variance². The data support strong correlation between the two, suggesting proportionality between the two (Sato *et al.* 2003).

²To be precise, the phenotype variable x is defined by $\log(\text{fluorescence})$, as will be discussed in Section 2.3.

2.2 Supports from numerical experiments

To confirm this relationship between the evolution speed and isogenic phenotypic fluctuation quantitatively, we have also numerically studied a model of reproducing cells consisting of catalytic reaction networks. Here the reaction networks of mutant cells were slightly altered from the network of their mother cells. Among the mutants, those networks with a higher concentration of a given, specific chemical component were selected for the next generation. Again, the evolution speed was computed by the increase of the concentration at each generation, and the fluctuation by the variance of the concentration over identical networks³. From extensive numerical experiments, the proportionality between the two was clearly confirmed (Kaneko and Furusawa 2006).

2.3 Remark on Gaussian type distribution

In the above experiment and numerical model, the fluorescence or the concentration obeys approximately the log-normal distribution, i.e. $\log(\text{fluorescence})$ or $\log(\text{concentration})$ obeys nearly the Gaussian distribution. In the following analysis that we borrow from statistical physics, the distribution is assumed to be nearly Gaussian. If the distribution is of the log-normal type, we adopt such quantity taking a logarithm, so that the distribution of the phenotypic variable is nearly Gaussian, in order to apply the theoretical discussion. In fact, Haldane suggested that most phenotypic variables should be defined after taking a logarithm. In the above analyses between fluctuation and response, all the quantities are defined and computed after taking a logarithm $\log(\text{fluorescence})$ and $\log(\text{concentration})$ are used to compute the evolution speed and the variance. The linear relationship between the two is confirmed by using these variables.

2.4 Fluctuation-response relationship

To understand the proportionality between the phenotypic fluctuation and the evolution speed, we first discuss a possible general relationship between fluctuation and response. Here, we refer to a measurable phenotype quantity (e.g. logarithm of the concentration of a protein) as a “variable” x of the system, while we assume the existence of a “parameter” which controls the change of the variable. The parameter corresponding to the variable x is indicated as a . In the case of adaptation, this parameter is a quantity to specify the environmental condition, while, in the case of evolution, the parameter is an index of genotype that governs the corresponding phenotypic variable.

³Again, the phenotypic variable x is defined by $\log(\text{concentration})$, and the variance is measured by the distribution of $\log(\text{concentration})$ (see Section 2.3).

Even among individuals sharing the identical gene (the parameter) a , the variables x are distributed. The distribution $P(x;a)$ of the variable x over cells is defined, for a given parameter a (e.g. genotype). Here the “average” and “variance” of x are defined with regards to the distribution $P(x;a)$ (see figure 1 for schematic representation of the fluctuation-response relationship).

Consider the change in the parameter value from a to $a \rightarrow a + \Delta a$. Then, the proposed fluctuation-response relationship (Sato *et al.* 2003; Kaneko 2006) is given by

$$\frac{\langle x \rangle_{a+\Delta a} - \langle x \rangle_a}{\Delta a} \propto \langle (\delta x)^2 \rangle, \quad (1)$$

where $\langle x \rangle_a$ and $\langle (\delta x)^2 \rangle = \langle (x - \langle x \rangle)^2 \rangle$ are the average and variance of the variable x for a given parameter value a , respectively.

The above relationship is derived by assuming that the distribution $P(x;a)$ is approximately Gaussian and that the effect of the change in a on the distribution is represented by a bi-linear coupling between x and a . With this assumption, the distribution is written by

$$P(x;a) = N_0 \exp\left(-\frac{(x - X_0)^2}{2\alpha(a)} + v(x,a)\right), \quad (2)$$

with N_0 a normalization constant so that $\int P(x;a) dx = 1$.

Here, X_0 is the peak value of the variable at $a = a_0$, where the term $v(x, a)$ gives a deviation from the distribution at $a = a_0$, so that $v(x, a)$ can be expanded as $V(x,a) = C(a-a_0)(x-X_0) + \dots$, with C as a constant, where \dots is a higher order term in $(a-a_0)$ and $(x-X_0)$ which will be neglected in the following analysis.

Assuming this distribution form, the change of the average value $\langle x \rangle$ following the change of the parameter from a_0 to $a_0 + \Delta a$ is straightforwardly computed, which leads to

$$\frac{\langle x \rangle_{a=a_0+\Delta a} - \langle x \rangle_{a=a_0}}{\Delta a} = C\alpha(a_0 + \Delta a). \quad (3)$$

Noting $\alpha = \langle (\delta x)^2 \rangle$, and neglecting deviation between $\alpha(a_0 + \Delta a)$ and $\alpha(a_0)$, we get

$$\frac{\langle x \rangle_{a=a_0+\Delta a} - \langle x \rangle_{a=a_0}}{\Delta a} = C \langle (\delta x)^2 \rangle. \quad (4)$$

This relationship is a general result that holds for Gaussian-like distributions if changes in parameters are represented by a “linear coupling term”, which brings about a shift of the average of the corresponding variable.

Remark: Although the above formula is formally similar to that used in statistical physics, it is not grounded on any established theory. It is a proposal here. We set up a phenomenological description so that the formula is consistent with the data obtained by experiments and numerical simulations on reaction network models. (However, such phenomenological description is the first necessary step in science, as the history of thermodynamics tells.) The choice of the distribution form is not derived from the first principle, but is based on several assumptions.

First, the distribution must be close to Gaussian, or the variable has to be scaled properly so that the corresponding distribution is nearly Gaussian. If the variance of the distribution of the variable in concern is not very large, by suitable choice of variable transformation, the distribution can be transformed to be nearly Gaussian. As mentioned in Section 2.3, log-normal distribution is often observed in phenotypic variables z in a biological system, say the concentration of some protein. In this case, one can just adopt $x = \log(z)$ as a phenotypic variable in concern.

Second, the choice of the coupling form in eq. (2) is also an assumption so that the fluctuation-response relation is consistent with experimental and numerical observations. With this bi-linear coupling form $C(x-X_0)(a-a_0)$, the relationship is obtained. In this sense, eq. (4) is not derived, but we choose a phenomenological distribution function (2) so that eq. (4) is reached to be consistent with the observations in experiments or numerical models. Here, neglect of higher-order terms as $(x-X_0)^k (a-a_0)^m$ (with $k, m > 1$) will be justified if the deviations from a_0 and X_0 are small, but the validity of such expansion in the distribution itself is a proposal here. In other words, possible dependence of C on the parameter a is discarded, so that C is regarded as a constant. This will be justified if the change in a in concern is not so large.

2.5 Plasticity-fluctuation relationship

First we apply the above relation to plasticity. Here we make the following correspondence:

- (i) a — parameter to characterize the environmental condition
- (ii) change in a — change in environment
- (iii) $\langle x \rangle$ — a variable characterizing a phenotypic state
- (iv) change in $\langle x \rangle$ — change in the average phenotype due to the environmental change
- (v) $\langle (\delta x)^2 \rangle$ — variance of the phenotypic distribution at a given fixed environmental condition.

Then, the relationship eq. (1) means that the plasticity is proportional to the fluctuation.

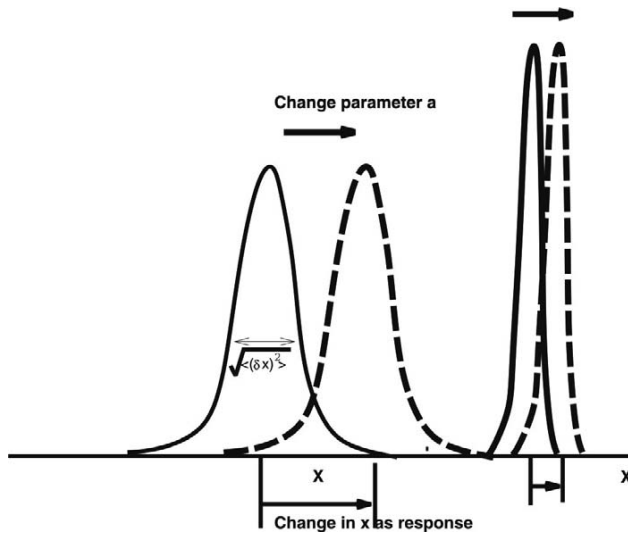


Figure 1. Schematic diagram on fluctuation-response relationship. The distribution function $P(x; a)$ is shifted by the change of the parameter a . If the variance is larger the shift is larger.

2.6 Evolutionary fluctuation-response relationship

This fluctuation-response relationship can be applied to evolution by making the following correspondence:

- (i) a — a parameter that specifies the genotype
- (ii) change in a — genetic mutation
- (iii) change in $\langle x \rangle$ — change in the average phenotype due to the genetic change, and
- (iv) $\langle (\delta x)^2 \rangle$ — variance of the phenotypic distribution over clones V_{ip} .

Then, the above relationship (1) suggests that the evolution speed of a phenotype (i.e. the change of the average phenotype per generation) divided by the mutation rate is proportional to V_{ip} , the variance of isogenic phenotypic fluctuation. This is what we have obtained in the experiment and simulations.

The above proposition can be interpreted as follows: As the degree of the fluctuations of the phenotype decreases, the change of the phenotype under a given rate of genetic change also decreases. In other words, the larger the phenotypic fluctuation, the larger the evolution speed is. In this sense, V_{ip} can work as a measure for the degree of evolvability.

3. Macroscopic approach II: Stability theory for genotype-phenotype mapping

The evolutionary fluctuation-response relationship mentioned above casts another mystery to be solved. There is an established relationship between evolutionary speed

and phenotypic fluctuation. It is the so-called fundamental theorem of natural selection by Fisher (1930) which states that evolution speed is proportional to V_g , the variance of the phenotypic fluctuation due to genetic variation. It is given by the variance of the *average* phenotype for each genotype over a heterogenic population. In contrast, the evolutionary fluctuation — response relationship, proposed above, concerns phenotypic fluctuation of isogenic individuals as denoted by V_{ip} . While V_{ip} is defined as a variance over clones, i.e., individuals with the same genes, V_g is a result of the heterogenic population distribution. Hence, the fluctuation-response relationship and the relationship concerning V_g by Fisher's theorem are not identical.

If V_{ip} and V_g are proportional through an evolutionary course, the two relationships are consistent. Such proportionality, however, is not self-evident, as V_{ip} is related to variation against the developmental noise and V_g is against the mutation. The relationship between the two, if it exists, postulates a constraint on genotype-phenotype mapping and may create a quantitative formulation of a relationship between development and evolution.

Here we formulate a phenomenological theory so that the evolutionary fluctuation-response relationship and Fisher's theorem is consistent (see figure 2). Recall that originally the phenotype is given as a function of gene, so that we considered the conditional distribution function $P(x; a)$. However, the genotype distribution is influenced by phenotype through the selection process, as the fitness for selection is a function of phenotype. Now, consider a gradual evolutionary process. Then, it could be possible to assume that some “quasi-static” distribution on genotype and phenotype is obtained as a result of feedback process from phenotype to genotype (see figure 2). Considering this point, we hypothesize that there is a two-variable distribution $P(x, a)$ both for the phenotype x and genotype a .

By using this distribution, V_{ip} , variance of x of the distribution for given a , can be written as $V_{ip}(a) = \int (x - \bar{x}(a))^2 P(x, a) dx$, where $\bar{x}(a)$ is the average phenotype of a clonal population sharing the genotype a , namely $\bar{x}(a) = \int P(x, a) x dx$. V_g is defined as the variance of the average $\bar{x}(a)$, over genetically heterogeneous individuals and is given by $V_g = \int (\bar{x}(a) - \langle \bar{x} \rangle)^2 p(a) da$, where $p(a)$ is the distribution of genotype a and $\langle \bar{x} \rangle$ as the average of $\bar{x}(a)$ over all genotypes.

Assuming the Gaussian distribution again, the distribution $P(x, a)$ is written as follows:

$$P(x, a) = \hat{N} \exp \left[-\frac{(x - X_0)^2}{2\alpha(a)} + C(x - X_0)(a - a_0) - \frac{1}{2\mu}(a - a_0)^2 \right], \quad (5)$$

with \hat{N} as a normalization constant. The Gaussian distribution $\exp(-\frac{1}{2\mu}(a - a_0)^2)$ represents the distribution of geno-

types at around $a = a_0$, whose variance is (in a suitable unit) the mutation rate μ . The coupling term $C(x - X_0)(a - a_0)$ represents the change of the phenotype x by the change of genotype a . Recalling that the above distribution (5) can be rewritten as

$$P(x, a) = \hat{N} \exp\left[-\frac{(x - X_0 - C(a - a_0)\alpha(a))^2}{2\alpha(a)} + \left(\frac{C^2\alpha(a)}{2} - \frac{1}{2\mu}\right)(a - a_0)^2\right], \quad (6)$$

the average phenotype value for given genotype a satisfies

$$\bar{x}_a \equiv \int xP(x, a)dx = X_0 + C(a - a_0)\alpha(a). \quad (7)$$

Now we postulate evolutionary stability, i.e. at each stage of the evolutionary course, the distribution has a single peak in (x, a) space. This assumption is rather natural for evolution to progress robustly and gradually. When a certain region of phenotype, say $x > x_{thr}$, is selected, the evolution to increase x works if the distribution is concentrated. On the other hand, if the peak is lost and the distribution is flattened, then selection to choose a fraction of population to increase x no longer works, as the distribution is extended to very low-fitness values (see also a remark at the end of this section). In the above form, this stability condition is given by

$$\frac{\alpha C^2}{2} - \frac{1}{2\mu} \leq 0, \text{ i.e.}$$

$$\mu \leq \frac{1}{\alpha C^2} \equiv \mu_{max}. \quad (8)$$

This means that the mutation rate has an upper bound μ_{max} beyond which the distribution does not have a peak in the genotype–phenotype space. In the above form, the distribution becomes flat at $\mu = \mu_{max}$, so that mutants with low fitness rate appear, as discussed as error catastrophe by Eigen (Eigen and Schuster 1979) (see also the remark at the end of this section). Recalling $V_g = \langle (\bar{x}(a) - X_0)^2 \rangle$ and eq.(6), V_g is given by $(\alpha C^2) \langle (\delta a)^2 \rangle$. Here, $(\delta a)^2$ is computed by the average over $P(x, a)$, so that it is obtained by

$$\hat{N} \int (a - a_0)^2 \exp\left[\left(\frac{C^2}{2\alpha(a)} - \frac{1}{2\mu}\right)(a - a_0)^2\right] da.$$

From this formula we obtain

$$V_g = \frac{\mu C^2 \alpha^2}{1 - \mu C^2 \alpha} = \alpha \frac{\frac{\mu}{\mu_{max}}}{1 - \frac{\mu}{\mu_{max}}}. \quad (9)$$

If the mutation rate μ is small enough to satisfy $\mu \ll \mu_{max}$, we get

$$V_g \sim \frac{\mu}{\mu_{max}} V_{ip}, \quad (10)$$

by recalling that $V_{ip}(a) = \alpha(a)$. Thus we get the proportionality between V_{ip} and V_g .

Note that if we choose the distribution consisting of heterogenic individuals taking a given phenotype value x only, the variance $\langle (a - a_0)^2 \rangle_{fixed-x}$ is given simply by μ . The variance of average phenotype over such population sharing the phenotype value x is defined as V_{ig} (Kaneko and Furusawa 2008). Then, by using eq. (7) we get $V_{ig} = (\alpha C)^2 \langle (a - a_0)^2 \rangle_{fixed-x} = \mu (\alpha C)^2$, instead of eq.(9). Accordingly, the inequality (8) is rewritten as

$$V_{ip} \geq V_{ig}, \quad (11)$$

while $V_{ip} = \frac{\mu}{\mu_{max}} V_{ig}$ is also obtained without assuming $\mu \ll \mu_{max}$. If the evolution process successively increases the selected value of x by generations, the distribution at each generation is centered at around a given phenotype. In such case, V_{ig} , instead of V_g , may work as a relevant measure for the phenotypic variance due to genetic change. Then the inequality (11) can work as a measure for stability.

Note again that the above relationships (10) and (11) are not derived from the first principle, but a phenomenological description to be consistent with numerical experiments. There are several assumptions to obtain the relationships.

First, whether genotype is represented by a continuous parameter a is not a trivial question, as gene is coded by a genetic sequence. Possible change by mutation to the sequence can be represented by a walk along a high-dimensional hyper-cubic lattice, and whether one can introduce a scalar parameter a instead is not self-evident. A candidate for such scalar parameter is Hamming distance from the fittest sequence, or a projection according to the corresponding phenotype (Sato and Kaneko 2007). We note that such scalar genetic parameter is often adopted in population genetics also.

Second, description by using a two-variable distribution in genotype and phenotype $P(x, a)$ is not trivial, either. As the gene controls the phenotype, use of the conditional distribution $P(x|a)$, i.e. the distribution of phenotype for given genotype a would be natural, to compute $P(x, a)$ as $p(a)P(x|a)$. In contrast, the eq. (5) takes symmetric form with regards to x and a . Here the form is chosen after taking into account of selection process based on phenotype x . This selection process introduces a feedback from phenotype to the genotype distribution as in figure 2. Considering quasi-static evolutionary process to select phenotype x , the form (5) is chosen for $P(x, a)$.

Third, the stability assumption is introduced for robust and gradual evolution, postulating that $P(x, a)$ has a single peak in (x, a) space at each generation. Fourth, existence of threshold mutation rate μ_{max} is implicitly assumed, beyond which the stability condition is not satisfied. In other words, existence of error catastrophe is implicitly assumed. Such catastrophe does not exist for every coupling

form in eq. (5). For example, if we adopted the distribution

$$\exp\left(-\frac{(x-c(a-a_0))^2}{2\alpha} - \frac{(a-a_0)^2}{2\mu}\right),$$

instead, the stability condition would always be satisfied, as long as $\alpha > 0$ and $\mu > 0$. In other words, we assumed existence of such loss of stability with the increase of the mutation rate, to choose the coupling form in eq. (5). (see also a remark below).

Fifth, to get the proportionality (10), additional assumption is made on the smallness of the mutation rate μ , as well as the constancy of μ and μ_{\max} through the evolutionary course.

Note again we have used the expansion up to the second order around a_0 and X_0 in eq. (5), i.e. the Gaussian-like distribution as well as the coupling term $C(x-X_0)(a-a_0)$. In fact, this specific form is not necessary. By taking a generalized form, the single-peaked distribution assumption leads to the Hessian condition for $\log P(x, a)$ around the peak at $a = a_0$ and $x = X_0$. This leads to the inequality (11)⁴. For example, when we take the coupling $C'(x-X_0)(a-a_0)/\alpha$ in eq. (5), the inequality (8) is replaced by $\mu < \alpha C'^2 = \mu_{\max}$, whereas the equations on V_g is replaced by $V_g = \mu C'^2 / (1 - \mu C'^2 / \alpha)$, so that the final form in eq. (9) is identical.

Remark: At $\mu = \mu_{\max}$, the distribution (5) becomes flat. In fact, such loss of stability of a fitness distribution roughly corresponds to error catastrophe by Eigen and Schuster (1979), where non-fitted mutants accumulate with the increase of mutation rate, due to their combinatorial explosion. When fitted genotypes are rare, they are not sustained against the increase of the mutation rate.

In our formulation, however, we cannot tell anything about the distribution when the inequality (8) (or equivalently eq. [11]) is not satisfied. In the formulation with Gaussian distribution, the distribution of phenotype is symmetric around the peak. However, near this catastrophic point, the variance increases so that the expansion up to the second order in eq. (5) will no longer be valid. In general, the genetic sequence giving rise to fitted phenotype is rare, and thus the distribution at $\mu \sim \mu_{\max}$ is expected to be extended mostly to the lower fitness side, due to possible higher order terms in the expansion of $a-a_0$ and $x-X_0$. (In other words, this asymmetry to the side of lower fitness values in the distribution is also expected from the projection to a one-dimensional scalar variable a from a genetic sequence.) Accordingly, it is expected that the evolution to select a higher fitness phenotype no longer works when the inequality is not satisfied, as discussed by Eigen as error catastrophe.

A remark should be made here: In the discussion of Eigen, phenotype is uniquely determined by the genotype. In contrast, the present error catastrophe is associated

with stochasticity in genotype-phenotype mapping, as demonstrated by $V_{ip} > 0$, which is a result of noise encountered during the developmental dynamics. In fact, in the example to be discussed in the next section, the catastrophe occurs as a result of the decrease in the noise strength in developmental dynamics. In this sense, the error catastrophe here is not identical with that by Eigen.

4. Microscopic approach: Gene regulation network model

As the above discussion on phenotypic variances on genetic and epigenetic origins is based on several assumptions, it is important to study the evolution of robustness and phenotypic fluctuations, by using a model with “developmental” dynamics of phenotype. To introduce such dynamical systems model, one needs to consider the following structure of evolutionary processes.

- (i) There is a population of organisms with a distribution of genotypes.
- (ii) Phenotype is determined by genotype, through “developmental” dynamics.
- (iii) The fitness for selection, i.e. the offspring number, is given by the phenotype.
- (iv) The distribution of genotypes for the next generation changes according to the change of genotype by mutation, and selection process according to the fitness.

During evolution *in silico*, procedures (i), (iii), and (iv) are adopted as genetic algorithms. Here, however, it is important that the phenotype is determined only after (complex) ‘developmental’ dynamics (ii), which are stochastic due to the noise therein.

We previously studied a protocell model with catalytic reaction network in which cells that have a higher concentration of a specific chemical are selected among the networks having different paths. Extensive numerical simulations confirm the inequality $V_{ip} > V_{ig}$, error catastrophe at $V_{ip} \sim V_{ig}$, and the proportionality between V_{ip} and V_g . Note that in the numerical evolution of this model, none of the five assumptions in the last section are adopted.

As another example, we have studied gene expression dynamics that are governed by regulatory networks (Glass and Kauffman 1973; Mjolsness *et al.* 1991; Salzar-Ciudad *et al.* 2000). The developmental process (ii) is given by this gene expression dynamics. It shapes the final gene expression profile that determines the evolutionary fitness. Mutation at each generation alters the network slightly. Among the mutated networks, we select those with higher fitness values.

To be specific, the dynamics of a given gene expression level x_i is described by:

$$dx_i/dt = \gamma(f(\sum_j^M J_{ij}x_j) - x_i) + \sigma\eta_i(t), \quad (12)$$

⁴Note V_g in (Kaneko and Furusawa 2006) needs to be interpreted as V_{ig} here, as remarked in Kaneko and Furusawa (2008).

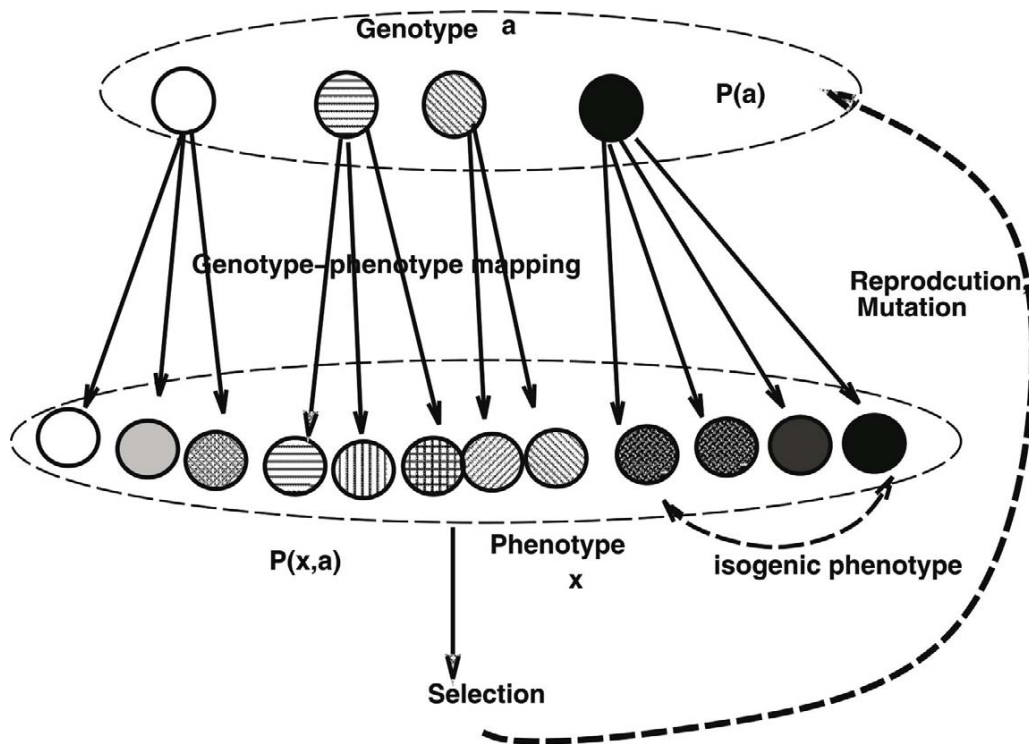


Figure 2. Schematic representation of genotype-phenotype relationship. The genotype a is distributed by $p(a)$, whereas there is a stochastic mapping from the genotype to phenotype as a result of developmental dynamics with noise. Thus the phenotype x is distributed even for isogenic individuals, so that the distribution $P(x,a)$ is defined. Mutation-selection process works on phenotype x , so that a feedback from $P(x,a)$ to the genotype distribution $P(a)$ is generated.

where $J_{ij} = -1, 1, 0$, and $\eta_i(t)$ is Gaussian white noise given by $\langle \eta_i(t) \eta_j(t') \rangle = \delta_{ij} \delta(t-t')$. M is the total number of genes. The function f represents gene expression dynamics following Hill function, or described by a certain threshold dynamics (Kaneko 2007, 2008).

The value of σ represents the strength of noise encountered in gene expression dynamics, originated in molecular fluctuations in chemical reactions. We prefix the initial condition for this “developmental” dynamical system (say, at the condition that none of the genes are expressed). The phenotype is a pattern of $\{x_j\}$ developed after a certain time.

The fitness F is determined as a function of x_i . Sometimes, not all genes contribute to the fitness. It is given by a function of only a set of “output” gene expressions. In the paper (Kaneko 2007), we adopted the fitness as the number of expressed genes among the output genes $j = 1, 2, \dots, k < M$, i.e. x_j greater than a certain threshold. Selection is applied after the introduction of mutation at each generation in the transcriptional regulation network, i.e. the matrix J_{ij} .

Among the mutated networks, we select a certain fraction of networks that has higher fitness values. Because the network J_{ij} determines the ‘rule’ of the dynamics, it is natural to treat J_{ij} as a measure of genotype. Individuals with different genotypes have different sets of J_{ij} .

As the model contains a noise term, whose strength is given by σ , the fitness can fluctuate among individuals sharing the same genotype J_{ij} . Hence the fitness F is distributed. This leads to the variance of the isogenic phenotypic fluctuation denoted by $V_{ip}(\{J\})$ for a given genotype (network) $\{J\}$, i.e.

$$V_{ip}(\{J\}) = \int dF \hat{P}(F; \{J\}) (F - \bar{F}(\{J\}))^2, \quad (13)$$

where $\hat{P}(F; \{J\})$ is the fitness distribution over isogenic species sharing the same network J_{ij} , and $\bar{F}(\{J\}) = \int dF \hat{P}(F; \{J\}) F$ is the average fitness. To check the relationship between V_{ip} and V_g , we either use the V_{ip} for $\{J\}$ that gives the peak value of the fitness distribution or the average $\bar{V}_{ip} = \int d\{J\} p(\{J\}) V_{ip}(\{J\})$, where $p(\{J\})$ is the population distribution of the genotype $\{J\}$. In both cases, the same result on the relationship between V_{ip} and V_g is obtained. Here we briefly describe the result with \bar{V}_{ip} . At each generation, there exists a population of individuals with different genotypes $\{J\}$. On the other hand, the phenotypic variance by genetic distribution is given by

$$V_{ig} = \int d\{J\} p(\{J\}) (\bar{F}(\{J\}) - \bar{F})^2, \quad (14)$$

where $\langle \bar{F} \rangle = \int p(\{J\}) \bar{F}(\{J\}) d\{J\}$ is the average of average fitness over all populations. (Here, as we continuously select fitted phenotypes, even if they are rare, the remaining population mainly consists only of those having nearly the optimal fitness value. Thus we expect that the variance by the above genetic algorithm roughly estimates V_{ig} rather than V_g , according to the formulation of the last section.)

Results from several evolutionary simulations of this class of models are summarized as follows:

- (i) There is a certain threshold noise level σ_c , beyond which the evolution of robustness progresses, so that both V_{ig} and \bar{V}_{ip} decrease. There, most of the individuals take the highest fitness value. In contrast, for a lower level of noise $\sigma < \sigma_c$, mutants that have very low fitness values always remain. There are many individuals taking the highest fitness value, whereas the fraction of individuals with much lower fitness values does not decrease. Hence the increase of the average fitness value over the total population stops at a certain level.
- (ii) At around the threshold noise level, V_{ig} approaches \bar{V}_{ip} and at $\sigma < \sigma_c$, $V_{ig} \sim \bar{V}_{ip}$ holds, whereas for $\sigma > \sigma_c$, $\bar{V}_{ip} > V_{ig}$ is satisfied. For robust evolution to progress, this inequality is satisfied.
- (iii) When noise is larger than this threshold, $V_{ig} \sim V_g \propto \bar{V}_{ip}$ holds (see figure 3), through the evolution course after a few generations. (Here as $V_{ig} \ll V_{ip}$, the difference between V_{ig} and V_g is negligible).

Thus, the results are consistent with those given by the phenomenological theory in the last section. Here, however, we should note that there is not a direct correspondence with the phenomenological distribution theory and the gene expression network model. So far no direct derivation of the former from the latter is available: The genotype here is given by the matrix $\{J\}$, and is not *a priori* represented by a scalar parameter a . The accumulation of low-fitness mutants with the decrease of noise level corresponds to the error catastrophe at $V_{ip} \sim V_{ig}$ but existence of such error catastrophe itself is not *a priori* assumed in the model. The proportionality of the two variances is not evident in the model, either. Indeed, as mentioned above, the proportionality is observed only after some generations, when evolution progresses steadily. This might correspond to the assumption for the quasi-static evolution of $P(x, a)$.

Why does the system not maintain the highest fitness state under a small phenotypic noise level with $\sigma < \sigma_c$? Indeed, the dynamics of the top-fitness networks that evolved under such low noise level have distinguishable dynamics from those that evolved under a high noise level. It was found that, for networks evolved under $\sigma < \sigma_c$, a large portion of the initial conditions reach attractors that give the highest fitness values, while for those evolved under $\sigma > \sigma_c$, only a

tiny fraction (i.e. in the vicinity of all-off states) is attracted to them.

When the time course of gene expression dynamics to reach its final pattern (attractor) is represented as a motion falling along a potential valley, our results suggest that the potential landscape becomes smoother and simpler through evolution and loses its ruggedness after a few dozen generations, for $\sigma > \sigma_c$. In this case, the 'developmental' dynamics gives a global, smooth attraction to the target (see figure 4). In fact, such type of developmental dynamics with global attraction is known to be ubiquitous in protein folding dynamics (Abe and Go 1980; Onuchic *et al.* 1995), gene expression dynamics (Li *et al.* 2004), and signal transduction network (Wang *et al.* 2006). On the other hand, the landscape evolved at $\sigma < \sigma_c$ is rugged. Except from the vicinity of given initial conditions, the expression dynamics do not reach the target pattern, as in the lower column of figure 4.

Now, consider mutation to a network to alter slightly a few elements $J_{i,j}$ of the matrix J . This introduces slight alterations in gene expression dynamics. In smooth landscape with global basin of attraction, such perturbation in dynamics makes only little change to the final expression pattern (attractor). In contrast, under the dynamics with rugged developmental landscape, a slight change easily destroys the attraction to the target attractor. Then, low-fitness mutants appear. This explains why the network evolved at a low noise level is not robust to mutation. In other words, evolution to eliminate ruggedness in developmental potential is possible only for a sufficiently high noise level.

We expect these relationships (i)–(iii) as well as the existence of the error catastrophe to be generally valid for systems satisfying the following conditions.

- (A) Fitness is determined through developmental dynamics.
- (B) Developmental dynamics is complex so that it may fail to produce the target phenotype that gives a high fitness value, when perturbed by noise.
- (C) There is effective equivalence between mutation to gene and noise in the developmental dynamics, with regards to phenotypic change.

Condition (A) is generally satisfied for any biological system. Even in a unicellular organism, phenotype is shaped through gene expression dynamics. The condition (B) is the assumption on the existence of 'error catastrophe'. A phenotypic state with a higher function is rather rare, and it is not easy to reach such a phenotype through the dynamics involving many degrees of freedom. In our model, the condition (B) is provided by the complex gene expression dynamics to reach a target expression pattern. In a system with (A)–(B), slight differences in genotype may lead to a large differences in phenotype, as the phenotype is determined after temporal integration of the developmental

dynamics where slight differences in genotype (i.e. in the rule that governs the dynamics) are accumulated through the course of development and amplified. This introduces “error catastrophe” as a result of noise in developmental dynamics.

Postulate (C) also seems to be satisfied generally; by noise, developmental dynamics are modulated to change a path in the developmental course, whereas changes introduced by mutation into the equations governing the developmental dynamics can have a similar effect. In the present model, the phenotypic fluctuation to increase synthesis of a specific gene product and mutation to add another term to increase such production can contribute to the increase in the expression level of a given gene in a similar manner.

5. Discussion

Our theory has several implications in development and evolution. We briefly discuss a few topics.

5.1 *Evolvability and its relationship with fluctuations: answer to question 1*

In section 2, we have proposed the proportionality between phenotypic plasticity and phenotypic fluctuation V_{ip} as a natural consequence of fluctuation-response relationship. Then we have proposed the proportionality between this epigenetic phenotypic fluctuation V_{ip} and the fluctuation due to genetic variation, V_g . Since the latter is proportional

to the evolution speed according to Fisher’s theorem, the phenotypic plasticity and evolvability are correlated through the phenotypic fluctuation. Although such relationship was discussed qualitatively over decades, quantitative conclusion has not been drawn yet. Here we have proposed such quantitative relationship by phenomenological distribution theory, to be consistent with the data from numerical and *in vivo* experiments.

Waddington proposed genetic assimilation in which phenotypic change due to the environmental change is later embedded into genes (Waddington 1953, 1957). The proportionality among phenotypic plasticity, V_{ip} , and V_g is regarded as a quantitative expression of such genetic assimilation, since V_g gives an index for the evolution speed.

Evolvability may depend on species. Some species called living fossil preserve their phenotype over much longer generations than others. An origin of such difference in evolvability could be provided by the degree of rigidity in developmental process. If the phenotype generated by developmental process is rigid, then the phenotypic fluctuation as well as phenotypic plasticity against environmental change is smaller. Then according to the theory here, the evolution speed will be smaller.

Evolvability depends not only on species but on each gene in a given species. Some proteins could evolve more rapidly than others. If we apply the fluctuation-response relationship in section 2 the expression level of each gene, correlation between the level of fluctuation of each gene expression and its evolution speed is expected to exist not only through the evolutionary course, but also for different genes in a given

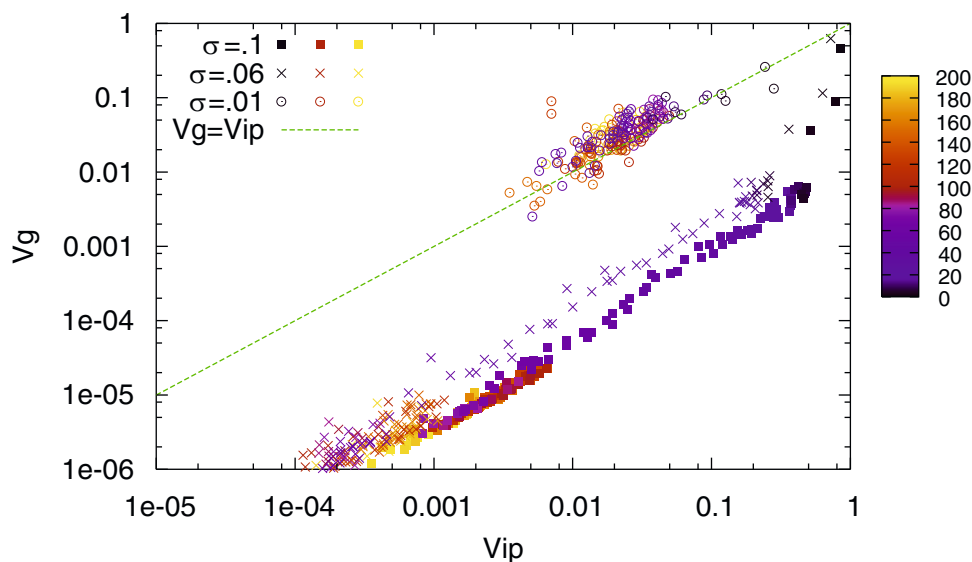


Figure 3. The relationship between V_g (V_{ig}) and \bar{V}_{ip} . V_g is computed from the distribution of fitness over different $\{J\}$ at each generation (eq. [14]), and \bar{V}_{ip} by averaging the variance of isogenic phenotype fluctuations over all existing individuals (eq. [13]). Computed by using the model eq. [12] (see [Kaneko 2007] for detailed setup). Points are plotted over 200 generations, with gradual colour change as displayed. $\sigma = 0.01$ (\circ), 0.06 (\times), and 0.1 (\blacksquare). For $\sigma > \sigma_c \approx 0.02$, both decrease with successive generations.

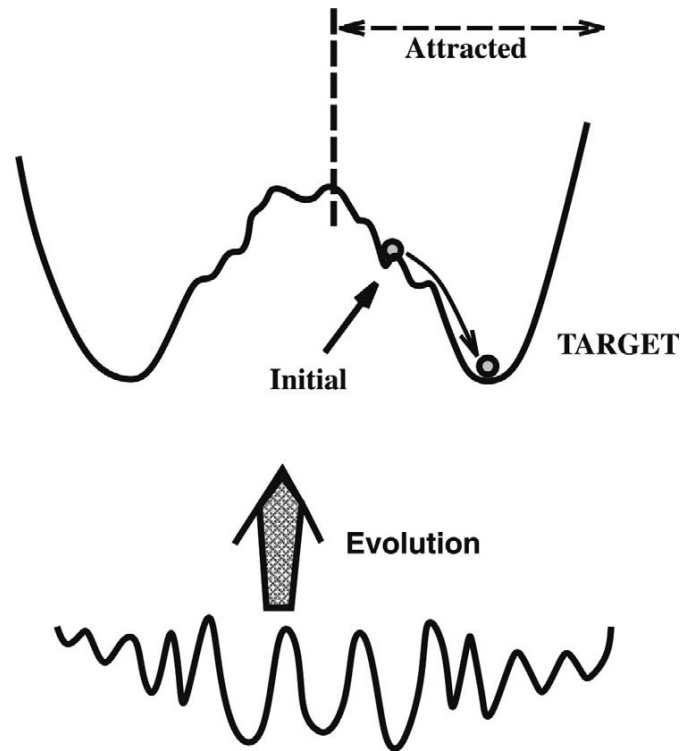


Figure 4. Schematic representation of the basin structure, represented as a process of descending a potential landscape. In general, developmental dynamics can have many attractors, and depending on initial conditions, they may reach different phenotypes, so that the developmental landscape is rather rugged. After evolution, global and smooth attraction to the target phenotype is shaped by developmental dynamics, if gene expression dynamics are sufficiently noisy.

generation. To discuss such dependence on each protein, we have studied the model discussed in section 4, consisting of expression levels of a variety of genes, and found the proportionality between V_g and V_{ip} over expressions of a variety of genes. Indeed, we have measured the variances for expression levels of each gene i , and defined their genetic and epigenetic variances $V_g(i)$ and $V_{ip}(i)$ for each. From several simulations, we have confirmed the proportionality between $V_g(i)$ and $V_{ip}(i)$ over many genes i (Kaneko 2008). Although direct experimental support is not available yet, recent data on isogenic phenotypic variance and mutational variance over proteins in yeast may suggest the existence of such correlation between the two (Landry *et al.* 2007; Lehner 2008).

5.2 Evolution of robustness and relevance of noise:

Answer to the question 2

As mentioned in the introduction, biological robustness is the insensitivity of fitness or phenotype to system's changes. Thus, the developmental robustness to noise is characterized by the inverse of V_{ip} , while the mutational robustness is characterized by the inverse of V_g . In Section 4, it is shown that the two variances decrease in proportion

through the evolution under a fixed fitness condition, if there exists a certain level of noise in the development. Robustness evolves, as discussed by Schmalhausen's stabilizing selection (Schmalhausen 1949) and Waddington's canalization (Waddington 1942, 1957). On the other hand, the proportionality between the two variances implies the correlation between developmental and mutational robustness. Note that this evolution of robustness is possible only under a sufficient level of noise in development. Robustness to noise in development brings about robustness to mutation.

Our result demonstrates the role of noise during phenotypic expression dynamics to evolution of robustness. Despite recent quantitative observations on phenotypic fluctuation, noise is often thought to be an obstacle in tuning a system to achieve and maintain a state with a higher function, because the phenotype may be deviated from a state with a higher function, perturbed by such noise. Indeed, questions most often asked are how a given biological function can be maintained in spite of the existence of phenotypic noise (Ueda *et al.* 2001; Kaern *et al.* 2005). In contrast, we have revealed a positive role of noise to evolution quantitatively. We also note that relevance of noise to adaptation (Kashiwagi *et al.* 2006; Furusawa and

Kaneko 2008) and to cell differentiation (Kaneko and Yomo 1999) is recently discussed.

In our theory, robustness in genotype and phenotype are represented by the single-peaked-ness in the distribution $P(x = \text{phenotype}, a = \text{genotype})$. If we write the distribution in a “potential form” as

$$P(x, a) = \exp(-U(x, a)), \quad (15)$$

the stability condition means existence of global minimum of the potential $U(x, a)$. With the increase in the slope of the attraction in the potential, the robustness increases. The smooth attraction in the developmental potential shown in Section 4 is a representation of canalization in the epigenetic landscape by Waddington, whereas the correlation in the attractions in phenotypic (x) and genetic (a) directions is one representation of genetic assimilation.

5.3 Future issues

The proportionality between the phenotypic variances of isogenic individuals and that due to genetic variation in Section 2 and Section 3 is not a result of derivation from the first principle, but is a result of phenomenological description based on several assumptions. Although numerical results on gene expression network and catalytic reaction network models support the relationship, there remains a large gap between the macroscopic phenomenological theory and microscopic simulations. It should be important to establish a statistical physics theory to fill the gap between the two (see also Sakata *et al.* [2009] for a study towards this direction).

All of the plasticity, fluctuation, and evolvability decrease in our model simulations through the course of evolution, under selection by a fixed fitness condition. Considering that none of the phenotypic plasticity, fluctuation, and evolvability vanishes in the present organisms, some process for restoration or maintenance of plasticity or fluctuation should also exist. To consider such process, the followings are necessary:

- (1) *Fitness with several conditions:* In our model study (and also in experiments with artificial selection), the fitness condition is imposed by a single condition. In nature, the fitness may consist in several conditions. In this case, we may need more variables in the characterization of the distribution function P , say $P(x, y, a, b)$ etc. In such multi-dimensional theory, interference among different phenotypic variable x and y may contribute to the increase or maintenance of fluctuations.
- (2) *Environmental variation:* If the environmental condition changes in time, the fluctuation level may be sustained. To mimic the environmental change, we have altered the condition of target

gene expression pattern in the model of Section 4 after some generation. To be specific, some of the target genes should be “off” instead of “on”. After this alteration in the fitness condition, both the fluctuations V_{ip} and V_g increase over some generations, and then decrease to fix the expression of target genes. It is interesting that both the expression variances increase keeping proportionality. At any rate, the restoration of fluctuation emerges as a result of the change in environmental condition. After this change, both the variances increase to adapt the new environmental condition (K. Kaneko, unpublished). Hence, the variances again work as a measure of plasticity. It will also be important to incorporate environmental fluctuation into our theoretical formulation.

- (3) *Interaction:* Another source to sustain the level of fluctuation is interaction among individuals, which may introduce a change in the ‘environmental condition’ that each individual faces with. Here as the variation in phenotypes is larger, the variation in the interaction will be increased. Due to the variation in the interaction term, the phenotypic variances will be increased. Then, mutual reinforcement between diversity in interaction and phenotypic plasticity may be expected, which will result in both the genetic and phenotypic diversity. Co-evolution for such reinforcement between phenotypic plasticity and genetic diversity will be an important issue to be studied in future.
- (4) *Speciation:* So far, we have considered gradual evolution keeping a single peak in phenotype distribution. On the other hand, the bimodal distribution should appear at the speciation. Previously we have shown that robust sympatric speciation can occur as a mutual reinforcement of bifurcation of phenotypes as a result of developmental dynamics and due to genetic variation (Kaneko and Yomo 2000; Kaneko 2002). It will be important to reformulate the problem in terms of the present distribution theory.
- (5) *Sexual reproduction:* Our simulation in the present paper was based on asexual haploid population. On the other hand, the phenomenological theory on the distribution could be applicable also to a population with sexual reproduction. In the sexual reproduction, however, recombination is a major source of genetic variation, rather than the mutation. Then, instead of the mutation rate, the recombination rate could control the error catastrophe. Here, the degree of genetic heterogeneity induced by recombination strongly depends on the genotype distribution at the moment. (For example, for an isogenic population,

the mutation can introduce heterogeneity, but the recombination only cannot produce the heterogeneity.) It would be interesting to extend our theory on evolutionary stability and phenotypic fluctuation to incorporate the sexual reproduction.

6. Conclusion

We have proposed general relationship among phenotypic plasticity and phenotypic fluctuations of genetic and epigenetic origins. Through experiments and numerical simulations, the relationship is confirmed. Increase of developmental and mutational robustness through evolution is demonstrated, which progresses keeping the proportionality between the two.

Recall Waddington's canalization, epigenetic landscape, and genetic assimilation (Waddington 1957). Our distribution function $P(\text{phenotype} = x, \text{genotype} = a) = \exp(-U(x, a))$ and its stability give one representation of the epigenetic landscape, and canalization. The decrease in fluctuations through evolution means that the potential valley is deeper, as described by canalization. The genetic assimilation is represented by the proportionality between the fluctuations of genetic and epigenetic origins. Note that the existence of the phenotype-genotype distribution $P(x, a) = \exp(-U(x, a))$ is based on mutual relationship between phenotype and genotype. The genotype determines (probabilistically) the phenotype through developmental dynamics, while the phenotype restricts the genotype through mutation-selection process. For a robust evolutionary process to progress, the consistency between phenotype and genotype is shaped, which is the origin of the distribution $P(\text{phenotype}, \text{genotype})$ assumptions, and the general relationships proposed here.

In formulating fluctuation-response relationship by Einstein, such "consistency" between microscopic and macroscopic descriptions was a guiding principle. We hope that quantitative formulation of Waddington's ideas based on Einstein's spirit will provide a coherent understanding in evolution-development relationship.

Acknowledgements

I would like to thank C Furusawa, T Yomo, K Sato, M Tachikawa, S Ishihara, S Sawai for continual discussions, an anonymous referee for important comments and E Jablonka, T Sato, S Jain, S Newman, and V Nanjundiah for stimulating discussions during the conference at Trivandrum.

The theoretical study here was inspired at 2005, a hundredth anniversary both of Einstein's miracle year and of Waddington's birth, advanced through the conference held in 2007, the 50th anniversary of the publication of the *Strategy of the Genes* by Waddington, and the manuscript is completed at the 200th anniversary of Darwin's birth.

I would like to express sincere gratitude to these giants for inspiration.

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ePublication: 4 September 2009