

Origin of the neutral and nearly neutral theories of evolution

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1. Proposal of the neutral theory

I was born in 1933 in a suburb near Toyota, the well known city of the automobile company. At that time, the Toyota company was quite insignificant, and people around me were mostly farmers. When I was in junior high school, I was interested in mathematics and physics, but no one encouraged me to enter these fields for further study. So I did my undergraduate work majoring in horticulture at the University of Tokyo. I could not find a professional job after graduation and became an editor of a publishing company in Tokyo. I was not happy since the job was tiresome. A lucky event happened after two years at the publishing company, when a well-known wheat geneticist, Hitoshi Kihara, hired me to work in his private institute. There I studied cytogenetics of wheat and sugar beet for four years. Then another lucky event came my way, a chance to study in the USA. I became a graduate student at the North Carolina State University, where I finally found my own professional field, namely population genetics. I finished my Ph D in 1966 under the late Ken-ichi Kojima and came back to Japan.

The late 1960s were a remarkable time for students of evolution and population genetics. Before then there had been only a few real cases where population genetics theory could be applied to evolutionary problems. But at that time biochemical data were beginning to accumulate and they needed to be looked at from the point of view of evolution. Motoo Kimura, who was already a well known population geneticist, was looking for data which he could handle with the theoretical apparatus of apply population genetics. After my Ph D course, I visited Kimura and asked him whether it might be possible for me to do a post-doctoral stint under him. He was a little reluctant but finally accepted me as a post-doctoral fellow of the Japan Society for the Promotion of Science. I started to work in his laboratory in the spring of 1967.

At that time, among other members of the Kimura laboratory there were Takeo Maruyama, who just finished his

Ph D at the University of Wisconsin under J F Crow, and Norikazu Yasuda who did his Ph D work under N Morton at the University of Hawaii. Maruyama was an expert in mathematics, almost finishing a Ph D in it. At that time, J F Crow visited Kimura during the summer almost every year, and Masatoshi Nei, who was at the Radiobiological Institute in Chiba, visited us every two or three weeks. There were very lively discussions among these scientists in the laboratory. The environment was excellent for young students, and made me very enthusiastic about studying population genetics and molecular evolution.

In 1965, Zuckerkandl and Pauling published their influential paper "Evolutionary divergence and convergence in proteins", in which they showed that proteins are evolving at what is approximately a constant rate (Zuckerkandl and Pauling 1965). This phenomenon, known as the "molecular clock", was totally unexpected on the basis of traditional evolutionary thinking. Kimura was much puzzled by the molecular clock, and tried to examine it in some detail.

At that time, the relevant raw data that were available pertained to amino acid sequences. Zuckerkandl and Pauling (1965) compared amino acid sequences of hemoglobin *a* and cytochrome *c* among several mammals and discovered the molecular clock. The first job that Kimura asked me to investigate was to check their analysis in detail. I found that the analysis was profound; in fact their approach has become the most standard one. If the two homologous amino acid sequences compared differ at d_{aa} amino acid sites among the total of n_{aa} sites, the fraction of sites with different amino acids is, $p_d = d_{aa}/n_{aa}$. By assuming that amino acid substitutions obey the Poisson law of statistics, the average number of amino acid substitutions per site (K_{aa}) is estimated by the formula

$$K_{aa} = -\log_e(1 - p_d). \quad (1)$$

Zuckerkandl and Pauling (1965) found that the evolutionary distance estimated by this formula increased almost linearly with divergence time based on fossil records.

After a careful reading of their paper, we decided that their conclusion must be true and evolutionary biologists should seriously consider their finding.

Kimura first paid attention on the number of mutant substitutions per generation in a whole genome. His interest came from the concept of the genetic load, particularly the load due to advantageous mutant substitutions (Haldane 1957). The load is called the cost of natural selection. Haldane estimated the amount of genetic death needed for an advantageous mutation to be fixed in the population, and argued that the rate of advantageous mutant substitutions would be upper bounded because too much genetic death would cause extinction of the species. Kimura extrapolated from the number of mutant substitutions per protein locus to the same number per total genome, and obtained roughly one nucleotide substitution per 2 years in mammalian lineages. He argued that this figure was much too large as compared to the Haldane's estimate, that an advantageous allele may be substituted in a population every 300 generations. This led him to propose the neutral theory of molecular evolution. Its essential basis was the assumption that much of what happened at the molecular (DNA or protein) level during evolution was independent of natural selection (Kimura 1968).

In the next year, King and Jukes (1969) published their provocatively titled article, "Non-Darwinian evolution" where they argued that most mammalian DNA does not encode proteins, so that the load argument of Kimura was not appropriate. In this article, various subjects from the molecular clock to the proportion of coding regions in the genome were discussed in relation to the neutral theory. In the same year, Kimura (1969) published another article, where he argued that the molecular clock was a strong piece of evidence for the neutral theory. He further predicted that genes in 'living fossils' may be expected to have changed by the same amount as corresponding genes in more 'ordinary' species. The prediction, now famous, was regarded as very unorthodox at the time. Calder (1973) remarked in his book, "*Life Game*", that evolution may be studied by biochemistry without any knowledge of fossils, and the investigation might be like playing games such that the place of the species of interest in the phylogenetic tree was totally unknown, but was estimable from the protein data. In 1976, Kimura received the Order of Culture from the Emperor of Japan (see figure 1a, which was taken a little later). Figure 1b shows the members of his laboratory in the middle of the 1970s.

2. Protein polymorphism as a phase of molecular evolution

Before the neutral theory was proposed, mutant substitution and polymorphism were thought to be separate pro-

cesses, i.e. the former was by selection for advantageous mutations, whereas the latter was kept by balancing selection. An exploration of the neutrality concept led Kimura and Ohta (1971) to advance the hypothesis that protein polymorphisms were simply phases of molecular evolution.

Let the rate of neutral mutations at a locus be ν per generation. In a diploid population of size N , a total of $2N\nu$

(a)



(b)



Figure 1. (a) Motoo Kimura with the Medal of Order of Culture from the Emperor of Japan, taken in 1978. (b) Members of the Kimura laboratory taken in the mid 1970s. From left to right, Ms H Shimizu (technician), T Yamazaki, Ms Y Ishii (secretary), T Ohta, M Kimura and T Maruyama.

new mutations appear in each generation. Among these mutations, the fraction, $1/(2N)$, survives and gets fixed in the population, in other words the rate (k) of neutral evolution, becomes

$$k = 2N\nu \times (1/2N) = \nu. \quad (2)$$

This means that the rate of molecular evolution is simply equal to the rate of mutation.

Contrary to selectively advantageous cases, a neutral mutant takes a long time to fix in the population. Kimura and Ohta (1969) showed that it takes $4N$ generations on the average. During the process of spreading, the population is polymorphic. Actually the expected heterozygosity was obtained by Malécot (1948) and Kimura and Crow (1964) even before the proposal of the neutral theory. They analysed the equilibrium between mutation and random drift, and the expected heterozygosity (H_e) was been shown to be

$$H_e = 4N\nu/(1 + 4N\nu). \quad (3)$$

Note that H_e is highly dependent on the product, $N\nu$.

During 1970s, there had been numerous studies to measure protein polymorphisms by electrophoresis. A remarkable observation was a narrow range of average heterozygosity among various species. Lewontin (1974) surveyed the heterozygosity of 20 or so species, and found that it ranged from 5.6% in the house mouse *Mus domesticus* to 18.4% in the fly *Drosophila willistoni*. He emphasized that such a small range can not be explained by the neutral theory, and that it was a strong evidence against the theory. This problem will be discussed later.

3. The nearly neutral theory

Almost ever since the proposal of the neutral theory by Kimura, I have had several questions about the theory. The first one was whether or not natural selection was so simple as to distinguish neutral mutations from selected ones. It seemed to me that there might be numerous mutations whose effects were so small that both random drift and selection influenced their behaviour. The second question was concerned with the molecular clock: the clock looked as if it depended on real (chronological) time rather than on generation number. This was contrary to the belief, then prevalent, that generation length provided the appropriate time scale over which mutation rates should be measured. If that were so, what could explain why the proteins in the human and mouse lineages (for example) – lineages which would have gone through vastly different generation numbers within the same length of chronological time – evolved at such similar rates? The third question concerned the narrow range of heterozygosity in many species as measured by electrophoresis. As explained before, under the neutral theory, the expected

heterozygosity must be dependent on the parameter, $N\nu$, the product of population size and mutation rate. Therefore the narrow range implied that $N\nu$ should not differ much among species. However, there seemed to be many species with greatly different population sizes.

I had been much puzzled by these three questions and realized that by bringing very slightly deleterious mutations into the neutral theory, these puzzles could be explained. The slightly deleterious mutation theory was published first by Ohta and Kimura (1971), and in more explicit form by me (Ohta 1973). It has later been called the nearly neutral theory.

The differences between the neutral and the nearly neutral theories are given below.

3.1 Molecular clock

Under the strictly neutral theory, the evolutionary rate is equal to the mutation rate (eq. 2). If many slightly deleterious or nearly neutral mutations are involved, the evolutionary rate is negatively correlated with the species population size. This is because, based on the Kimura's result, for slightly deleterious mutations the fixation probability decreases as a function of the product of population size and selection coefficient, s (Kimura 1962). The prediction may be roughly expressed by the following formula, by measuring the evolutionary rate per generation, k_g ,

$$k_g \propto \nu_g/N, \quad (4)$$

where ν_g is the mutation rate per generation. The evolutionary rate per year, k , becomes, by letting g be the generation length in terms of the year,

$$k \propto \nu_g/(Ng). \quad (5)$$

Now, if organisms with large body size tend to have small N , and vice versa, the product, Ng , would be relatively invariant among species. This formula tells us that the nearly neutral theory would fit better than the neutral theory to the molecular clock, provided that ν_g is constant among different species.

3.2 Narrow range of heterozygosity

The narrow range of heterozygosity referred to above may be explained by incorporating slightly deleterious mutations into the model. By considering protein polymorphisms measured by electrophoresis, Ohta and Kimura (1975) used the stepwise mutation model as follows.

Mutation rate	–	$\nu/2$	$\nu/2$	$\nu/2$	$\nu/2$	–
Allelic state	–	$A_{-2} \leftrightarrow$	$A_{-1} \leftrightarrow$	$A_0 \leftrightarrow$	$A_1 \leftrightarrow$	A_2 –
Frequency	–	x_{-2}	x_{-1}	x_0	x_1	x_2 –
Fitness	–	$1 - s$	$1 - s$	1	$1 - s$	$1 - s$ –

In this model, by increasing N , the frequency distribution of alleles in the population changes from the neutrality prediction to the deterministic mutation-selection balance. The average homozygosity (H_o) under neutrality for Ns much less than unity is,

$$H_o = 1/\sqrt{(1 + 8Nv)}. \quad (6)$$

H_o under deterministic mutation-selection balance becomes

$$H_o = \sqrt{1 + (v/s)^2} \{ \sqrt{1 + (v/s)^2} - v/s \}^2. \quad (7)$$

When measured by electrophoresis, the frequency spectrum is characterized by the most common allele surrounded by the less common alleles. If Ns is small, the spectrum is irregular, and as Ns gets larger, it becomes more stable. In any case, the model nicely explains the narrow range of heterozygosity over many species.

In the same year, I published a paper on contrasting patterns of protein polymorphisms between *Drosophila* and humans (Ohta 1975). In this paper, I showed that there was an excess of rare alleles as compared to the neutral prediction both in *Drosophila* and in human populations. But in the former, alleles with frequencies less than 10% were in excess, whereas only alleles of less than 1% frequency were in excess in the latter. I suggested that in *Drosophila*, the frequency spectra might be close to the equilibrium between mutation and selection, whereas in humans such an equilibrium has not been reached.

There were disagreements on these interpretations. Neutralists proposed that changes of population size could explain the facts. In particular, Nei and his colleagues proposed that occasional bottlenecks might be responsible for the narrow range of heterozygosity among species, and polymorphisms of *Drosophila* populations were not in equilibrium and contain limited heterozygosity by the small effective population size through bottlenecks (Nei 1975). In other words, heterozygosity decreased at the bottleneck, and has not yet increased to the level expected by the present population size in *Drosophila*.

Subsequently more data on polymorphisms accumulated, and it was found that there were some species lacking protein polymorphisms, i.e. heterozygosity was practically zero. For example, no polymorphism was found in 21 protein loci in elephant seal (Bonnell and Selander 1974). Hence Kimura and other neutralists thought that there was no lower bound on the range of heterozygosity, and data on polymorphisms were compatible with the neutral theory.

Also Kimura realized that the effective population size might not become large because of local extinction and recolonization with structured population. Together with Maruyama, he showed that, if the rate of local extinction and recolonization of colonies is higher than the rate of migration between colonies, the effective population size

can be much smaller than the total size (Maruyama and Kimura 1980). Kimura therefore argued that the effective size of *Drosophila* should be much smaller than it looked.

On the other hand, in 1970s, many selectionists considered that most protein polymorphisms were maintained by some kind of balancing selection. Note, however, that there was no way to prove or disprove the selection theory. The choice of parameters such as the intensity and type of selection was arbitrary, and any data could be explained by choosing parameter values.

One indelible remark on the range of near-neutrality is the role of a molecular chaperone such as Hsp90 on the protein folding mechanism. Hsp90 helps stabilization of important proteins of signal transduction. Rutherford and Lindquist (1998) found that mutant heterozygotes at this locus of the fruitfly show various morphological abnormalities when the temperature is high, because the folding of protein signal transducers becomes unstable. In other words, some amino acid changes of signal transducers cause unstable folding under high temperature, but are protected by Hsp90 under ordinary conditions. They are selected only under stress and/or when Hsp is insufficient. Hsp90 represents a good example where selective neutrality depends upon genetic as well as external environmental conditions.

4. Reconciling morphological evolution with the nearly neutral theory

It has been said that under the neutral theory, molecular evolution and morphological evolution are dichotomous, i.e. the former occurs by random drift with almost uniform rate and the latter, by natural selection depending on environmental changes. However, genes should be responsible for morphological evolution. How can we reconcile such a dichotomy?

It is now recognized that gene regulation is crucial for morphogenesis, and our knowledge of gene regulation has expanded rapidly in the recent ten or so years (see Davidson 2001; Carroll *et al* 2001; Wilkins 2001). It is thought that expression of many genes is quantitatively regulated via interaction between transcription factors and regulatory elements. Usually the regulatory elements are located upstream of the coding regions. The most abundant mutations of the regulatory elements are nucleotide substitutions in binding sites for transcription factors. Under mutation and selection for maintaining expression patterns, the *cis*-regulatory elements are in constant turnover within the allowed latitude of selection and drift (Carroll *et al* 2001; Ludwig *et al* 2000). The stripe 2 element of the gene *even-skipped* of *Drosophila* species has been studied in detail. Ludwig *et al* (2000) constructed a chimera between the stripe 2 element of *D. melanogaster* and that of *D. pseudoobscura*. They compared the

expression pattern of a reporter gene downstream from the chimeric element with that downstream from the native element. While the two native elements gave normal expression patterns, the chimeric element did not. Their finding indicates that the regulatory elements are differentiating via slightly deleterious plus compensatory mutant substitutions within the latitude allowed by drift and selection (Ludwig *et al* 2000).

Such a turnover of regulatory elements is analogous to the nearly neutral evolution of proteins. In order to understand the relationship of adaptive protein evolution under near-neutrality, I put forward a model of mutant effects on fitness by considering environmental diversity (Ohta 1972). In this model, a continuous distribution of mutant effects on fitness that are very weak was assumed, and it was argued that the probability of a mutant being acceptable in evolution, i.e. neutral or advantageous, increases as the environment becomes more uniform. This is because the average effect is negative and the variance effect becomes larger as environment gets more uniform. The model was modified later so that it was more easily handled by assuming a normal distribution of mutant effects on fitness (Ohta and Tachida 1990; Tachida 1991). Let s be the standard deviation of the normal distribution, and N be the effective population size as before. Then the selection intensity may be measured by s , and the magnitude of drift by the reciprocal of N . So roughly speaking, if s is smaller than $1/N$, both drift and selection influence the mutant behaviour and the population mean moves erratically (Ohta and Tachida 1990; Tachida (1991).

As compared with the amino acid altering mutations, the base changes at the binding sites of regulatory elements would be strongly influenced by environmental factors that include both genetic backgrounds and external environments. This is because many quantitative traits are controlled by gene regulation (Mackey 1995), and are important targets of natural selection. Then the variance of the distribution of the fitness effects would be larger than in the case of amino acid altering substitutions. Furthermore, the distribution of fitness effects would differ among regulatory elements of various kinds of proteins. For an important protein, i.e. deep in the regulatory network, the mean is negative and the variance is not large because few alterations can be tolerated. On the other hand, shortly after a gene has originated by duplication, the mean would be close to the neutrality expectation (zero point of figure 2) and the variance would be large. Thus the turnover rate is higher for relatively young genes than for ancient genes.

Rockman and Wray (2002) surveyed polymorphisms at regulatory regions in the human genome and found that polymorphisms are abundant as compared with coding regions. They also found that the frequency distribution of alleles does not fit the neutral prediction and argued

that various types of selection such as geographically heterogeneous selection, balancing selection because of antagonistic pleiotropy and others, may be working. The effects of various types of selection would be compatible with the present proposal that the variance of the fitness effects would be larger for mutations at the binding sites of the regulatory elements than for amino acid altering mutations.

How is the distribution influenced by genetic and environmental factors? If a species with small population size occupies a simple environment, the variance of mutant effects on fitness becomes large as compared with a species with large size occupying diverse environments. This is because, if the environment is diverse, the fitness effect is averaged over many environmental conditions. Figure 2 gives comparison of the fitness distribution of these two cases.

So far, our discussion has been about the turnover of already existing regulatory elements. Even more interesting is new recruitment (cooption) of gene expression, which is the reassignment of regulatory genes to different positions in the networks than they occupied ancestrally (Carroll *et al* 2001; Davidson 2001). Mammalian Hox genes that are expressed at various tissues and developmental times are good examples.

The kinds and quantities of transcription factors are regulated and differ in space and time within developing organisms. Regulatory elements of genes for tissue-specific proteins coevolve with the tissue distribution of transcription factors. According to Carroll *et al* (2001), a new regulatory element may evolve either by *de novo* formation or by duplication followed by modification of preexisting elements. The probability of attaining the status of a new regulatory element seems to be not so small; cooption would be constantly occurring and being tested by natural selection. Most such mutations would have weak effects and are thought to be subject to both random drift and selection. It appears that drift and selection can not be separated in the initial stage of cooption. Once a new chain of gene recruitments starts, positive selection would become effective.

Through an interdisciplinary approach as presented above, it would be possible to unify the traditional evolutionary biology and molecular genetics. So far, the two fields have looked as if there has been little connection between them. Also, evolution at the molecular level and at the morphological level have been thought to be dichotomous. We are now beginning to understand real processes at the molecular level underlying evolution of enormous diverse forms of organisms. Here the neutral theory provides the starting point for assessing evolutionary forces, i.e. it has been and will be the null hypothesis of numerous evolutionary studies at the gene level for clarifying effects of natural selection and interaction systems.

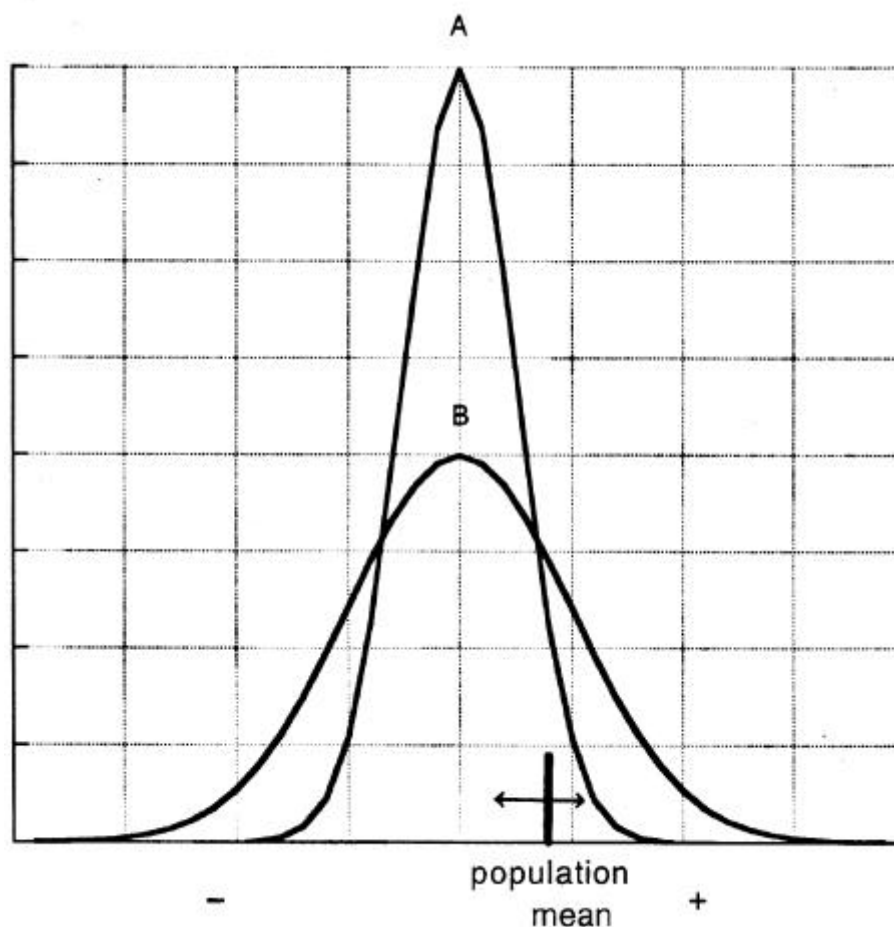


Figure 2. Frequency distribution of the fitness effects of new mutations (from Ohta 2002). Curve A is for a large population that occupies a heterogeneous environment, and curve B is for a small population that occupies a simple (homogeneous) environment. The population mean moves by drift and selection.

Kimura, in his late years, often told me that the nearly neutral theory might be more realistic than the strictly neutral theory, but that the latter was certainly more useful than the former. He was completely right in this statement. In any case, he did not like the complicated nearly neutral theory. I have been lucky in that rapid progress of molecular evolutionary studies has taken place during my career. Kimura passed away in 1994, and did not have a chance to see the new development on gene regulation. We had never expected such a rapid progress 30 years ago when we met strong objections to the neutral theory.

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