Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 10676–10680, November 1993
Evolution

An examination of the generation-time effect on molecular evolution

(nearly neutral theory/negative vs. positive selection/synonymous vs. nonsynonymous substitutions)

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Communicated by Motoo Kimura, August 9, 1993

ABSTRACT **By using DNA sequences of 17 mammalian genes, the generation-time effect is estimated separately for synonymous substitutions and nonsynonymous substitutions. Star phylogenies composed of rodentia, artiodactyla, and primates are examined. The generation-time effect is found to be more conspicuous for synonymous substitutions than for nonsynonymous substitutions, by using the methods of (*i*) Nei and Gojobori, (*ii*) Li, and (*iii*) Ina. The proportion of accepted amino acid substitutions in evolution is estimated to be about twice as large in the primate lineage as in the rodent lineage. This result is in accord with the nearly neutral theory of molecular evolution.**

Clarification of the pattern of nucleotide change in evolution is fundamental for understanding evolutionary mechanisms. One approach is to compare gene sequences that form a star phylogeny and to estimate the number of substitutions in each lineage (ref. 1, p. 77). For the study of mammalian phylogeny, this method is criticized because the mammalian radiation may not form a star phylogeny (2). However, if only three mammalian species are analyzed, they automatically form a star phylogeny. For DNA sequence analysis, it is desirable to estimate the numbers separately for synonymous and nonsynonymous substitutions, since the numbers are directly related to the theoretical interpretation. About 20 years ago, I predicted (3) that the rate of DNA evolution reflects generation number more strongly than does the rate of protein evolution, based on the nearly neutral theory of molecular evolution. In this paper, results of DNA sequence analysis of the mammalian star phylogeny composed of primates, artiodactyla, and rodentia are presented, and this prediction will be shown to hold for 17 genes examined.

SEQUENCE ANALYSIS

I obtained nucleotide sequences from the genetic data bases maintained at the National Institute of Genetics (Japan), which include GenBank, DNA Data Bank of Japan, and European Molecular Biology Laboratory. The sequences used in the analysis are listed in Table 1. They have been chosen with the following conditions: the coding region is not small and the protein function has not changed for a long time. Note that, if the function changes, the ordinary pattern of synonymous vs. nonsynonymous substitutions will be violated (51, 52).

For acquisition and analysis of the data, I used the ODEN package created by Ina (53) at the National Institute of Genetics (Japan). The method of Nei and Gojobori (54), which is incorporated into the ODEN package, was used for estimating the numbers of synonymous and nonsynonymous substitutions. This method divides the nucleotide substitutions into synonymous and nonsynonymous categories, and then the number of multiple hits was estimated under the assumption of random mutability among the four kinds of bases. Because this assumption is often not satisfied, the method was not completely satisfactory. Recently, Li (55) and Pamilo and Bianch (56) invented a better method. In this method, the problem of bias in transitional vs. transversional substitutions is overcome by taking the weighted average of these changes, at 2-fold and 4-fold degenerate sites, for estimating the number of synonymous substitutions. Let K_i, A_i, and B_i be the numbers of nucleotide substitutions and transitional and transversional substitutions, respectively, at the i-fold degenerate sites of the two sequences being compared. Also let L_i be the number of i-fold degenerate sites. Then the number of synonymous substitutions per site, K_s, is,

$$K_s = (L_2A_2 + L_4A_4)/(L_2 + L_4) + B_4. \quad [1]$$

The number of nonsynonymous substitutions per site, K_n, is

$$K_n = A_0 + (L_0B_0 + L_2B_2)/(L_0 + L_2). \quad [2]$$

The program for calculating K_s and K_n was provided by W.-H. Li, and it was implemented here by Y. Ina.

Li (55) did not give formulas for calculating the numbers of synonymous and nonsynonymous sites. According to the suggestion of Ina (personal communication), the following formulas should be used. By denoting these numbers S and N,

$$S = A_4L_2/K_4 + L_4 \quad [3]$$

and

$$N = B_4L_2/K_4 + L_0. \quad [4]$$

Ina (personal communication) has invented another more efficient method for estimating the numbers of synonymous and nonsynonymous substitutions. His method brings Kimura's two-parameter model (57) into the Nei–Gojobori method (54). Through extensive simulations, Ina has shown that his method gives even better estimates than Li's method, since the transitional substitution rate often considerably exceeds random expectation.

From the estimated divergence of pairwise sequence comparisons among primates, artiodactyla, and rodentia, the branch length of the star phylogeny was estimated as in Kimura (ref. 58; see also ref. 59). Let d_{pa}, d_{pr}, and d_{ar} be the estimated divergences between primates and artiodactyla, between primates and rodentia, and between artiodactyla and rodentia, respectively. Also let d_p, d_a, and d_r be the branch length of primates, artiodactyla, and rodentia lineages of the star phylogeny. Then, we have (58, 59)

$$d_p = (d_{pa} + d_{pr} - d_{ar})/2,$$

$$d_a = (d_{pa} + d_{ar} - d_{pr})/2,$$

and

$$d_r = (d_{pr} + d_{ar} - d_{pa})/2. \quad [5]$$

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Table 1. DNA sequences used in the study

Protein	Accession number			Refs.
	Primates	Artiodactyla	Rodentia	
Albumin	V00494	M73993	J00698	4, 5, E. W. Holowachuk (personal communication)
Lactate dehydrogenase A	X02152	D90143	M27554	6–8
Fibrinogen γ	X02415	X15556	X05860	9–11
Acetylcholine receptor α	Y00762	X02509	X03986	12–14
Acetylcholine receptor β	X14830	X00962	M14537	15–17
Growth hormone	J00148	M27325	K03232	18–20
Growth hormone receptor	X06562	X54429	M33324	21–23
Prolactin	V00566	J00022	X02892	24–26
IGF-I	M37484	X15726	X06107	27–29
IGF-II	M17862	X53553	M14951	30–32
IGF binding protein 1	Y00856	X54979	M89791	33, 34
IGF binding protein 3	M35878	M76478	M33300	35–37
Interleukin 1α	M15329	M37210	D00403	38–40
Interleukin 1β	M15840	M37211	M15131	38, 41, 42
Interleukin 2	V00564	M12791	K02797	43–45
Interleukin 6	M29150	X57317	J03783	46, 47, 49
Interleukin 7	J04156	X64540	X07962	48–50

IGF, insulin-like growth factor.

RESULTS AND DISCUSSION

A total of 17 gene loci have been analyzed. As representatives among them, phylogenies of albumin and lactate dehydrogenase are shown in Fig. 1. The estimated total numbers of nonsynonymous and synonymous substitutions are shown beside each branch. Roman type represents the numbers estimated by Nei and Gojobori (54), italic type represents numbers estimated by Li (55), and boldface italic type represents numbers estimated by Ina. The estimated total numbers do not differ much under the three methods. However, the estimated numbers of synonymous and nonsynonymous sites differ considerably by the three methods: the number of synonymous sites is larger and the number of the nonsynonymous sites is smaller by the methods of Li and Ina than by the Nei–Gojobori method. As the result, the number of synonymous substitutions per site is considerably smaller with the methods of Li and Ina than with the Nei–Gojobori method.

Li's previous method (60) has also been tried, but the results are not given here because the estimated numbers are very close to the values obtained by Nei and Gojobori. According to Ina (personal communication), the most recent methods of Li and of Ina are more efficient than the Nei–Gojobori method, as they give values that are very close to the true divergences in Ina's simulations.

From Fig. 1, it is also clear that the rodent line is the most divergent among the three lineages for synonymous substitutions but not necessarily so for nonsynonymous substitutions. This is in accord with previous observations on other loci (59, 61). Let me now examine the total numbers of nonsynonymous and synonymous substitutions of 17 loci.

Fig. 2 shows the numbers. Again, roman type represents the values obtained by the Nei–Gojobori method, italic type represents values obtained by Li's more recent method, and boldface italic type represents values obtained by Ina's method. The characteristic pattern of synonymous substitutions is very clear. To examine statistical significance of the different patterns between synonymous and nonsynonymous substitutions, I performed the χ^2 test of independence between lineages and substitution types. For the test, uncorrected numbers of different sites are used, since they are closer to the observed numbers than the corrected divergences. Note that Nei–Gojobori and Ina methods give the same values for the uncorrected numbers. Also note that the numbers are estimated values because the paths of codon changes may include various possibilities. Nevertheless, a χ^2

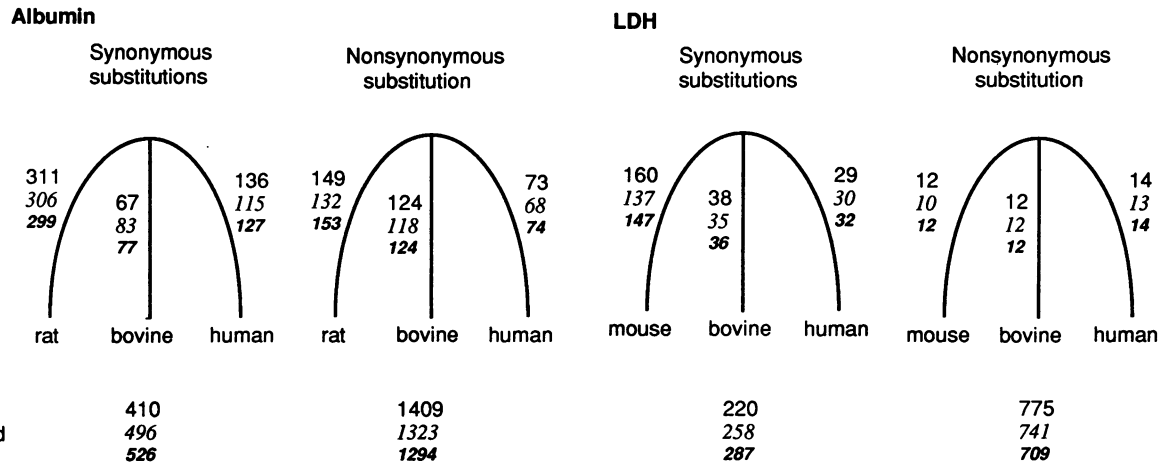

FIG. 1. Star phylogenies of albumin and lactate dehydrogenase A (LDH). The numbers beside each branch are the total number of substitutions estimated as follows. Roman type represents the value estimated by the method of Nei and Gojobori (54), italic type represents the value estimated by the method of Li (55), and boldface italic type represents the value estimated by the method of Ina.

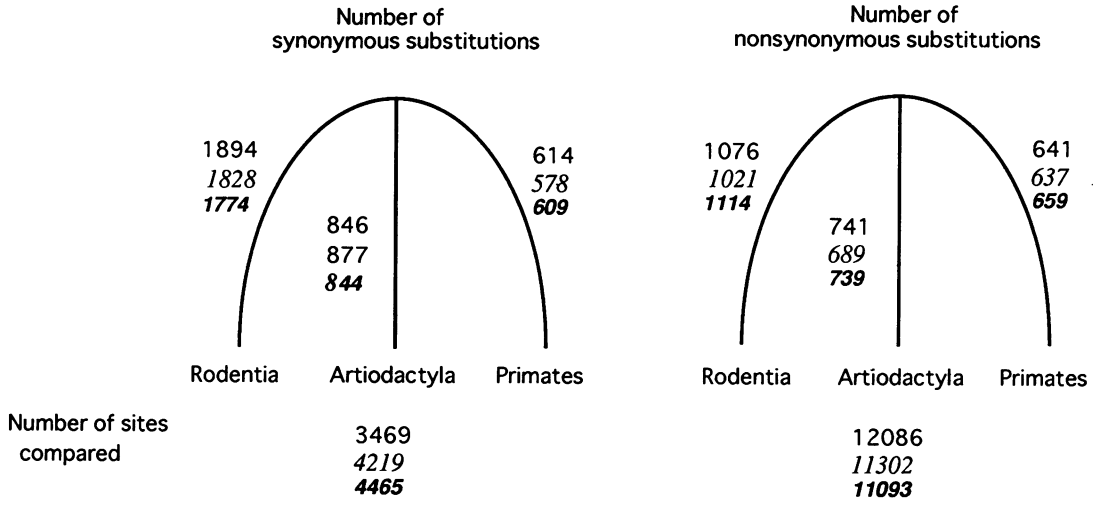

FIG. 2. Star phylogenies of 17 genes. The numbers beside each branch are as in Fig. 1.

test based on these estimates would be a conservative test, and the significance is meaningful. The total numbers of different sites in pairwise comparisons among the three lineages of the 17 genes were calculated for synonymous and nonsynonymous substitutions by the Nei–Gojobori and Ina methods. From the resulting values, the branch lengths of the three lineages were obtained as before (Eq. 5). They are given in Table 2. The χ^2 value was 21.2 with 2 df and is highly significant. Thus, the difference in patterns between synonymous and nonsynonymous substitutions is statistically significant.

By using the result of Li *et al.* (61), Gillespie (59) estimated the lineage effect by a weighting factor. This factor is the characteristic divergence of each lineage and the average is constrained to equal 1. From the branch lengths of the star phylogenies in Fig. 2, I obtained weight factors for synonymous and nonsynonymous substitutions. The comparison of Gillespie's results and the present results is given in Table 3. The difference between the previous and the present estimates is not large, but the pattern of a large synonymous weight factor for the rodent lineage and a small weight factor for the primate lineage is a little more pronounced for the present estimates.

By examining the divergence pattern of several genes among marsupials, rodents, and primates, Easteal (2) argued that there is no evidence of systematic variation in evolutionary rates among the orders. However, the synonymous divergences between marsupial and eutherian genes are close to or more than 100%, and so rate differences may not be detected even if there are some differences. Detection would be particularly difficult, if there is some nonrandomness either by mutation or by selection in synonymous base substitutions (62). In addition, the loci he examined include some gene families for which the pattern of synonymous and nonsynonymous substitutions may be violated as discussed below. Furthermore, genealogical relationships may not agree with species relationships in gene families with diverse members.

Table 2. Branch lengths based on the uncorrected numbers of different sites and χ^2 value for testing independence between the lineages and the substitution types

Substitution lineage type	Branch length		
	Primate	Artiodactyla	Rodentia
Synonymous	503.1	595.9	1062.1
Nonsynonymous	596.4	653.6	917.4

χ^2 = 21.2 with 2 df.

At any rate, the pattern found for synonymous substitutions represents generation-time effects. One can note that generation-time effects are smaller for nonsynonymous substitutions than for synonymous substitutions. In fact, the rodent/primate weight factor ratio is about 3 for synonymous substitutions but only 1.6 for nonsynonymous substitutions. This result is in accord with the nearly neutral theory of molecular evolution (3, 63, 64). In other words, if one assumes that most synonymous substitutions belong to the neutral class, whereas most nonsynonymous substitutions belong to the nearly neutral class, the present result may be easily explained. Note that a negative correlation is expected between evolutionary rate and population size for nearly neutral mutations and that rodent species are thought to have larger population sizes than primate species (refs. 3 and 64; for a recent verification of this relationship, see ref. 65). Originally, this negative correlation was thought to be due to very slightly deleterious mutations that cannot spread in a large population but are able to replace the other alleles in a small population. A more realistic model has been invented that incorporates both slightly advantageous and disadvantageous mutations (66, 67). The negative correlation is again expected under this version of the nearly neutral model, provided that selection is mainly for keeping structure and function of gene products, i.e., purifying selection.

Table 3. Weight factors and the ratio of the number of nonsynonymous substitutions to that of synonymous substitutions per site

	Rodentia	Artiodactyla	Primates
Weight factor			
Synonymous			
Nei–Gojobori	1.694	0.757	0.549
Li-93	1.671	0.802	0.528
Ina-1	1.649	0.784	0.566
Gillespie (data by Li)	1.611	0.762	0.627
Nonsynonymous			
Nei–Gojobori	1.313	0.905	0.782
Li-93	1.305	0.881	0.814
Ina-1	1.330	0.883	0.787
Gillespie (data by Li)	1.279	0.885	0.830
Ratio			
Nei–Gojobori	0.162	0.251	0.300
Li-93	0.209	0.293	0.411
Ina-1	0.253	0.352	0.436
Gillespie (data by Li)	0.166	0.242	0.279

Li-93, Li's most recent method (55); Ina-1, Ina's most recent method 1 (personal communication).

Table 4. Synonymous vs. nonsynonymous substitution rates among duplicated genes

Gene	Species	Substitution rate		Ratio
		Synonymous	Nonsynonymous	
Interferon α	Mouse (6 genes)	0.200	0.076	0.378
	Human (5 genes)	0.111	0.053	0.486
Cytochrome P450*	Mouse, rat	0.116	0.061	0.367
	Human	0.235	0.080	0.341

*Data are from table II of Gotoh (68). Values for the conserved region are used.

In Table 3, the ratio of the number of nonsynonymous substitutions to synonymous substitutions per site is also given. Note that this ratio is the proportion of acceptable amino acid substitutions. The estimated value of the proportion varies among lineages and also by statistical methods. The highest estimate is obtained by the method of Ina, which gives the most satisfactory result in simulation studies (Y. Ina, personal communication).

The present result shows that the proportion of acceptable amino acid substitutions in the primate lineage is about twice as large as that in the rodent lineage. The proportion in the artiodactyl lineage is between the two values but close to that of the primate lineage. One has to note here that this result holds for those genes whose function has been fixed for a long time and does not apply to duplicated genes and other genes whose function has been modified in the evolutionary course of the lineages studied (52). As an example of duplicated gene families, let us examine the pattern of sequence divergence in the interferon α gene family and the cytochrome P450 family. Table 4 gives the comparison of the patterns in the mouse–rat gene family and the human gene family. As can be seen from the table, the ratio is only slightly higher in the human genes than in the mouse genes for interferon α and almost the same for cytochrome P450. In other words, the generation-time effect on synonymous substitutions disappears for these gene families, indicating that purifying selection is not the only type of selection. It is likely that, to increase functional diversity among duplicated genes, slightly advantageous amino acid substitutions contribute to the pattern here.

A final comment is the problem of a molecular clock in relation to the present result. It seems to be generally accepted that nucleotide substitution has slowed down in primates and has been elevated in rodentia (69). The problem here is the true length of the rodent lineage. Although it would be difficult to estimate the rate difference accurately, testing such as that developed by Muse and Weir (70) will be helpful in the future. Now the question is: which of the synonymous substitutions and the nonsynonymous substitutions obey the chronological clock? I suggest that nonsynonymous substitutions more closely follow the clock, because the generation-time effect is likely to be reduced for the nearly neutral mutations by cancellation between the generation-time effect and the population-size effect (3, 63). This suggestion may be applicable only to mammals, and more studies will be needed to obtain the generation-time effect of other taxa.

I thank Mr. Yasuo Ina for his help on using the most recent method of Li and for letting me use his own method for estimating the rates of synonymous and nonsynonymous substitutions. Thanks are also due to him and to Drs. W.-H. Li, B. S. Weir, J. Tomizawa, H. Tachida, and Mrs. T. Steen for their valuable comments on the manuscript. This is contribution no. 1968 from the National Institute of Genetics, Japan.

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