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Molecular Evolutionary Analyses of the Rh Blood Group Genes and Rh50 Genes in Mammals

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Takashi Kitano, Satoshi OOta and Naruya Saitou (1999) Molecular evolutionary analyses of the Rh blood group genes and Rh50 genes in mammals. *Zoological Studies* **38**(4): 379-386. All mammals probably possess the Rh blood group gene and its homologous Rh50 gene coded membrane proteins. In this study, we compared nucleotide sequences of Rh and Rh50 genes for primates and rodents. We found that the rates of synonymous substitutions of both genes were roughly constant, and the divergence time between mouse and rat was estimated to be about 30 million years ago (mya). Heterogeneity of the evolutionary rate between primates and rodents was suggested. We also compared amino acid changes of outer-, inner-, and trans-membrane regions of the Rh and Rh50 proteins. Amino acid changes of outer-membrane regions were more frequent except for primate Rh genes. To obtain an improved estimation of divergence time, it is better to have multiple calibration points for the molecular clock. The time of gene duplication that produced the Rh and Rh50 genes was estimated to be about 340-380 mya.

Key words: Rh and Rh50 genes, Relative rates, Divergence time.

The human Rh blood group plays important roles in transfusion and clinical medicine, including haemolytic diseases of newborns, autoimmune diseases, and mild haemolytic anemia. Rh polypeptides were observed to be phosphorylated 30-32 kD membrane proteins by using SDS-PAGE and immunoprecipitation (Gahmberg 1982, Moore et al. 1982), and the Rh blood group system was shown to be composed of 2 closely linked *D* and *CE* loci (Mouro et al. 1993). A protein was obtained together with the Rh gene product on immunoprecipitation with anti-Rh antibodies from human, and named the 50kD glycoprotein (Moore and Green 1987). This glycoprotein was considered to form a heterotetramer with Rh blood group gene products on erythrocyte membranes (Eyers et al. 1994). The nucleotide sequence of the human 50kD glycoprotein (Rh50) was determined, and its amino acid sequence was homologous with that of the human Rh gene (Ridgwell et al. 1992). That protein was also predicted to have the 12 trans-membrane domains which are similar to those of the Rh blood group gene product (Avent et al. 1990).

Nucleotide sequences of Rh genes in nonhuman primates have been reported (Mouro et al. 1994, Salvignol et al. 1994 1995, etc.). We recently analyzed those published Rh blood group genes of primates and found more nonsynonymous substitutions than synonymous ones, clear evidence of positive Darwinian evolution (Kitano and Saitou, 1999). To see if a similar evolutionary pattern also exists in rodents, we determined nucleotide sequences of Rh and Rh50 genes in mouse and rat as well as that of the Rh50 gene for the crab-eating macaque (Kitano et al. 1998). Results indicated that the Rh50 gene has been evolving about 2 times more slowly than the Rh blood group gene. Matassi et al. (1999) also obtained a similar result.

In this study, we compare evolutionary rates of Rh and Rh50 genes for primates and rodents. We also analyze the evolutionary relationship of these genes and examine the effect of numbers of OTUs (operational taxonomic units) for estimation of divergence time.

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MATERIALS AND METHODS

Nucleotide sequence data for Rh, Rh50, and their related genes were retrieved from the DDBJ/EMBL/GenBank international nucleotide sequence database. Human RhD (X63097; Le Van Kim et al. 1992), human RhcE (X54534, M34015; Avent et al. 1990, Cherif-Zahar et al. 1990; both are identical), crab-eating macaque Rh (L37054; Salvignol et al. 1995), mouse Rh (AB015189; Kitano et al. 1998), rat Rh (AB015191; Kitano et al. 1998), human Rh50 (X64594; Ridgwell et al. 1992), crab-eating macaque Rh50 (AB015467; Kitano et al. 1998), mouse Rh50 (AB015192; Kitano et al. 1998), rat Rh50 (AB015194; Kitano et al. 1998), and the Rh related genes for nematode *Caenorhabditis elegans* (Z74026-B0240.1 and U64847-F08F3.3; Wilson et al. 1994.) and that for sponge (Y12397; Seack et al. 1997) were used.

CLUSTAL W version 1.6 (Thompson et al. 1994) was used for multiple alignments. The go/0 program (OOta and Saitou 1997) was used for constructing phylogenetic trees by the maximum likelihood method (Felsenstein 1981). The PredictProtein server (EMBL) was used for analyses of trans-membrane helix location of these proteins.

RESULTS AND DISCUSSION

Evidence for higher rates of nucleotide substitution in rodents than in primates

We previously estimated numbers of synonymous (d_S) and nonsynonymous (d_N) substitutions for Rh and Rh50 genes (Table 2 of Kitano et al. 1998). Figure 1 shows a comparison of d_S between Rh and Rh50 genes based on their results. Because d_S values were similar between Rh and Rh50 genes, they were plotted close to the line of an angle of 45° (d_S for Rh = d_S for Rh50). This suggests that these genes evolve at similar rates in terms of d_S, and a molecular clock (constancy of evolutionary rate) exists for both Rh and Rh50 genes. Numbers in parentheses in this figure are relative evolutionary distances.

It has been suggested that mutation rates of rodents are higher than those of primates (Wu and Li 1985, Gu and Li 1992). We estimated relative evolutionary rates between primate and rodent lineages and the divergence time between mouse and rat. Figure 2A shows the scheme for this procedure. The divergence time between primates and rodents was assumed to be 122 million years ago (mya) based on the study of Easteal et al. (1995). Relative evolutionary distances between mouse and rat, and between human and macaque were 0.35 and 0.1, respectively, when the distance between rodents and primates was 1.0. If we assume that the relative evolutionary rate (R) between primate and rodent lineages is

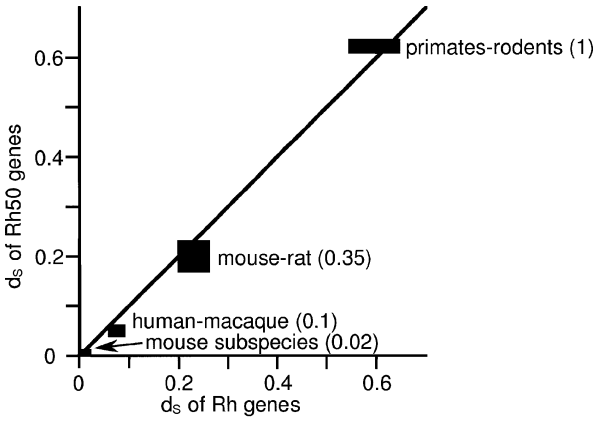

Fig. 1. Comparison of synonymous substitutions between Rh and Rh50 genes of primates (human and crab-eating macaque) and rodents (mouse and rat). Boxes show points of d_S with standard errors for Rh and Rh50 genes. Numbers in parentheses are relative rates.

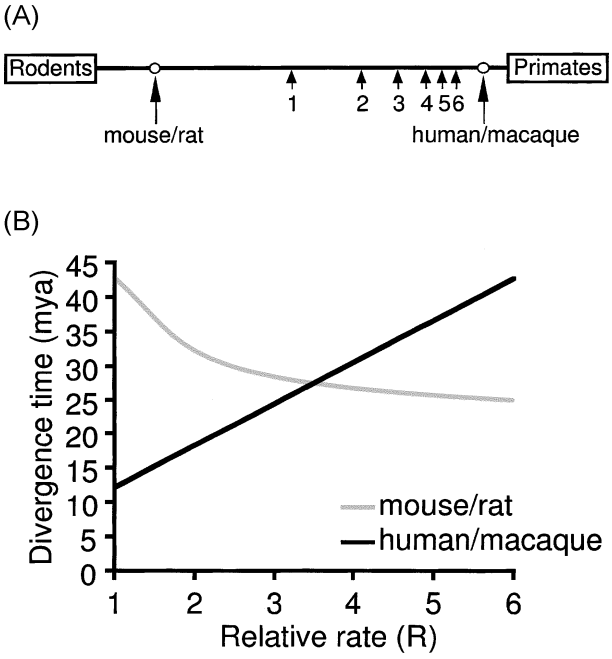

Fig. 2. Examination of relative evolutionary rate (R) between primate and rodent lineage. (A) The scheme for this procedure. Numbers show R for each assumed divergence point between primate and rodent lineage. (B) The relationship between R and divergence time.

equal (indicated by the arrow with number 1 in Fig. 2A), divergence times between human and macaque and between mouse and rat are estimated to be 12.2 mya {= (0.1/1) × [122(mya)/2] × (2/1)} and 42.7 mya {= (0.35/1) × [122(mya)/2] × (2/1)}, respectively. If we assume R = 2 (2 times higher in rodents than primates; indicated by arrow 2 in Fig. 2A), the divergence times between human and macaque and between mouse and rat are estimated to be 18.3 mya {= (0.1/1) × [122(mya)/2] × (3/1)} and 32.025 mya {= (0.35/1) × [122(mya)/2] × (3/2)}, respectively. These values are given by the following formulas:

$$T_{hm} = (D_{hm}/D_{pr}) \times (T_{pr}/2) \times (R + 1)$$
$$= (0.1/1) \times (122/2) \times (R + 1), \quad (1)$$
$$T_{mr} = (D_{mr}/D_{pr}) \times (T_{pr}/2) \times ([R + 1]/R)$$
$$= (0.35/1) \times (122/2) \times ([R + 1]/R), \quad (2)$$

where T_{hm}, T_{mr}, and T_{pr} are divergence times (mya) between human and macaque, between mouse and rat, and between primates and rodents, respectively. D_{hm}, D_{mr}, and D_{pr} are relative evolutionary distances between mouse and rat (0.35), between human and macaque (0.1), and between primates and rodents (1.0), respectively.

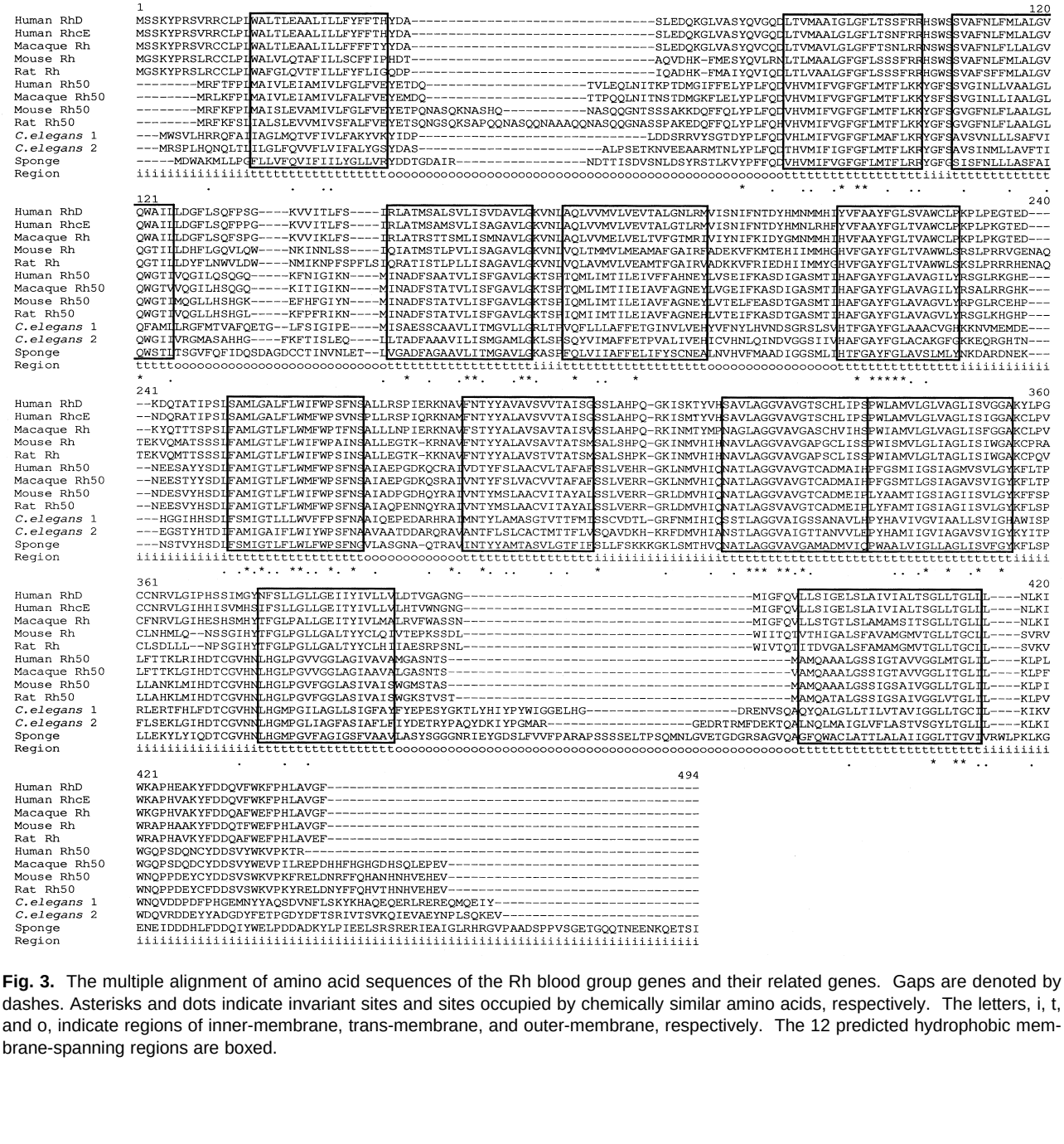

Fig. 3. The multiple alignment of amino acid sequences of the Rh blood group genes and their related genes. Gaps are denoted by dashes. Asterisks and dots indicate invariant sites and sites occupied by chemically similar amino acids, respectively. The letters, i, t, and o, indicate regions of inner-membrane, trans-membrane, and outer-membrane, respectively. The 12 predicted hydrophobic membrane-spanning regions are boxed.

Figure 2B shows the relationship among R, T_{mr}, and T_{hm}. If we assume the divergence time between human and macaque to be 23 mya (Kumar and Hedges 1998), R becomes approximately 2.8, and the divergence time between mouse and rat becomes ca. 30 mya. This suggests that the mutation rate in rodents is about 3 times higher than that in primates. In this case, rate of synonymous substitutions (per site per year) of primate and rodent lineage are estimated to be about 1.6×10^{-9} and 4.4×10^{-9}, respectively.

The divergence time between mouse and rat is controversial. Wilson et al. (1977) argued that it can be anywhere between 5 and 35 mya, whereas Jacobs and Pilbeam (1980) estimated it to be 8-14 mya. Recently, Kumar and Hedges (1998) estimated it to be 41 mya based on 343 gene comparisons. This value is substantially higher than our result. In their study, however, the variance of the divergence time between mouse and rat was larger than that between human and macaque, especially for the upper part. In any case, our result is shorter than Kumar and Hedges's (1998) estimate.

Comparison of numbers of amino acid substitutions for different regions of Rh and Rh50 genes

Figure 3 shows the multiple alignments of amino acid sequences of Rh blood group genes and their related genes. The amino acid sequence lengths of Rh genes for human D, human cE, crab-eating macaque, mouse, and rat are 417, 417, 417, 418, and 422, respectively. The amino acid sequence lengths of Rh50 genes for human, crab-eating macaque, mouse, and rat are 409, 428, 438, and 450, respectively. The amino acid sequence lengths of Rh related genes for *C. elegans* 1 (Z74026-B0240.1), *C. elegans* 2 (U64847-F08F3.3), and sponge are 457, 463, and 523, respectively. Twelve membrane-spanning regions are shown by boxes.

We analyzed patterns of amino acid changes for 3 regions (trans-membrane, inner-membrane, and outer-membrane) of primates (human vs. crab-eating macaque) and rodents (mouse vs. rat) Rh and Rh50 genes (Table 1). Numbers of amino acid substitutions were estimated by using Kimura's (1983) method. Numbers of amino acid substitutions were more or less similar among the 3 regions in primate Rh genes. It is interesting that the number of amino acid substitutions of trans-membrane regions of primate Rh genes was higher than those of others. This suggests that primate Rh genes have lower selective constraints than the other genes.

Using a one-sided *t*-test (at the 5% level) we found in primate Rh50 genes, the number of amino acid substitutions of outer-membrane regions is significantly greater than that of trans-membrane regions. In rodent Rh genes, the number of amino acid substitutions of outer-membrane regions is significantly (at the 1% level) greater than those of inner- and trans-membrane regions. In rodent Rh50 genes the number of amino acid substitutions of outer-membrane regions is also significantly greater than those of other regions (5% and 1% for inner- and trans-membrane regions, respectively). It is also noted that outer-membrane regions of rodent Rh and Rh50 genes contain 4 gaps, and 1 and 11 gaps, respectively. These observations are compatible with the results of numbers of amino acid substitutions.

Effect of the number of sequences on the estimation of the gene duplication time for Rh and Rh50 genes

Figure 4 shows the phylogenetic tree for Rh

Table 1. Comparisons of numbers of amino acid substitutions per site (d_A) and amino acid differences (P_A in %) for each region of Rh and Rh50 genes

	Rh		Rh50	
	$d_A \pm SE$	P_A	$d_A \pm S.E.$	P_A
Primates				
trans-membrane	0.280 ± 0.041	23.3 (52/223)	0.081 ± 0.020	7.6 (17/223)
inner-membrane	0.248 ± 0.054	21.1 (23/109)	0.159 ± 0.044	14.3 (14/98)
outer-membrane	0.233 ± 0.059	20.0 (17/85)	0.194 ± 0.052	17.1 (15/88)
Rodents				
trans-membrane	0.143 ± 0.027	13.0 (29/223)	0.056 ± 0.016	5.4 (12/223)
inner-membrane	0.170 ± 0.043	15.2 (17/112)	0.131 ± 0.036	12.0 (14/117)
outer-membrane	0.513 ± 0.102	37.4 (31/83)	0.282 ± 0.062	23.5 (23/98)

Numbers of different sites/numbers of sites compared are shown in parentheses.

blood group genes and their related genes by using the maximum likelihood method by the go/0 program of OOta and Saitou (1997). Because these regions do not include gaps and are relatively conserved, only amino acid sequences of membrane-spanning regions (223 sites) were used for tree reconstruction (Fig. 3). The root was located by assuming the Rh-like protein of sponge as an outgroup. There are 3 clusters in this tree: Rh50 genes of mammals, Rh genes of mammals, and 2 genes of *C. elegans*. The branch lengths of Rh50 genes are much shorter than those of Rh genes, indicating a lower evolutionary rate in the Rh50 gene than in the Rh gene. It is interesting that after the gene duplication (indicated by a closed diamond) which produced Rh and Rh50 genes, the Rh gene lineage started to evolve more rapidly than the Rh50 lineage. Kitano et al. (1998) obtained essentially the same tree by using the neighbor-joining method (Saitou and Nei 1987).

To examine the effects of numbers of sequences for estimation of the divergence time, we reconstructed 4 trees (Fig. 5) for Rh and Rh50 genes of primates and rodents by using the go/0 program of OOta and Saitou (1997), and estimated the divergence time between Rh and Rh50 genes. The 223 amino acid sites for membrane-spanning regions (Fig. 3) were used for construction of trees. The root for each tree was located by assuming the Rh-like protein of sponge (Seack et al. 1997) as an outgroup. Numbers of amino acid substitutions of single lineage were obtained by applying the method of Ishida et al. (1995) to each tree (Table 2). Because numbers of amino acid substitutions for Rh were consistently 2-3 times higher than those for Rh50, a rough molecular clock exists for both genes. Therefore, we estimated evolutionary rates of Rh and Rh50 genes by using regression through the origin. Divergence times between human and crab-eating macaque, between mouse and rat, and between primates and rodents were assumed to be 23 (Kumar and Hedges 1998), 30 (estimated in this study), and 122 (Easteal et al. 1995) mya, respectively, and they were used for calibration of the molecular clock.

In the case of tree A, the divergence time between Rh and Rh50 genes is estimated to be 130-170 mya. This period roughly corresponds to the

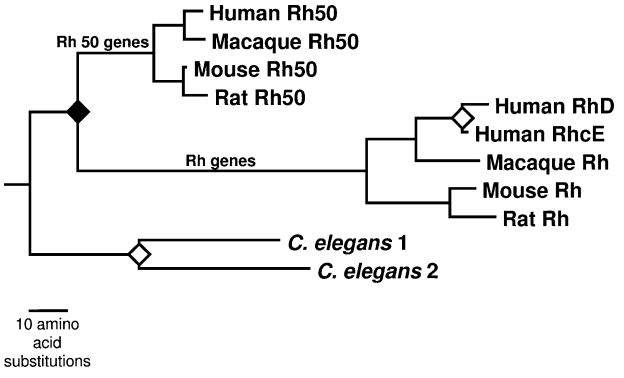

Fig. 4. The phylogenetic tree of the Rh blood group genes and their related genes. Only amino acid sequences of membrane-spanning regions (223 sites; see Fig. 3) are used. The root was located by assuming the Rh-like protein of sponge as an outgroup. Diamonds on interior nodes indicate gene duplication. The closed diamond indicates the gene duplication which produced between Rh and Rh50 genes.

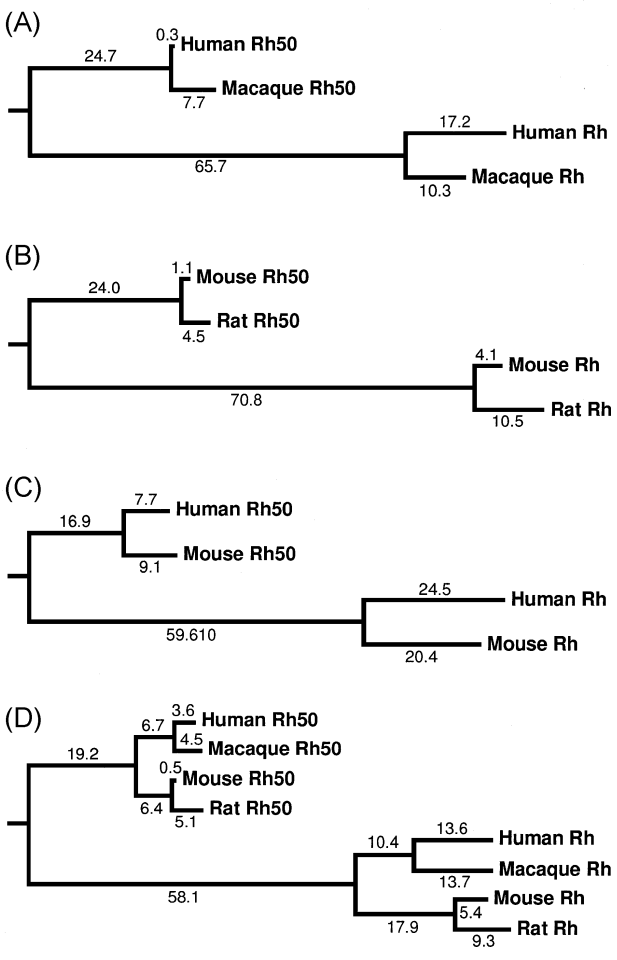

Fig. 5. Four trees examining the effects of numbers of OTUs for estimation of the divergence time between Rh and Rh50 genes. Numbers on branches show numbers of amino acid substitutions estimated from the maximum likelihood method. The root of each tree was located by assuming the Rh-like gene of sponge as an outgroup. (A) Rh and Rh50 genes for human and crab-eating macaque were used. (B) Rh and Rh50 genes for mouse and rat were used. (C) Rh and Rh50 genes for human and mouse were used. (D) Rh and Rh50 genes for human, crab-eating macaque, mouse, and rat were used.

early Mesozoic before the mammalian radiation. Because these values are close to the divergence time between primates and rodents, these are probably underestimations. Acceleration of evolutionary rate for the primate lineage by positive selection may have affected these values. In the case of tree B, the divergence time is estimated to be 290-320 mya. This period roughly corresponds to the late Paleozoic. These values are about 2 times higher than those of tree A. In the case of tree C, the divergence time is estimated to be 370-450 mya. In the case of tree D, the divergence time is estimated to be 340-380 mya. This period roughly corresponds to the middle Paleozoic, and the divergence between land vertebrates and amphibian lineage occurred around that period. The ranges of these values are smaller than those of tree C. It is clear from these comparisons that many calibration points for the molecular clock are needed to obtain a better estimation of divergence times. Figure 6 shows comparisons between amino acid substitutions and divergence times from tree D of figure 5. It indicates evolutionary constancy of Rh and Rh50 genes except in primates. It is interesting that evolutionary rates of Rh and Rh50 genes are accelerated on the primate lineage. In any case, one should be very careful when estimating the divergence time of genes.

In conclusion, we performed evolutionary analyses of the Rh blood group genes and its homologous Rh50 genes in the present study. First, we compared synonymous substitutions of Rh and Rh50 genes in primates and rodents. As a result, we obtained the result that the mutation rate in rodents is about 3 times higher than that in primates, and we could estimate the divergence time of mouse and rat. Second, we compared amino acid changes of outer-, inner-, and trans-membrane regions of the Rh and Rh50 proteins. Amino acid changes of outer-membrane regions were more frequent than others except for primate Rh genes. Finally, we made the multiple alignment for these genes and constructed the phylogenetic tree. The time of the gene duplication that produced the Rh and Rh50 genes was estimated to be about 340-380 mya. To obtain a more accurate estimation of divergence time, it is better to have multiple calibration points for the molecular clock.

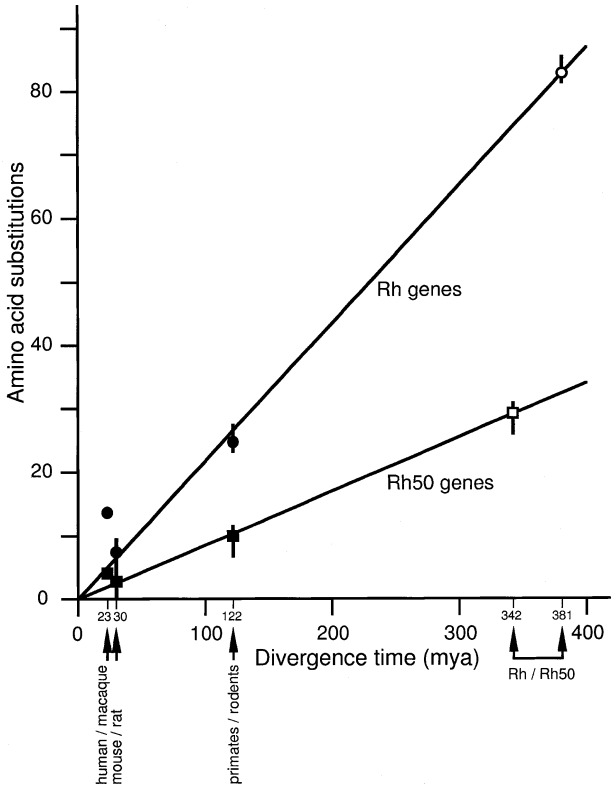

Fig. 6. Comparisons between amino acid substitutions and divergence times from tree D of figure 5. Closed circles and closed squares show points for Rh and Rh50 genes, respectively. Divergence times between Rh (open circle) and Rh50 (open square) genes are estimated by using regression through the origin.

Table 2. Numbers of amino acid substitutions and divergence times

Diverging node: Divergence time (mya):	human/macaque (23)	mouse/rat (30)	primates/rodents (122)	Rh/Rh50	time[a]
Rh (Tree A)	13.73	–	–	79.44	133
Rh (Tree B)	–	7.29	–	78.10	321
Rh (Tree C)	–	–	22.46	82.07	446
Rh (Tree D)	13.66	7.38	24.68	82.79	381
Rh50 (Tree A)	3.99	–	–	28.71	165
Rh50 (Tree B)	–	2.78	–	26.83	290
Rh50 (Tree C)	–	–	8.43	25.33	366
Rh50 (Tree D)	4.06	2.79	9.94	29.15	342

[a]Divergence time (mya) between Rh and Rh50 genes estimated from numbers of amino acid substitutions of each tree of figure 5.

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哺乳動物之 Rh 血型基因和 Rh50 基因之分子進化分析

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所有的哺乳動物大概都有負責生產膜蛋白的 Rh 血型基因以及其同源的 Rh50 基因。本文比較靈長類動物和囓齒類動物的 Rh 和 Rh50 基因的核苷酸序列，發現這兩個基因同義取代速率大約相同，推算小鼠類和大鼠類大約在三千萬年前分歧進化出來。此外，靈長類動物和囓齒類動物的進化速率不一致。比較 Rh 和 Rh50 蛋白質的膜外、膜內和穿膜區段的胺基酸變化，發現除了靈長類動物的 Rh 基因外，膜外區段最容易改變。此外，想要較精確的估算進化分歧時間，最好在分子鐘上有多個的校正點，依此原則估算基因重複產生 Rh 和 Rh50 基因的時間，約在三億四千萬至三億八千萬年前。

關鍵詞：Rh 和 Rh50 基因，相對速率，分歧時間。

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