Phylogenetic Relationships among Japanese, Rhesus, Formosan, and Crab-eating Monkeys, Inferred from Restriction-Enzyme Analysis of Mitochondrial DNAs[1]

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Mitochondrial DNA (mtDNA) polymorphisms in four species of macaques, i.e., Japanese monkey (*Macaca fuscata*), rhesus monkey (*M. mulatta*), Formosan monkey (*M. cyclopis*), and crab-eating monkey (*M. fascicularis*), were analyzed to study phylogenetic relationships. When 17 restriction enzymes of 6-bp recognition were used, 42–49 sites were observed in the samples. The estimated number of nucleotide substitutions per site among Japanese, rhesus, and Formosan monkeys ranges from 0.0318 to 0.0396, and that between the crab-eating monkey and the other monkeys from 0.0577 to 0.0653. These findings suggest that the crab-eating monkey diverged from the other three ~1.5–3.0 Myr before the present (Mybp) and that the Japanese, rhesus, and Formosan monkeys diverged ~0.9–1.8 Mybp, although the branching order cannot be determined conclusively.

Introduction

Having adapted to diverse environments, species of macaques, genus *Macaca,* are widely distributed throughout Southeast Asia, East Asia, and other areas of southern Asia as well as neighboring islands. Fooden (1980) classified them into 19 species that could be arranged into four species groups on the basis of morphological traits. Phylogenetic relationships among the species of macaques have been studied by using fossil records (Delson 1980) and blood protein polymorphisms (Darga et al. 1975; Nozawa et al. 1977; Cronin et al. 1980).

While species of macaques can be distinguished from each other by their morphological traits, some of them are sympatric and interbreed (Fooden 1964; Bernstein and Gordon 1980; Eudey 1980). This implies that they have not speciated completely and are in the process of speciation. Therefore, genetic studies of macaques are of interest in understanding the processes of evolution.

Recent advances in molecular biology have made it possible to investigate polymorphisms of DNA sequences within and between species. In particular, since the evolutionary rate of base substitutions for mitochondrial DNA (mtDNA) is several times greater than that for nuclear DNA (Brown 1983), its polymorphisms of sequences frequently have been used to investigate genetic relationships both within species and between closely related species (Brown 1980; Brown et al. 1982; Ferris et al. 1983*b;*

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Cann et al. 1984; Horai et al. 1984; Hixson and Brown 1986; Horai and Matsunaga 1986). Using restriction-enzyme analysis of mtDNA, George (1982) investigated phylogenetic relationships among seven species of Old World monkeys that include three species of macaques. We have already analyzed mtDNA polymorphisms in 10 Japanese monkeys (*M. fuscata*) and have found a considerable divergence of mtDNA sequences (Hayasaka et al. 1986). In the present paper, we extend our investigation to three other species of macaques, the rhesus monkey (*M. mulatta*), the Formosan monkey (*M. cyclopis*), and the crab-eating monkey (*M. fascicularis*). The four species are considered to be closely related and compose the fascicularis species group defined by Fooden (1980). Results of our analysis suggest that the Japanese, rhesus, and Formosan monkeys are closely related to each other while the crab-eating monkey is distantly related to them.

Material and Methods

mtDNAs were isolated from the livers of 10 Japanese monkeys, one rhesus monkey, one Formosan monkey, and one crab-eating monkey according to a method described by Drouin (1980) and Hayasaka et al. (1986). The Japanese monkeys were derived from four local populations as described elsewhere (Hayasaka et al. 1986). The liver samples of a rhesus monkey of Indian origin, a Formosan monkey, and a crab-eating monkey from Mindanao Island of the Philippines were provided by Drs. J. Suzuki (Primate Research Institute, Kyoto University), H. Inagaki (Japan Monkey Centre), and T. Tanaka (Shizuoka Laboratory Animal Center), respectively.

Approximately 1-μg portions of mtDNA were digested by three units each of 17 restriction enzymes: *Ava*I, *Bam*HI, *Bgl*II, *Bst*EII, *Cla*I, *Dra*I, *Eco*RI, *Hae*II, *Hin*cII, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Sca*I, and *Xba*I, all of which recognize 6-bp sequences. Digested mtDNAs were separated by electrophoresis in 1.2% agarose horizontal slab gels for 16 h at 1.5 V/cm. Lambda phage DNAs digested by *Eco*RI and *Hin*dIII were also run in gels as a size standard. One hour after the initiation of electrophoresis, 50 ml of ethidium bromide solution (2 μg/ml) was overlaid on the surface of the gels. After electrophoresis, restriction fragments of mtDNA were visualized and photographed under ultraviolet light and sizes of fragments were measured from the photographs.

We mapped the recognition sites by means of double digestion analysis. The average number of nucleotide substitutions per site or of nucleotide diversities was estimated using equations (21) and (28) of Nei and Tajima (1983). Phylogenetic trees were constructed using the unweighted-pair-group method (Sneath and Sokal 1973) and the neighbor-joining method (Saitou and Nei 1987).

Results

In our previous paper (Hayasaka et al. 1986), we identified four different types of mtDNA in 10 Japanese monkeys. Among the 10 monkeys there was a length polymorphism involving ~200 bp, with the sizes of long and short mtDNA estimated to be 17.0 and 16.8 kb, respectively. We found that the length of mtDNA for rhesus, Formosan, and crab-eating monkeys was approximately the same as the short one (see below). In the following comparison of restriction-fragment patterns, we used mtDNA type III (short mtDNA) as representative of Japanese monkeys.

Figure 1 shows the cleavage patterns of enzymes that produce one or two patterns in the four species. Digestion by *Kpn*I showed a monomorphic restriction pattern among the four species. The cleavage patterns differ from each other by site gain/loss

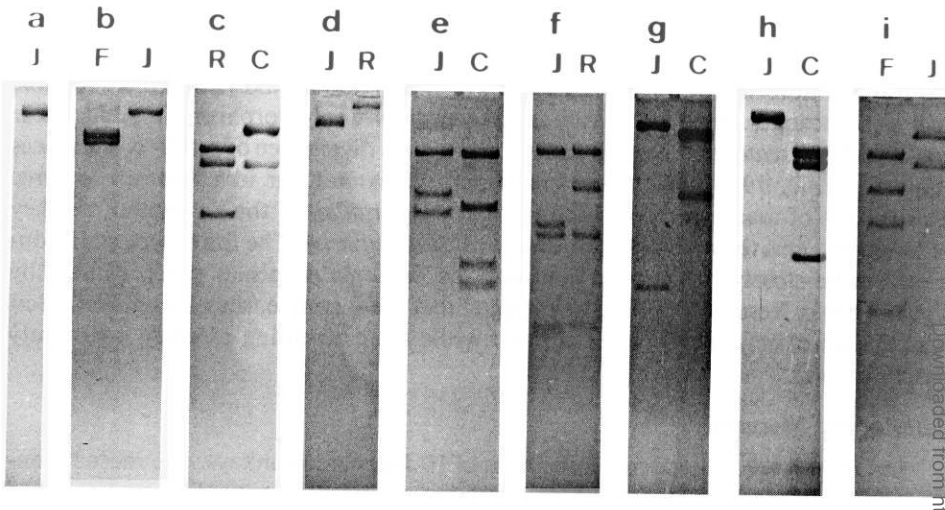


FIG. 1.—Cleavage patterns of nine enzymes: a, *Kpn*I; b, *Eco*RI; c, *Bgl*II; d, *Pvu*II; e, *Sca*I; f, *Hae*II; g, *Cla*I; h, *Sac*I; and i, *Xba*I. J, R, F, and C on the top represent Japanese, rhesus, Formosan, and crab-eating monkeys, respectively. With *Eco*RI and *Xba*I, cleavage patterns for the rhesus and crab-eating monkeys are the same as those for the Japanese monkey. With *Bgl*II, cleavage patterns for the Japanese and Formosan monkeys are the same as that for the rhesus monkey. With *Sca*I, *Cla*I, and *Sac*I, cleavage patterns for the rhesus and Formosan monkeys are the same as those for the Japanese monkey. With *Pvu*II and *Hae*II, cleavage patterns for the Formosan and crab-eating monkeys are the same as those for the rhesus monkey.

(*Eco*RI, *Bgl*II, *Pvu*II, and *Hae*II), by site gain and site loss (*Cla*I and *Sca*I), or by two site gains/losses (*Sac*I and *Xba*I).

Figure 2 shows the cleavage patterns of the enzymes that produce three different patterns in the four species. The cleavage patterns differ from each other by, at most, two site gains/losses (*Bam*HI and *Pst*I), three site gains/losses (*Bst*EII and *Ava*I), or by four site gains/losses (*Eco*RV).

Figure 3 shows the cleavage patterns of enzymes that produce four different patterns in the four species. The cleavage patterns differ from each other by, at most, four site gains/losses (*Dra*I and *Hinc*II) or by two site gains/losses (*Hin*dIII).

Previously, we could not detect 0.1-kb *Sca*I fragments and 0.2-kb *Bam*HI fragments in Japanese monkeys, owing to the limited resolution of the gels used (Hayasaka et al. 1986). However, side-by-side comparison of samples confirmed the existence of these fragments in some samples, as shown in tables 1 and 2.

For all samples the sizes of restriction fragments produced by digestions with each enzyme are shown in table 1. Relative positions of recognition sites for the 17 enzymes determined by double digestion analysis are listed in table 2. All four types of mtDNA observed in the 10 Japanese monkeys are included in this table. The position of each site is expressed as the distance (in kilobases) from the *Eco*RI site shared by all samples. All 11 sites in the region from 9.7 kb to 11.9 kb are unvaried among the samples examined. This region corresponds approximately to the two subunits of ribosomal RNA genes. Therefore, there may be some functional constraints that make these sites invariable.

Table 3 shows the estimated average number of nucleotide substitutions per site between each pair of the samples. The values for Japanese monkeys in this table are different from those in our previous paper (Hayasaka et al. 1986, table 3) because we

Table 1

Restriction-Fragment Sizes of mtDNAs Produced by 17 Restriction Enzymes for the Four Species of Macaques

Enzyme and Species	Fragment Size(s) (kb)
*Kpn*I:	
J R F C	16.8
*Eco*RI:	
J R C	16.8
F	8.8 8.0
*Bgl*II:	
J R F	7.2 6.0 3.6
C	10.8 6.0
*Pvu*II:	
J	16.8
R F C	No cut
*Sca*I:	
J R F	7.9 4.7 4.1 (0.1)
C	7.9 4.2 2.5 2.2
*Hae*II:	
J	7.6 3.4 3.1 1.4 1.3
R F C	7.6 4.7 3.1 1.4
*Cla*I:	
J R F	14.6 2.2
C	11.9 4.9
*Sac*I:	
J R F	16.4 0.4
C	7.4 6.4 2.6 0.4
*Xba*I:	
J R C	10.4 6.4
F	7.0 4.7 3.4 1.7
*Bam*HI:	
J	7.2 5.9 3.5 (0.2)
R	13.1 3.7
F C	13.1 3.5 (0.2)
*Pst*I:	
J	16.8[a]
R C	16.8[a]
F	No cut
*Bst*EII:	
J	16.8
R	12.8 3.0 1.0
F C	No cut
*Ava*I:	
J R	7.4 6.1 3.3
F	8.5 5.0 3.3
C	7.4 6.1 2.8 0.5
*Eco*RV:	
J	16.8
R F	12.9 3.9
C	16.1 0.7
*Dra*I:	
J	6.3 4.3 1.9 1.6 1.6 1.1
R	6.0 5.4 1.9 1.9 1.6
F	6.0 3.8 1.9 1.6 1.6 0.7 0.5 (0.4) (0.3)
C	6.3 3.0 2.0 1.9 1.6 1.6 (0.4)

Table 1 (*Continued*)

Enzyme and Species	Fragment Size(s) (kb)						
*Hinc*II:							
J	9.2	3.3	2.2	2.1			
R	11.3	3.3	2.2				
F	7.2	4.1	3.3	2.2			
C	6.6	3.3	2.8	2.0	1.3	0.6	(0.2)
*Hind*III:							
J	9.9	4.2	1.0	0.9	0.8		
R	9.9	3.5	1.5	1.0	0.9		
F	9.9	5.9	1.0				
C	5.7	5.0	4.2	1.0	0.9		

NOTE.—J = Japanese monkey; R = rhesus monkey; F = Formosan monkey; and C = crab-eating monkey. Figures in parentheses refer to the sizes of the fragments that cannot be detected in the gels but whose existence is confirmed by detailed analysis.

[a] Although mtDNAs of the Japanese, rhesus, and crab-eating monkeys are cut at a single site by *Pst*I, the positions of the sites differ between the Japanese monkeys and the others.

have since detected two additional recognition sites that had not been found previously. We also did not use *Acc*II in the present study. Each sample has 42–49 recognition sites, corresponding to ~1.5%–1.8% of the genome. Types I and II for Japanese monkeys differ only by a length polymorphism of ~200 bp, as mentioned above, and therefore they have the same set of recognition sites (table 2) and a nucleotide diversity of zero (table 3). The number of nucleotide substitutions within the Japanese monkeys is less than that between any pair of the different species. The number of nucleotide substitutions among the Japanese, rhesus, and Formosan monkeys is approximately

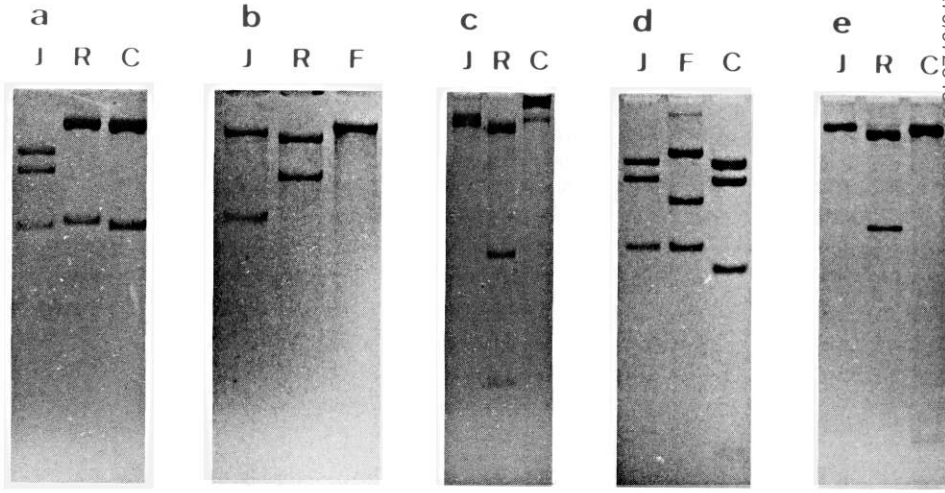


FIG. 2.—Cleavage patterns of five enzymes: a, *Bam*HI; b, *Pst*I digested doubly with *Kpn*I; c, *Bst*EII; d, *Ava*I; and e, *Eco*RV. Abbreviations for samples are as in fig. 1. With *Bam*HI and *Bst*EII, cleavage patterns for the Formosan monkey are the same as those for the crab-eating monkey. With *Pst*I, the cleavage pattern for the crab-eating monkey is the same as that for the rhesus monkey. With *Eco*RV, the cleavage pattern for the Formosan monkey is the same as that for the rhesus monkey. With *Ava*I, the cleavage pattern for the rhesus monkey is the same as that for the Japanese monkey.

Table 2
Recognition Sites for Each mtDNA of the Four Species of Macaques

Position, Enzyme	J1	J2	J3	J4	R	F	C
0.0,[a] *Eco*RI	+	+	+	+	+	+	+
0.0, *Dra*I						+	+
0.1, *Hin*dII			+	+	+		+
0.2, *Hin*cII							+
0.4,[a] *Dra*I	+	+	+	+	+	+	+
0.8, *Hin*cII						+	+
0.9,[a] *Ava*I	+	+	+	+	+	+	+
1.0,[a] *Hin*dIII ...	+	+	+	+	+	+	+
1.0,[a] *Bgl*II	+	+	+	+	+	+	+
1.2,[a] *Sca*I	+	+	+	+	+	+	+
1.3, *Sca*I	+	+	+	+	+	+	
2.0, *Eco*RV ...	+	+	+	+	+	+	
2.1, *Hin*cII							+
2.2, *Bst*EII						+	
2.3,[a] *Dra*I	+	+	+	+	+	+	+
2.7 *Bam*HI	+	+	+	+		+	+
2.9,[a] *Bam*HI ...	+	+	+	+	+	+	+
2.9, *Hin*cII	+	+					
3.0, *Sac*I	+	+	+		+	+	+
3.4,[a] *Sac*I	+	+	+	+	+	+	+
3.2, *Bst*EII						+	
3.6, *Pvu*II			+				
3.6, *Xba*I	+	+	+		+	+	+
7.1,[a] *Hin*cII	+	+	+	+	+	+	+
7.6, *Hin*cII	+	+					
8.0, *Eco*RI						+	
8.3, *Xba*I	+	+				+	
8.8, *Bam*HI ...			+	+			
9.4, *Ava*I						+	
9.7,[a] *Hae*II	+	+	+	+	+	+	+
10.0,[a] *Xba*I	+	+	+	+	+	+	+
10.2,[a] *Dra*	+	+	+	+	+	+	+
10.3,[a] *Kpn*I	+	+	+	+	+	+	+
10.4,[a] *Hin*cII	+	+	+	+	+	+	+
10.9,[a] *Hin*dIII ...	+	+	+	+	+	+	+
11.1,[a] *Hae*II	+	+	+	+	+	+	+
11.5,[a] *Cla*I	+	+	+	+	+	+	+
11.8,[a] *Dra*I	+	+	+	+	+	+	+
11.8,[a] *Bgl*II	+	+	+	+	+	+	+
11.9,[a] *Hin*dIII ...	+	+	+	+	+	+	+
12.4, *Sac*I							+
12.5, *Hin*cII			+	+			
12.5, *Kpn*I	+	+					
12.7, *Hin*dIII ...			+				
12.8, *Bam*HI ...	+	+					
13.3,[a] *Sca*I	+	+	+	+	+	+	+
3.9, *Dra*I	+	+	+	+		+	+
4.2, *Dra*I					+	+	
4.3, *Pst*I					+		+
4.6, *Bgl*II	+	+	+	+	+	+	
4.9,[a] *Hin*cII	+	+	+	+	+	+	+
5.1, *Hin*cII							+
5.4,[a] *Sca*I	+	+	+	+	+	+	+

Table 2 (*Continued*)

Position, Enzyme	J1	J2	J3	J4	R	F	C
5.9, *Eco*RV ...					+	+	
6.0, *Sac*I							+
6.2, *Bst*EII	+	+	+	+	+		
6.4, *Pst*I	+	+	+	+			
6.6, *Cla*I							+
6.6,[a] *Hae*II	+	+	+	+	+	+	+
6.7, *Hin*dIII ...							+
7.0, *Ava*I	+	+	+	+	+		+
13.4, *Eco*RV ...							+
13.4, *Xba*I						+	
13.5, *Hin*dIII ...					+		
13.6, *Bst*EII	+	+					
13.7, *Cla*I	+	+	+	+	+	+	
14.1, *Eco*RV ...							+
14.4,[a] *Ava*I	+	+	+	+	+	+	+
14.5, *Hae*II	+	+	+	+			
14.8, *Dra*I							+
14.9, *Ava*I							+
15.6, *Dra*I						+	
15.8,[a] *Hae*II	+	+	+	+	+	+	+
15.8, *Sca*I							+
16.0,[a] *Bam*HI ...	+	+	+	+	+	+	+
16.1, *Dra*I	+	+	+	+		+	

NOTE.—J1, J2, J3, and J4 = Japanese monkey types I, II, III, IV, respectively, in Hayasaka et al. (1986). Other abbreviations are as in table 1. + = Presence of recognition site. Sites mapped to the same position by two enzymes are ambiguous in their exact orders.

[a] Sites shared by all samples.

the same, ranging from 0.0318 to 0.0396. That between the crab-eating monkey and the other species is between 0.0577 and 0.0653.

Figures 4*a* and 4*b* show the phylogenetic trees constructed using the unweighted-pair-group method (Sneath and Sokal 1973) and the neighbor-joining method (Saitou and Nei 1987), respectively. Both trees give the same order of divergence among the four species in which the four types of Japanese monkeys form a cluster, and the crab-eating monkey is the most distant relative to the others. These trees show that the Formosan monkey diverged from the lineage leading to Japanese and rhesus monkeys before the latter two species diverged. However, an exact branching order for these three species cannot be conclusively determined by this analysis, because of both the small differences in estimated nucleotide diversity among these species and the considerable sampling error (table 3) due to the small number of recognition sites. If we assume an average nucleotide substitution rate of $2\text{–}4 \times 10^{-8}$/site/year (Ferris et al. 1983*b*), the Japanese, rhesus, and Formosan monkeys would have diverged ~1.8–0.9 Mybp and the divergence time between these three monkeys and the crab-eating monkey would be 3.0–1.5 Mybp.

Discussion

Delson (1980) discussed phylogenetic relationships among species of macaques by using fossil data. The phylogeny suggested from our analysis is compatible with Delson's (1980, fig. 6 of chap. 2) in terms of topology but not for divergence times. Our estimates for divergence times are generally greater than those given by Delson.

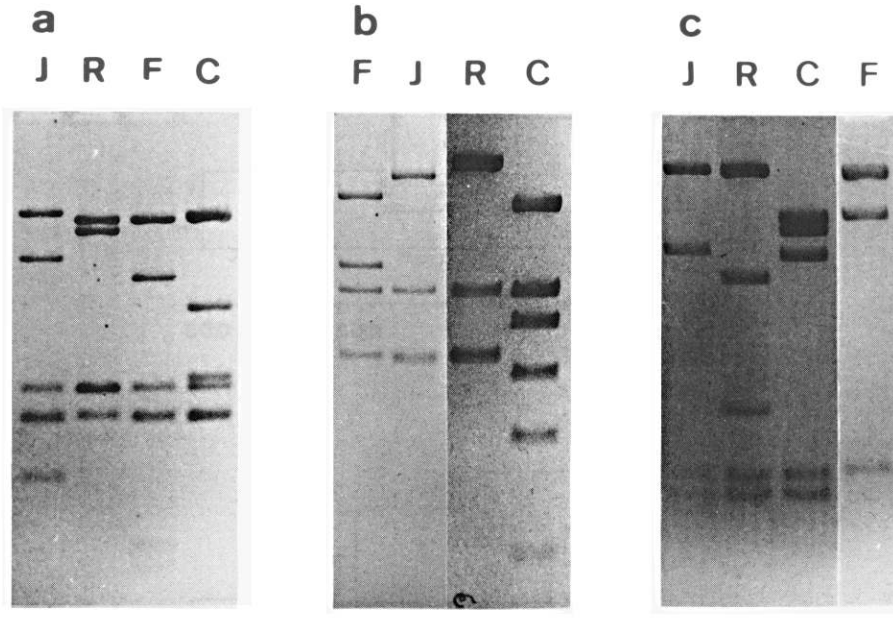


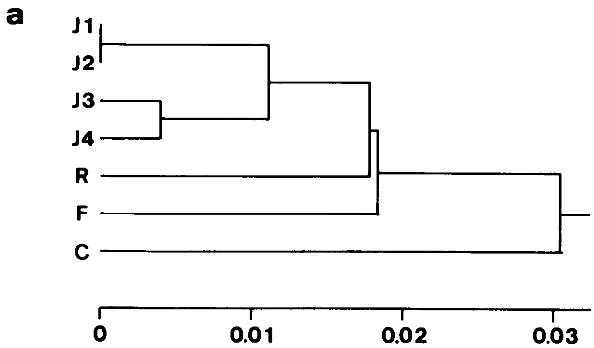
FIG. 3.—Cleavage patterns of three enzymes: a, *Dra*I; b, *Hinc*II; and c, *Hin*dIII. Abbreviations for samples are as in fig. 1.

For example, he estimated the divergence time for Japanese and rhesus monkeys to be 0.3 Mybp while we estimated it to be 0.9–1.8 Mybp. As Delson mentioned, this discrepancy might result from the ambiguity of his estimation of divergence times. Alternatively, it is also possible that we overestimated the divergence times. In the present study we have estimated the divergence time for genes; and genes might have diverged prior to the divergence of species. The population of common ancestors

Table 3
Number of Recognition Sites in Each mtDNA, Recognition Sites Shared by Each Pair, and Average Number of Nucleotide Substitutions per Site

	J-1	J-2	J-3	J-4	R	F	C
J-1	47	47	41	39	36	38	33
J-2	0	47	41	39	36	38	33
J-3	0.0217	0.0217	46	42	37	37	34
	(0.0070)	(0.0070)					
J-4	0.0228	0.0228	0.0080	42	35	35	32
	(0.0073)	(0.0073)	(0.0041)				
R	0.0385	0.0385	0.0318	0.0335	43	36	33
	(0.0100)	(0.0100)	(0.0089)	(0.0095)			
F	0.0349	0.0349	0.0376	0.0396	0.0365	46	34
	(0.0093)	(0.0093)	(0.0098)	(0.0103)	(0.0097)		
C	0.0653	0.0653	0.0581	0.0613	0.0577	0.0581	49
	(0.0139)	(0.0139)	(0.0129)	(0.0137)	(0.0130)	(0.0129)	

NOTE.—The number of recognition sites in each mtDNA is shown on the diagonal. The number of recognition sites shared and the average number of nucleotide substitutions per site are shown above and below the diagonal, respectively. The numbers in parentheses are SEs of the number of nucleotide substitutions. Abbreviations are as in table 2.



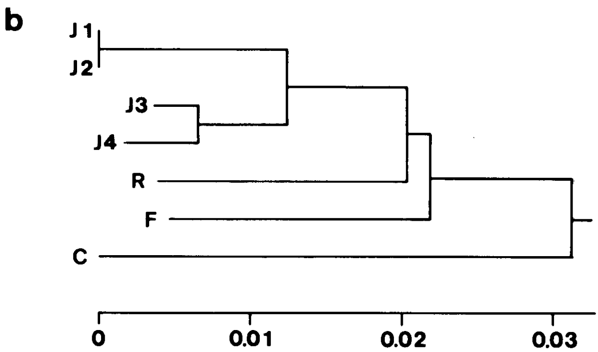


FIG. 4.—Phylogenetic trees constructed from the recognition-site data by using (a) the unweighted-pair-group method and (b) the neighbor-joining method. J1, J2, J3, J4, R, F, and C represent the Japanese monkeys types 1–4, rhesus, Formosan, and crab-eating monkeys, respectively. Scales below the figures represent branch lengths (in number of nucleotide substitutions per site).

might have already been polymorphic (Takahata and Nei 1985; Horai et al. 1986), as suggested by substantial intraspecific nucleotide diversity among Japanese monkeys.

Nozawa et al. (1977, fig. 5) obtained a different phylogenetic relationship among the four species by using blood protein polymorphisms detected from gel electrophoresis. In their phylogeny crab-eating, rhesus, and Formosan monkeys form a cluster and Japanese monkeys are placed at some distance from them in evolution, while in the present analysis the Japanese, rhesus, and Formosan monkeys form a cluster and the crab-eating monkey is placed at some distance from the others. Some authors also reported discrepancies between nuclear and mitochondrial phylogenies in different taxonomic groups (Brown and Simpson 1981; Ferris et al. 1983*b;* Lansman et al. 1983; DeSalle and Giddings 1986). These discrepancies might have been caused by a different mechanism of inheritance between nuclear DNA and mtDNA, since mtDNA is inherited maternally, unlike nuclear DNA (Giles et al. 1980). Hybridization between species after their speciation, introgression of mtDNA across species boundaries, or both of these factors might have resulted in such discrepancies in divergence estimates. Introgression of mtDNA has been found in some animal populations (Ferris et al. 1983*a;* Powell 1983; Spolsky and Uzzell 1984).

Japanese and Formosan monkeys are confined to the Japanese and Formosan Islands, respectively. The distribution of rhesus and crab-eating monkeys overlaps

only in the limited area of Indochina (Fooden 1980). In the past, however, these species might have had a large overlapping distribution; this is partly because the Asian continent has been contiguous with its neighboring islands from time to time (Delson 1980; Eudey 1980). Moreover, hybrids between rhesus and crab-eating monkeys have been observed in Thailand (Fooden 1964; Eudey 1980). Interspecific hybrids of macaques have also been observed in captivity and are known to be fertile (Bernstein and Gordon 1980). Therefore, it is probable that hybridization and introgression among macaques has occurred in the past.

DeSalle and Giddings (1986) also argued that discrepancies among divergence estimates might have been caused by hybridization or introgression, and they favor the former possibility on the basis of geographic data. Lansman et al. (1983) suggested that asymmetric dispersal by sex could also be a factor leading to differing estimates of divergence. Most male macaques emigrate from their native populations and move between populations or live solitarily, while female macaques usually stay in their native populations for their entire lives (Sugiyama 1976). It is possible that bisexually inherited nuclear genomes might have been homogenized by the migration of males, while maternally inherited mtDNA might reflect the past events of population splitting more accurately.

Our estimates have some sampling errors (table 3) owing to the small number of recognition sites in each sample; thus our constructed phylogeny may change when we examine more samples, because of substantial intraspecific nucleotide diversity, as mentioned above. However, intraspecific nucleotide diversity among the Japanese monkeys ranges from 0 to 0.0228, while that between any pair of the four species is ≥ 0.0318 (table 3) in the present study. Comparison of nucleotide sequences of the homologous 0.9-kb *Hin*dIII fragments of mtDNA from the Japanese, rhesus, and crab-eating monkeys (data not shown) shows that the Japanese and rhesus monkeys are more closely related than either of them is to the crab-eating monkey. Since comparison of the 0.9-kb nucleotide sequences shows less variance than the restriction-enzyme analysis and can detect every base change, the phylogenetic relationship that we obtained probably reflects the actual mtDNA phylogeny as far as these three species are concerned. Determination of the nucleotide sequence for the homologous fragment from the Formosan monkey may make the relationships among the Japanese, rhesus, and Formosan monkeys clearer.

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