

Molecular Phylogeny of Macaques: Implications of Nucleotide Sequences from an 896-Base Pair Region of Mitochondrial DNA

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We determined the nucleotide sequences of an 896-base pair region of mitochondrial DNA (mtDNA) from 20 primates representing 13 species of macaques, a baboon, and a patas. We compared these sequences and the homologous sequences from four macaques and a human against each other and deduced the phylogenetic relationships of macaques. The results from the phylogenetic analyses revealed five groups among the macaques: (1) Barbary macaque, (2) two species of Sulawesi macaques, (3) Japanese, rhesus, Taiwanese, crab-eating, and stump-tailed macaques, (4) toque, pig-tailed, and lion-tailed macaques, and (5) Assamese and bonnet macaques. The phylogenetic position of Tibetan macaque remains ambiguous as to whether it belongs to the fourth or fifth group. Phylogenetic trees revealed that Barbary macaque diverged first from the other Asian macaques. Subsequently, the four groups of Asian macaques diverged from one another in a relatively short period of time. Within each group, most of the species diverged in a relatively short period of time following the divergence of the groups. Assuming that the Asian macaques diverged from the outgroup Barbary macaque three million years ago (MYA), the divergence times among groups of Asian macaques were estimated at 2.1-2.5 MYA and within groups at 1.4-2.2 MYA. The intraspecific nucleotide diversity observed among three rhesus macaques was so large that they did not form a monophyletic cluster in the phylogenetic trees. Instead, one of them formed a cluster with Japanese and Taiwanese macaques, whereas the other two formed a separate cluster. This implies that either polymorphisms of mtDNA sequences that existed before the divergence of these three species (ca. 700,000 years ago) have been retained in rhesus macaques or introgression has occurred among the three species.

Introduction

Macaques, genus *Macaca*, are distributed widely in East and Southeast Asia, Indian subcontinent, Afghanistan, and surrounding islands, as well as limited areas in North Africa. They are classified into at least 14 morphologically distinguishable species. Thus, in terms of speciation and distribution, they are considered a successful genus.

Based on morphological characteristics, Fooden (1980) classified the macaques into four species groups: *silenus-sylvanus* group, including Barbary (*M. sylvanus*), lion-tailed (*M. silenus*), and pig-tailed macaques (*M. nemestrina*) and species of Sulawesi macaques (*M. nigra* and *M. tonkeana* in this study); *sinica* group, including toque (*M. sinica*), bonnet (*M. radiata*), Assamese (*M. assamensis*), and Tibetan macaques (*M. thibetana*); *arctoides* group, including stump-tailed macaque (*M. arctoides*); and *fascicularis* group, including Japanese (*M. fasciata*), rhesus (*M. mulatta*), Taiwanese (*M. cyclopis*), and crab-eating macaques (*M. fascicularis*). Delson (1980) proposed a slightly different classification. His classification gives *M. sylvanus* its own group and includes *M. arctoides* in the *sinica* group.

During the past decade, usefulness of mitochondrial DNA (mtDNA) sequences in analyzing phylogenetic relationships between closely related species has been demonstrated. However, there has not been a thorough phylogenetic analysis of macaques using mtDNA sequences. We determined nucleotide sequences of an 896-base pair (bp) region of mtDNA from 20 individ-

uals of 13 species of macaques, a baboon, and a patas, all of which belong to family Cercopithecidae. From 5' to 3' on the light strand, the 896 bp region contains the 3' end (458 bp) of the NADH dehydrogenase subunit (ND) 4 gene, the tRNA^{His} gene, the tRNA^{Ser(AGY)} gene, the tRNA^{Leu(CUN)}, and the 5' end (239 bp) of the ND5 gene. We analyzed the phylogenetic relationships of macaques using these sequences as well as homologous sequences of four macaques and human determined previously, and we describe the results from these analyses in this paper.

Materials and Methods

Samples

MtDNA was purified from liver samples of two individuals each of Japanese (#2 and #3; see below), rhesus (#2 and #3; see below), and crab-eating macaques (#2 and #3; see below) and one individual each of bonnet and Taiwanese macaques and hamadryas baboon (*Papio hamadryas*) as described previously (Hayasaka et al. 1986). Total DNA was isolated from blood samples from two crab-eating macaques, one each of stump-tailed, pig-tailed, lion-tailed, Tibetan, Assamese, toque, Celebes (*M. nigra*), and Tonkean macaques (*M. tonkeana*) and a B-cell line of patas (*Erythrocebus patas*) as described in Sambrook, Fritsch, and Maniatis (1989). The two Japanese macaques originated from Takahama and Shiga populations in Japan, the two crab-eating macaques (#4 and #5; see below) and the Assamese macaque from Thailand, and the pig-tailed macaque from Borneo, whereas origins of the other samples were unknown.

Nucleotide Sequences

The nucleotide sequence of baboon was determined by a conventional method (Sambrook, Fritsch, and Man-

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iatis 1989). The 896-bp Hind111 fragment and smaller fragments derived from restriction digestion of the original fragment were cloned into the plasmid pUC19. Template single-stranded DNA was prepared by the alkali-lysis method.

Nucleotide sequences of the other animals were determined using DNA fragments amplified by the polymerase chain reaction (PCR) (Saiki et al. 1988).

The sequences of the stump-tailed, pig-tailed, lion-tailed, Tibetan, toque, bonnet, Celebes, and Tonkean macaques were determined from template DNA prepared by asymmetrical PCR (Gyllensten and Erlich 1988). The first symmetrical PCR reaction mixture consisted of 1 X PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.31, 1.5 mM MgCl₂, 0.01% gelatin), 0.8 mM each of four dNTPs, 0.2 μM each of a pair of primers, 0.5 μl of AmpliTaq (units/ml) (Perkin-Elmer-Cetus), and 5 ng of purified mtDNA or 100 ng of total DNA in a volume of 50 μl. DNA fragments were amplified through 30 cycles of 15 s at 94°C, 15 s at 45°C, and 1 min at 72°C. The PCR products were gel-purified by Suprec-01 columns (Takara). The second asymmetrical PCR reaction mixture consisted of 1 X PCR reaction buffer, 0.8 mM each of four dNTPs, 1 μM of one primer, 0.01 μM of the other primer, 0.5 μl of AmpliTaq, and 2 μl of the first PCR product in a volume of 50 μl. DNA fragments were amplified through 35 cycles of 15 s at 94°C, 15 s at 45°C, and 1 min at 72°C. Products of the second PCR were purified by PCR Purification Kit (Qiagen) and dissolved in 20 μl of ddH₂O.

The sequences of the Assamese, Japanese, rhesus, and Taiwanese macaques, patas, and the two crab-eating macaques (#2 and #3) were determined from template DNA prepared by the Dynabeads M-280 (Dyna) biomagnetic separation method for PCR products (Hultman et al. 1991). The PCR reaction consisted of 1 X PCR buffer, 0.2 μM each of dNTPs, 60 pM of a biotinylated primer (p302; see below), 60 pM of a nonbiotinylated primer (p207; see below), 0.5 μl of AmpliTaq, and 2.5 ng of purified mtDNA or 50 ng of total DNA in a volume of 50 μl. DNA fragments were amplified through 35 cycles of 15 s at 94°C, 15 s at 50°C, and 1 min at 72°C. Forty microliters of the PCR product was mixed with 40 μl of Dynabeads suspension, and single-stranded DNA was prepared according to the manufacturer's instructions. A quarter of the single stranded DNA was used in a sequencing reaction.

The sequences of the template DNA prepared by the methods mentioned above were determined by the dideoxy-chain termination method (Sanger, Nicklen, and Coulson 1977) using α-³²P-dCTP (Amersham), Sequenase V2.0 Kit (United States Biochemicals), and the universal and mtDNA-specific synthetic primers (see below).

The sequences of the other two crab-eating macaques (#4 and #5) were determined using an automatic DNA sequencer (373S; Applied Biosystems, Inc.). The fragment was amplified under the same conditions as for the biomagnetic separation method. The PCR product was filtered through Sephacryl S-400 columns (Pharmacia) to remove unincorporated nucleotides and primers. One-tenth of the PCR product was used for single sequencing reactions. The Prism Dye-Terminator kit (ABI/PEC) and synthetic primers were used for the sequencing reaction.

The nucleotide sequences of the oligonucleotide primers used in the PCR and sequencing reactions were p17 (ctttt atttg gagtt gcacc), p19 (ggtgc aactc caaat aaaag), p61 (ggact tcaaa ctcta ctccc), p126 (tityc ctgrt cccay atcac), p127 (yctrg grttt acytc ctgta), p128 (ctctc agccg atgaa gagtt), p139 (atagt ggggg gtagg gcaag), p142 (gtaat ygyag cctyc ctgat), p143 (gttra tgaay agtgc rattg), p207 (ctgat taatg tttgg gctcg), p300 (aatat gttt ctctg gtgaa), and p302 (ctgta agcca catrg ccctt), where y and r represent pyrimidines (t and c) and purines (a and g), respectively.

The sequences reported in this paper have been deposited in the DDBJ database (accession numbers D85268-D85291).

Analyses

In addition to the 20 sequences determined in this study, the four homologous sequences of macaques determined in our previous study (Hayasaka, Gojobori, and Horai 1988) and that of human (Anderson et al. 1981) were included in the phylogenetic analyses. The previously determined sequences of macaques included a Japanese macaque from the Gagyusan population in Japan, a rhesus macaque of Indian origin, a crab-eating macaque of Philippine origin, and a Barbary macaque (*M. sylvanus*). The number of nucleotide substitutions per site (nucleotide diversity) was estimated by the method of Tamura and Nei (1993) for each pair of sequences. Phylogenetic trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei 1987; "treenj" program in package ODN v1.1.1, Ina 1992) and the maximum parsimony (MP) method ("DNA-PARS" program in the package PHYLIP v3.5, Felsenstein 1993). A bootstrap test with 1,000 repetitions was also conducted by "DNAPARS" using "SEQBOOT" and "CONSENSE" programs in PHYLIP for the MP trees and by NJBOOT2 (kindly provided by Dr. K. Tamura) for the NJ trees.

Results and Discussion

Nucleotide Sequences

Twenty-five homologous sequences are aligned in figure 1. We observed no length differences in the 25

FIG. 1.—Alignment of the nucleotide sequences of the 896-bp region of mtDNA. Whole nucleotides are shown for the Japanese macaque 1 sequence. For the other sequences, nucleotides different from those in the Japanese macaque 1 sequence are indicated. < and > above the aligned sequence represent the 5' and 3' ends of the genes, respectively. The genes are abbreviated as ND4 and ND5 for the NADH dehydrogenase subunit 4 and 5 genes, respectively, and tRNA-His, tRNA-Ser, and tRNA-Leu for tRNA genes for histidine, serine (codon AGY), and leucine (codon CUN), respectively. Alternative initiation codon of ND5 gene is marked by ***.

120
 Japanese 1 AAGCTTTCCGGCGCAACCACTCCTTATGATCGCTCACGGACTCACCTCTCCATATATTTCTGCCTAGCCAATTCAAACATATGAACGCCTCACAAACCTACCATACTACTGTCCTCCGAGG
 Japanese 2
 Japanese 3
 Rhesus 1 T C T A
 Rhesus 2 T G C C G
 Rhesus 3 T G C C G
 Taiwanese
 Crab-eating 1 C C A C G G T G G T A
 Crab-eating 2 C C C A C G G T C G G T A
 Crab-eating 3 C C T C A C G G T G C G T A
 Crab-eating 4 C C G A C G G C T G T A
 Crab-eating 5 C C G A C C G C T G G
 Stump-tailed C T C A C C G C G T C C T G C A G
 Tibetan C T T C C T G C T C T G A A T
 Assamese C T T C C T G C T C C C A A
 Bonnet TG T T T C C T T G C T C C T G A A T
 Toque C T T A C T T G C T T C C T G C T A A
 Pig-tailed C T A C T T T C G C T T A T
 Lion-tailed C T A T G T I C G C T A T
 Celebes C T A T G T T G T A
 Tonkean C T G A T G T T G T A
 Barbary C T T A G T C T T G C C C G C T A
 Baboon C T T G T C C T T C G C C G C T T A
 Patas C T T C A C C G C T A G C T A G C
 Human CA GT T C A C G T A C AT CTA A C C C GT GTT A G C T A

240
 Japanese 1 ACTTCAAATCTACTTCCACTAACAGCCCTTTGATGATTAACAGCAAGCCCTTACTAACCTTGCCCTACCCCCACTATCAATCTACTAGGTAACCTTTGTAAATCGAACCTCATTC
 Japanese 2 C C C
 Japanese 3 C C
 Rhesus 1 C T C C G C C A G T
 Rhesus 2 C C C C A G T
 Rhesus 3 C C C C A G T
 Taiwanese C G C C
 Crab-eating 1 T T G C C C T T A T T
 Crab-eating 2 T T G C C C G A T T
 Crab-eating 3 T T T C C C C A T T
 Crab-eating 4 T G T T C C G T T A T T
 Crab-eating 5 T G T T C C G C T A T T
 Stump-tailed C C C G T T C G
 Tibetan C C T C C C T C C A A
 Assamese C C T A C C C C A A
 Bonnet C C C C C C G T A A TG
 Toque C T C G T A T C G C G T C C C A A
 Pig-tailed C C G C T C C C C T T
 Lion-tailed C C C C C C T T
 Celebes C C C C C C C T T
 Tonkean C C A C C C C T T
 Barbary C C C C C C T T T
 Baboon C G C C T A T C G C T G C C T A T G C C A T G G C C T A G T T
 Patas GC T TG CG AT C T A A CA G C T TA G T T
 Human CT C T T C TCT CG C T T C T C G A C GC A T A G

360
 Japanese 1 CTGATCCCATATCACCATTATGCTAACAGGACTTAACATATTAATACGGCCCTACTCTCTCCACATATTCACCTACAACACAACGAGAACACTCACACATCACAATAA
 Japanese 2 G C C G
 Japanese 3 G G G
 Rhesus 1 G AT T C C C G
 Rhesus 2 T G G C T C C G T T C T
 Rhesus 3 T G G T C C T T T C T
 Taiwanese G T G C
 Crab-eating 1 G T G C T C C A G T GT C T T
 Crab-eating 2 G T G C T C C A G T T C C T
 Crab-eating 3 G T G C T C C A G T T C C T
 Crab-eating 4 G AT C T C G C A T T T C C T
 Crab-eating 5 G T C T C G C A G T T C C T
 Stump-tailed T T T C A C C A T T T C C T
 Tibetan T T A C G C A T T G T C
 Assamese T T A C G C A T T T
 Bonnet C TG AT C C A C T T
 Toque G T G A G C C A T C T G
 Pig-tailed T T A C C A T T G G T G C T
 Lion-tailed T T G A C A T C T G G G T G C T
 Celebes T T A C C A T T T T
 Tonkean C T A C A A T TG T C T
 Barbary C C A G C A T C G G C T
 Baboon A A T AG C C C A C T G G C T C
 Patas T T C A T G C C C A T C T T T C A T C C T
 Human AA TC CA A T C C G C A A C T T C T G CT C C T A

ND4 >> tRNA-His 480
 Japanese 1 GCCCCCTTCACAGAGAAACACATTAATATTCATACACCTCGCTCCAATTAATCTCTATCCCTCAACCCCAACATCATCTGGGGTTTACTCTCTGTAGATATAGTTAACTAAAC
 Japanese 2 T T
 Japanese 3 T G
 Rhesus 1 A T C C C T T
 Rhesus 2 A T T T C G T T A A T
 Rhesus 3 A T T T C G T T A A
 Taiwanese A T
 Crab-eating 1 A T A
 Crab-eating 2 A T G T G A A
 Crab-eating 3 A T C T G A A
 Crab-eating 4 A T T A T
 Crab-eating 5 A T A
 Stump-tailed A T T T A A A TC
 Tibetan A T T A C T T A A G C
 Assamese A T T T C C T T A C T A
 Bonnet A T T T C C T A A C
 Toque AA T T T A A T A A
 Pig-tailed A T T G T T A A C T A
 Lion-tailed A T C G C T T T A C TC A
 Celebes A T T G T T A A T A
 Tonkean A T T G T T A A T A
 Barbary A A T T C T T T T A A T A
 Baboon A G T C TT T C C T T T T A AC TC T C
 Patas T A G T C C T T C C T T T G T A A T AG TC
 Human A T A CC C G C T AT C C C C G TACC T T A C

tRNA-His >< tRNA-Ser tRNA-Ser >< tRNA-Leu 600

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Japanese 1 ACTAGATTGTGAATCTAACCATAGAGACTCACCACCTCTTATTACCGAGAAAACTCGCAAGGACTGCTAACCCATGTACCCGTACCTAAAATACGGTTTCTCACTTTTAAAGGATA
Japanese 2 T G
Japanese 3 T
Rhesus 1 T T
Rhesus 2 T C T
Rhesus 3 T C T
Taiwanese T G T
Crab-eating 1 T T G CT T
Crab-eating 2 T G T G CT T
Crab-eating 3 T G C G CT T
Crab-eating 4 T G T AG T T
Crab-eating 5 T G T AG T T
Stump-tailed T G T AG T T
Tibetan T T AG C T
Assamese T T AG C T
Bonnet T G T AG C T
Toque T T C CT
Pig-tailed T T C G T
Lion-tailed T T C T
Celebes T GG G CT
Tonkean T G CT
Barbary T C T AG T T
Baboon GT C T AG C T
Patas C G T G CAGT T
Human TC G A C G T G C G A A T CC A GT C AC T C
    
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tRNA-Leu >< NDS *** 720

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Japanese 1 ACAGCTATCCATGACCTTAGGAGTCAA AACATTGGTGCAACTCAAATAAAAGTAATAATCATGCACACCCCCATCATTATAACAACCCCTTATCTCCCTAACTCTCCCAATTTTGCC
Japanese 2 T
Japanese 3 T
Rhesus 1 T T A
Rhesus 2 C A C
Rhesus 3 C A A
Taiwanese T T A G C T
Crab-eating 1 T A A C G C T
Crab-eating 2 C A A C G C T
Crab-eating 3 G G A A G C T
Crab-eating 4 G G A A C C T
Crab-eating 5 G G A A C C T
Stump-tailed T T A T C C G T
Tibetan C T T T A C C T
Assamese C T T T A C G T
Bonnet C T T T A CC G
Toque C G T T T A CGC T
Pig-tailed C G TG T A C C T
Lion-tailed C G TG G T A C C T
Celebes G T T A C
Tonkean G T T A T
Barbary G T T A T C
Baboon GT AC T T A T CGC G C T
Patas T G C T T A G CG T T CA
Human GT CCC TT C TA T A CC CCCT G CT T C C CC A
    
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840

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Japanese 1 ACCCTCATCAACCCCTTACAAAACGTCATACCCAGATTACGTAAAAACAACCGTAATATATGCTTTCATCATCAGCCTCCCTCAACAACCTTATTTCATCTCTCAAACCAAGAAACA
Japanese 2 C
Japanese 3 C
Rhesus 1 C C G
Rhesus 2 C C G
Rhesus 3 C C G
Taiwanese C C T T C C
Crab-eating 1 C C T T C C CC C
Crab-eating 2 C G T T A C C C G
Crab-eating 3 C T T C C C G
Crab-eating 4 G T T C C C CT
Crab-eating 5 G C T CT C C T G
Stump-tailed T G C G C T T
Tibetan T C A C T CT T TC G A T CTGG G
Assamese G T C T C T CT T CA CT
Bonnet T T C G C T T G CT T CA CT
Toque A A C C A C T T G T T CT T TC G A T TGG G
Pig-tailed T T GT CA CT A C T C T T CT T TC G A T TGG G
Lion-tailed T T TG A T A C T A C G T C C TAT C CC T CT
Celebes T T A C T A C G T T CT TA T C A T CT
Tonkean T T CA AC T T A C G T T C T TA T A T
Barbary T TG CA C ACT T A C T G TA C T TATC C A T
Baboon TG CA C ACT T A C T G TA C T TATC C A T
Patas T T A T AGT T A C T TAC C C TAT C T C C A C TCT T GG
Human G T A AACT CC T T C TT CGC CCA C T T T TT C C AA G G CT G GTT
    
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896

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Japanese 1 ACCATTTGGAGCTGACATTGAATAATGACCCAAACACTAGACCTAACGCTAAGCTT
Japanese 2 A G
Japanese 3 A G T
Rhesus 1 A A A
Rhesus 2 A AG T A
Rhesus 3 A AG T A
Taiwanese A A A G
Crab-eating 1 A T CA T A
Crab-eating 2 T T CA T A G
Crab-eating 3 T C T CA T T A
Crab-eating 4 A T A T A
Crab-eating 5 T T A T G A
Stump-tailed T T A T A
Tibetan G A CAG G T T A
Assamese G A C G A G T A T A T
Bonnet C G A G A T G A
Toque G A T CA G T A A
Pig-tailed G A T CA G T T A
Lion-tailed A C G A T T AG A
Celebes A T T A
Tonkean G G A G T A
Barbary T C A G C CA AG AT
Baboon C C A T A
Patas T A T G C CA A T AT
Human TT C C A C GCC CA ACCC G CT C
    
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Table 2
Comparison Between the Nucleotide Diversity per 100 Sites Derived from the Nucleotide Sequences and Restriction Enzyme Analysis

Compared Sequences	Sequence Data	Restriction Data
Japanese 1 and 2 ^a .	1.59	2.17 (1)
Japanese 1 and rhesus 1 ^a	3.72	3.18 (1)
Japanese 2 and rhesus 1 ^a	3.48	3.85 (1)
Japanese 1 and Taiwanese ^a	3.49	3.76 (1)
Japanese 2 and Taiwanese ^a	3.01	3.49 (1)
Rhesus 1 and Taiwanese ^a	3.24	3.65 (1)
Japanese 1 and crab-eating 1 ^a	9.26	5.81 (1)
Japanese 2 and crab-eating 1 ^a	9.51	6.53 (1)
Rhesus 1 and crab-eating 1 ^a	10.37	5.77 (1)
Taiwanese and crab-eating 1 ^a	8.29	5.81 (1)
Bonnett and Assamese	8.72	7.8-8.1 (2)
Bonnet and toque	14.28	6.8-7.0 (2)
Bonnet and stump-tailed.	12.60	7.0 (2)
Bonnet and Tibetan.	11.46	6.8 (2)
Bonnet and pig-tailed	14.82	7.9 (2)
Assamese and toque.	9.83	4.7-5.0 (2)
Assamese and stump-tailed	9.93	6.8-7.2 (2)
Assamese and Tibetan	6.05	7.1-8.0 (3)
Assamese and pig-tailed.	11.26	1.3-1.5 (2)
Toque and stump-tailed	13.92	2.4-3.7 (3)
Toque and Tibetan	7.95	8.7-9.7 (2)
Toque and pig-tailed.	8.75	6.2-7.1 (3)
Stump-tailed and Tibetan.	11.22	5.3-5.9 (2)
Stump-tailed and pig-tailed	11.37	5.1-5.8 (2)
Tibetan and pig-tailed.	8.51	7.7-7.9 (2)
Assamese and crab-eating	9.50-1 1.33	10.2 (2)
Assamese and rhesus	10.33-12.25	6.0-7.0 (3)
Tibetan and crab-eating	11.57-13.16	5.3 (2)
Tibetan and rhesus	11.88-12.91	6.6-6.8 (3)
Pig-tailed and crab-eating.	11.83-12.82	8.0 (2)
Pig-tailed and rhesus	12.29-13.32	6.8-7.5 (3)
Crab-eating and rhesus.	9.79-11.16	7.6-8.0 (3)
Crab-eating and stump-tailed.	10.19-11.12	5.9 (3)
Rhesus and stump-tailed.	11.15-12.19	6.8-7.5 (3)
Humans	0.30 (4)	7.2-7.7 (3)
Common and pygmy chimpanzees	4.71 (4)	0.32-0.36 (5.6)
Gorillas	0.11 (4,8)	2.9-4.3 (7)
Bornean and Sumatran orangutans	4.58 (4,8)	0.55 (7)

^a Identical samples were compared in these comparisons. Sources of the values are (1)Hayasaka et al. (1988), (2) Hoelzer, Hoelzer, and Melnick (1992), (3) Zhang and Shi (1993). (4) Horai et al. (1995), (5) Cann, Stoneking, and Wilson (1987), (6) Brown (1980), (7) Ferris et al. (1981), (8) Brown et al. (1982).

sequences. Thus, the lack of the DHU-arm of the tRNA^{Ser(AGY)} has been conserved among them. At the 3' end of the tRNA^{Leu(CUN)} gene, a stretch of 24 nucleotides has been completely conserved. In fact, this stretch of sequence has been completely conserved among 50 mammalian sequences, with an exception of a base substitution in the rat sequence (data not shown). This conservation implies the functional importance of this stretch of sequence. However, its function has yet to be identified.

As reported by Brown et al. (1982) for the orangutan sequence and by Hayashi et al. (1995) for the gibbon sequences, we observed heterogeneity in the initiation codon and/or initiation site of the ND5 gene in the macaque and patas sequences. The GTA codon was ob-

served at the putative initiation site in the sequences of pig-tailed and lion-tailed macaques and the ACA codon in Celebes macaque and patas. In addition, the ATG codon has been conserved among the macaques, baboon, and patas at an alternative initiation site (***) in fig. 1) suggested by Brown et al. (1982), which is located two codons downstream of the putative initiation site.

Nucleotide Diversity

The number of pairwise nucleotide differences is shown in table 1. The nucleotide sequences of rhesus 2 and 3 differ from each other at only two sites, whereas they differ from rhesus 1 at more than 6% of the sites. The sequence of rhesus 1 is much more similar to those

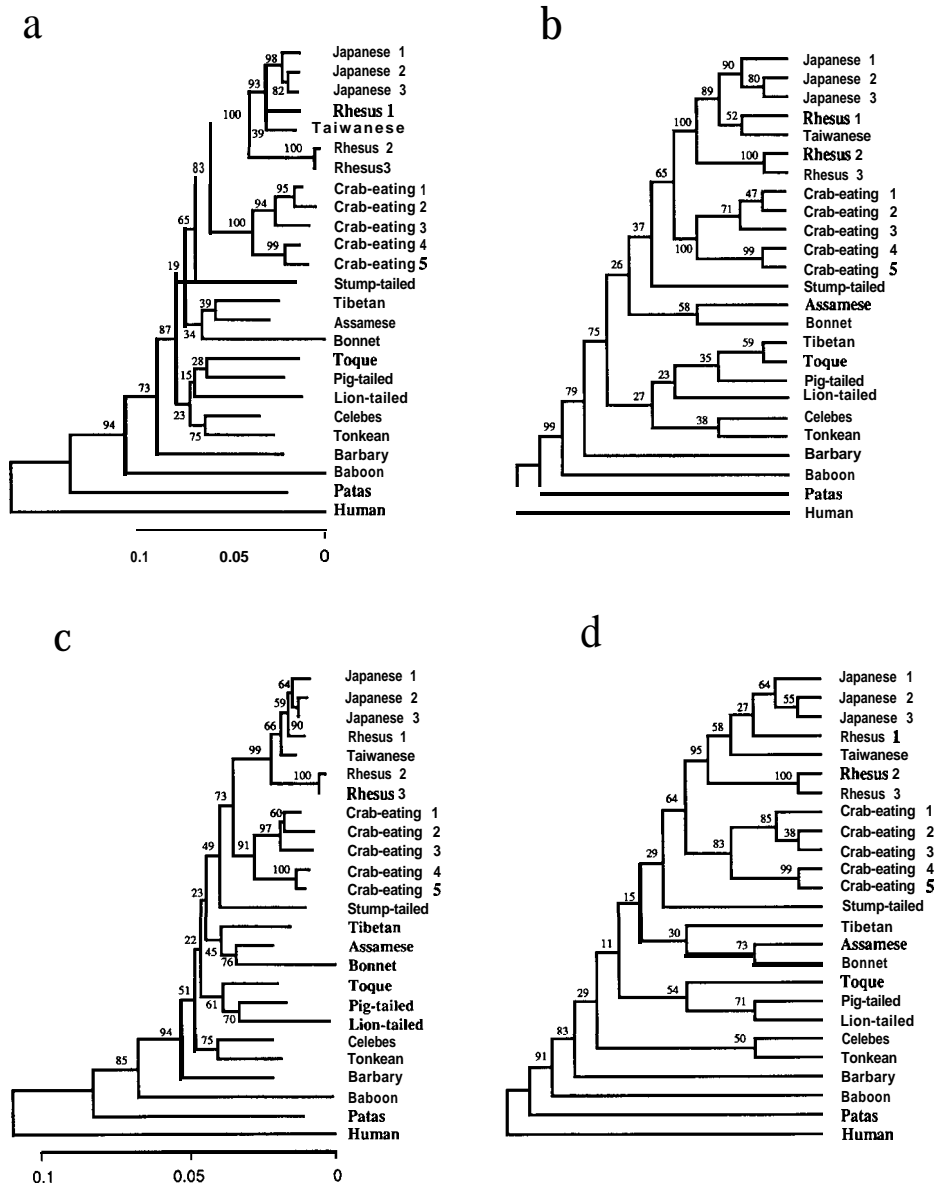


FIG. 2.—Phylogenetic trees were constructed by neighbor-joining (treenj) (a and c), and maximum parsimony (DNAPARS) (b and d). The trees in a and b were constructed from the data of all the 896 sites, whereas the others were from the data of 664 sites (see text). The trees shown in b and d represent the consensus parsimony tree from the bootstrap test of 1,000 repetitions. Figures on the branches in each tree represent percent bootstrap probabilities.

of Japanese and Taiwanese macaques than to the other two rhesus sequences (table 1). The sequences of crab-eating macaques 4 and 5 from Thailand are more similar to each other (1.90%) than either of them is to the sequences of the other three crab-eating macaques (4.02%–5.47%). The sequences of crab-eating macaques 1, 2, and 3 differ from each other at 2.01%–3.68% of the sites. Thus, in terms of mtDNA sequence, rhesus and crab-eating macaques each consisted of two groups. For the crab-eating macaques, the groups seem to reflect their geographic origins, Philippines (#1) and Thailand (#4 and #5), though the origins of two (#2 and #3) are unknown. On the other hand, among hominoids nucleotide differences of 4.35% and 4.46% were observed between two subspecies of orangutans and between two

species of chimpanzees, respectively, in this 896-bp region (Brown et al. 1982; Horai et al. 1995; see table 2). The two groups of rhesus macaques are slightly more differentiated than the two groups of crab-eating macaques, which are as differentiated as the two subspecies of orangutans or the two species of chimpanzees.

Compared to rhesus and crab-eating macaques, the nucleotide differences among the three Japanese macaques (1.00%–1.90%) are small. This may be a result of the small sample sizes. However, analyses of blood protein polymorphisms revealed that genetic heterozygosity within Japanese macaques was smaller than corresponding values within rhesus and crab-eating macaques (Nozawa et al. 1977, 1982; Kawamoto, Ischak, and Supriatna 1984). Japanese macaques are distributed

Table 3
Systematics of Macaques Proposed by Different Authors

	AUTHOR(S) AND METHOD					
	Fooden (1980)	Delson (1980)	Melnick & Kidd (1985)	Fooden & Lanyon (1989)	Zhang & Shi (1993)	This Study
	Morphology	Morphology	Blood Protein Polymorphism	Blood Protein Polymorphism	Restriction Analysis of mtDNA	Nucleotide Sequences of mtDNA
<i>fascicularis</i> group.	Japanese Rhesus Taiwanese Crab-eating	Japanese Rhesus Taiwanese Crab-eating	Japanese Rhesus Taiwanese Crab-eating	Japanese Rhesus Taiwanese Crab-eating	Japanese Rhesus Taiwanese Crab-eating	Japanese Rhesus Taiwanese Crab-eating
<i>sinica</i> group.	Toque Assamese Tibetan Bonnet	Toque Assamese Tibetan Bonnet Stump-tailed	Toque Bonnet	Toque Assamese Tibetan Bonnet Stump-tailed	Assamese Tibetan	Assamese (Tibetan) ^a Bonnet
<i>arctoides</i> group.	Stump-tailed		Stump-tailed		Stump-tailed	Stump-tailed
<i>silenus</i> group.	Pig-tailed Lion-tailed Sulawesi Barbary	Pig-tailed Lion-tailed Sulawesi	Pig-tailed Sulawesi	Pig-tailed	Pig-tailed	Pig-tailed Lion-tailed Toque (Tibetan)
<i>sylvanus</i> group		Barbary		Sulawesi ^b		Sulawesi ^b Barbary

^a Ambiguity remains for the phylogenetic position of Tibetan macaque.

^b Sulawesi macaques from their own group separate from the other species of *silenus* group.

in a limited range of the Japanese archipelago, whereas rhesus and crab-eating macaques have a wide distribution range that covers the Asian continent and surrounding islands. Thus the area of distribution and genetic diversity seem to correlate in these macaques.

The nucleotide diversities estimated from comparisons among the 896-bp sequences and a restriction enzyme analysis of whole mtDNA are compared (table 2). In general, the values estimated from the sequences are larger than those estimated from the restriction data. This difference could imply that the 896-bp region is more variable than other parts of mtDNA. However, when identical samples are compared between the two methods (^a in table 2), the two estimates correspond quite well for diversity values less than 5%, whereas they differ considerably for values greater than 5%. For the comparisons of the hominoids, where the diversity values are less than 5%, the two values also correspond well with each other. These observations suggest that restriction analysis is likely to underestimate the nucleotide diversity when it exceeds 5%.

Phylogenetic Relationships

Phylogenetic trees constructed by the two methods are shown in figure 2. The NJ tree (a) and MP consensus tree (b) share the same topology except for the position of Tibetan macaque. In order to avoid possible multiple substitutions at the third codon positions in the protein genes, we also constructed the trees using the sequence data from tRNA genes and the first and second positions of the protein genes (664 bp). The topologies of the NJ and MP consensus trees deduced from the 664-bp data set differ slightly from that of the NJ tree from the

896-bp data set. One significant difference concerns the position of the Sulawesi macaques (Celebes and tonkean macaques). They are a sister group of a cluster of toque, lion-tailed, and pig-tailed macaques in the 896-bp data set, whereas they are a sister group of all the other Asian macaques in the 664-bp data set.

These observations suggest the existence of six groups among the 13 species of macaques. Group 1 consists of Barbary macaque, Group 2 the two Sulawesi macaques (Celebes and tonkean macaques), Group 3 toque, lion-tailed, and pig-tailed macaques, Group 4 Assamese and bonnet macaques, Group 5 stump-tailed macaque, and Group 6 Japanese, rhesus, Taiwanese, and crab-eating macaques. Although Tibetan macaque often formed a cluster with Group 4 (see figs. 2a, 2c, and 2d), there remains the possibility that it belongs to Group 3 (see fig. 2b); thus, its phylogenetic position among the macaques remains ambiguous.

All the trees in figure 2 suggest that Barbary macaque diverged first from the other Asian macaques, and support for this phylogenetic position of Barbary macaque is relatively strong in the consensus parsimony trees (figs. 2b and 2d). In all the trees, Groups 5 and 6 form a cluster, which then forms a cluster with Group 4, although support for this clustering is not strong in the consensus parsimony trees (figs. 2b and 2d). Because the bootstrap values for internodal branches leading to some groups are not high, information from longer mtDNA sequences as well as from nuclear DNA will be necessary to infer the exact phylogeny of macaques.

Based on morphological characteristics, Fooden (1980) classified the macaques into four species groups:

Table 4
Divergence Time (in Million Years Ago [MYA])
Estimated from the NJ Tree for the 896-bp Data Set with
a Reference Divergence Time of Three MYA Between the
Barbary and Asian Macaques

Branching Point	Divergence Time (MYA)
(Group 2 + Group 3)–(Group 4 + Group 5 + Group 6).....	2.42-2.48
Group 2–Group 3	2.10-2.36
Group 4–(Group 5 + Group 6).....	2.23-2.48
Group 5–Group 6 (stump-tailed)	2.15-2.28
Group 2	
Celebes-Tonkean	1.56–1.73
Group 3	
Toque-pig-tailed.....	1.80-1.94
(Toque + pig-tailed)–lion-tailed.....	2.1 1-2.23
Group 4	
Tibetan-Assamese.....	1.43-1.55
(Tibetan + Assamese)–bonnet.....	1.79-2.19
Group 5	
Japanese 2–Japanese 3.....	0.19-0.22
(Japanese 2 + Japanese3)–Japanese 1	0.33–0.37
Rhesus 1–Taiwanese	0.63–0.70
Japanese–(rhesus 1 + Taiwanese)	0.65–0.73
Rhesus 2–rhesus 3	0-0.08
(Japanese + rhesus 1 + Taiwanese)–(rhesus 2 + rhesus 3).....	1.00-1.29
Crab-eating 1–crab-eating 2	0.27-0.5 1
(Crab-eating 1 + carb-eating 2)–crab-eating 3 ...	0.55-0.76
Crab-eating 4–crab-eating 5	0.30-0.46
(Crab-eating 1 + crab-eating 2 + crab-eating 3)–(crab-eating 4 + crab-eating 5).....	0.91-1.13
(Japanese + rhesuses + Taiwanese)–crab-eatings	1.83-2.01

silenus-sylvanus, *sinica*, *arctoides*, and *fascicularis*. Delson (1980) proposed a slightly different classification, which gives *M. sylvanus* its own group and includes *M. arctoides* in the *sinica* group. Groupings of macaques as deduced in this study and in previous studies are summarized in table 3. All of the studies support monophyly of the *fascicularis* group. A close relationship between Assamese and bonnet macaques was also observed in all studies in which the two species were analyzed. Our present study differs from the others in the following aspects. Four other studies placed toque macaque in the *sinica* group. However, all of our phylogenetic trees clearly placed toque macaque in the *silenus* group, in which this macaque formed a monophyletic cluster together with both pig-tailed and lion-tailed macaques.

Despite high intraspecific nucleotide diversity, the five crab-eating macaques form a monophyletic cluster, and this cluster is strongly supported by the bootstrap analysis in our phylogenetic trees. On the other hand, the three rhesus macaques do not form a monophyletic cluster. Instead, rhesus 1 forms a cluster with Japanese and Taiwanese macaques, and this cluster becomes a sister group to a cluster of rhesuses 2 and 3. This clustering pattern is strongly supported by the bootstrap test, and the phylogenetic tree that forces a monophyletic

clustering of rhesus macaques requires nine more substitutions (1,082 vs. 1,073) and is significantly worse (Felsenstein 1993) than the most parsimonious tree. These results imply that either polymorphisms of mtDNA sequences that existed before the divergence of these three species have been retained in rhesus macaques or introgression has occurred between the three species. High nucleotide diversity within the rhesus and Assamese macaques also has been noted from restriction enzyme analyses of mtDNA (Hoelzer, Hoelzer, and Melnick 1992; Melnick et al. 1993; Zhang and Shi 1993).

Divergence Time

Divergence times were estimated from the NJ tree (fig. 2a). We chose the reference divergence time of three million years ago (MYA) between Asian and Barbary macaques based on the observation that no fossil macaques older than 3 MYA have been discovered in Asia (Delson 1980). This divergence time was also supported by the number of synonymous substitutions observed in the present data, given an evolutionary rate for synonymous sites in primate mtDNA. When we estimated pairwise synonymous substitutions (K_s) between Barbary macaque and all other Asian macaques, we obtained the average value 0.265 ± 0.034 . Applying the synonymous substitution rate of $3.89 \times 10^{-8}/\text{site}/\text{year}$ (Horai et al. 1995), we obtained a divergence time between the Barbary and Asian macaques of 3.4 ± 0.4 MYA, which is nearly the same as from fossil evidence. Divergence time of each branching point in the tree was estimated as a proportion of the three million years indicated by the total branch length connecting the reference divergence point to each species descended from the node. Therefore, more than one estimate can be obtained for each branching point.

The divergence times thus obtained are listed in table 4. The values in the table suggest that the divergence of the groups of Asian macaques took place between 2.1 and 2.5 MYA. This divergence was followed by divergence among the species within the groups, which took place in a time period estimated between 1.4 and 2.2 MYA. The initial divergence within the cluster of Japanese, rhesus, and Taiwanese macaques and the final divergence among the three species were estimated at around 1.0 and 0.7 MYA, respectively. Thus, most of the species of Asian macaques emerged in a relatively short period of time between 1.4 and 2.5 MYA. Similar divergence times were estimated from the NJ tree based on the 664-bp data set (data not shown).

Around 2 MYA, fossil records indicate that macaques became abundant in numbers and species in Asia (Delson 1980). This paleontological evidence coincides with the date of speciation obtained from our analysis.

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