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Species identification based on the point mutations of histo-blood group ABO genes by PCR-RFLP and direct sequencing

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Abstract Using the PCR-restriction fragment length polymorphism (RFLP) and direct sequencing methods, 181 bp DNA fragments at the ABO blood group locus of human and eight different primates were examined. Through PCR amplification and digestion of the product with Hha-1 restriction enzyme, each of the amplified fragments of human, chimpanzee and green monkey was cut into two fragments of 147 and 34 bp, and the corresponding fragment of the other primates was digested into three fragments of 115, 34 and 32 bp. The 181 bp fragments of chimpanzee and green monkey were digested with Mva-1 into three fragments of 82, 58 and 41 bp, and 69, 58 and 54 bp, while the fragments of human and the other primates were separated into two fragments of 123 and 58 bp. Thus, using Hha-1 and Mva-1, human PCR-amplified product of a part of the ABO gene could be discriminated from those of the other primates examined. In addition, by direct sequencing of the 181 bp DNA fragment, two Mva-1 recognition sites and one Hha-1 recognition site were found in the fragments of chimpanzee and green monkey, one Mva-1 site and one Hha-1 site were detected in humans, and one Mva-1 site and two Hha-1 sites in the other primates. These results corresponded well with the data of PCR-RFLP. This method has a good potential for species identification at the DNA level.

Key words Species identification · Primates · ABO blood group · PCR-RFLP · Direct sequencing

Introduction

For forensic species identification, blood components such as serum albumin, immunoglobulin, hemoglobin, M and N substances, and phosphoglucose isomerase have been used (Robinson and Osterhoff 1983; Oshima and Hara 1984; Pex and Wolfe 1985; Takayasu et al. 1988; Yamamoto et al. 1989). With the advance of DNA analysis, gene-detection procedures using DNA fingerprints or PCR have also been available for species identification (Tajima et al. 1989; Blackett and Keim 1992; Guglich et al. 1993, 1994; Soteriou et al. 1995). In the ABO blood group system, A, B and H antigens were found on the red blood cells of pongidae, old world monkeys and new world monkeys (Landsteiner and Miller 1925a, b; Candela 1940). The red blood cells of chimpanzee for example showed human-like A phenotype and those of macaque family were B phenotype and thus there was no ABO phenotypes found exclusively in humans.

Recently, DNA sequences of human ABO glycosyltransferases (including $\alpha 1 \rightarrow 3$N-acetylgalactosaminyltransferase (A transferase) and $\alpha 1 \rightarrow 3$ galactosyltransferase (B transferase)) were determined (Yamamoto et al. 1990) and used for ABO genotyping and paternity testing (Hashimoto and Nakanishi 1993; Fukumori et al. 1995; Nishimukai et al. 1996). Phylogeny based on the ABO glycosyltransferase sequences in several animals has also been presented (Kominato et al. 1992), although species identification from the view point of the ABO glycosyltransferase sequence was not yet successful.

In the present study, we have developed a novel method to discriminate humans, chimpanzees and green monkeys from other primates, and to distinguish humans from chimpanzees and green monkeys, with PCR-restriction fragment length polymorphism (RFLP) based on the point mutations of histo-blood group ABO genes. Furthermore, we have directly sequenced the essential 181 bp fragment at ABO gene locus of several species of primates, and the sequencing data were compared with that of humans.

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Table 1 Human and primates examined

Species	Number	Species	Number
Human			
AA	1	OO	2
AO	3	BO	2
BB	1	AB	1
Pongidae			
Chimpanzee	20*	White-hand gibbon	2
Old world monkey			
Hamadryas baboon	2	Japanese monkey	2
Crab-eating monkey	2	Green monkey	3
Rhesus monkey	2		
New world monkey			
Tufted capuchin monkey	2		

* including 6 individuals (2: parents, 4: their children) with blood relationship

Materials and methods

Specimens

Human DNA specimens were extracted from 10 individuals (8 males, 2 females) with the phenol-chloroform method (Table 1). The primate DNA specimens (8 different species; total 35 individuals) were obtained from the Primate Research Institute of Kyoto University, Inuyama, Aichi, Japan. The species and the number of individuals examined are also listed in Table 1, and the primates examined had no blood relationship except 6 chimpanzees; 2 parents and their 4 children.

PCR-RFLP

PCR was carried out in 50 µl volumes containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton X-100, 100 µM dNTP, 2.5 units of Taq DNA polymerase (Takara Biotechnology, Ohtsu, Japan), 1.0 µM of each primer and 250 ng of template DNA. After preheating at 95° C for 5 min, 30 PCR cycles were carried out as follows; denaturation at 96° C for 24 s, annealing at 62° C for 30 s, and extension at 72° C for 1.5 min. The final cycle ended with an additional extension of 5 min at 72° C. The two oligonucleotides 3f and 3r were used as the primer pair for specific amplification of the 181 bp DNA fragment which corresponds to nucleotides 562–742 on the A transferase cDNA. The nucleotide sequences of each primer (Sasaki et al. 1994) are as follows;

3f: 5′-CGCATGGAGATGATCAGTGACTTC-3′

3r: 5′-GCTCGTAGGTGAAGGCCTCCC-3′

In order to determine the minimum amount of template DNA required for this method, PCR amplification was performed with each of the following amounts of human DAN to the PCR solution: 0.01, 0.1, 1.0, 10, 100 and 250 ng.

For the amplification products 10 µl was digested with 5 units of the restriction enzymes Mva-1 and Hha-1 (Takara Biotechnology, Ohtsu, Japan) at 37° C for 2 h. The digested products were then analyzed by electrophoresis in 12% polyacrylamide gels in TBE buffer (50 mM Tris-HCl, 50 mM boric acid, 1 mM EDTA, pH 8.0) at 300 V for 1 h. After electrophoresis, DNA bands were visualized by silver staining (Goldman and Merril 1982).

Direct DNA sequencing

The amplified products were purified in 6 M urea-polyacrylamide gels (12%) and dialysis membranes. After purification, the second PCR was performed for 25 cycles under the following conditions; denaturation at 96° C for 30 s, annealing at 50° C for 15 s, and extension at 60° C for 4 min. The PCR solution contained 9.5 µl of the premix solution from the Dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Branchburg, USA), 500 ng of the purified DNA fragment, and 3.2 pmol of only one primer (3f). The volume of the reaction mixture was adjusted to 20 µl with distilled water. Sequencing analysis was performed using the 373A DNA sequencing system (Applied Biosystems, Foster, USA).

Results

PCR-RFLP

Before digestion with restriction enzyme

With the primers 3f and 3r, each 181 bp DNA fragment was specifically amplified for the DNA specimens of human and the primates tested. The amount of the template DNA in the range of 0.01–250 ng was sufficient to amplify the 181 bp fragment.

Digestion with Hha-1

Digestion with Hha-1 produced 147 and 34 bp fragments in the amplified 181 bp DNA fragments of human, chimpanzee and green monkey. The 181 bp fragments of the other primates except for chimpanzee and green monkey were digested into three bands of 115, 34, and 32 bp fragments (Fig. 1). Thus, human, chimpanzee and green monkey showed a different band pattern from the other primates after Hha-1 digestion.

Digestion with Mva-1

Digestion with Mva-1 produced both 123 and 58 bp DNA fragments in all amplified human DNA and the primates DNA except for chimpanzee and green monkey (Fig. 2). However, three DNA fragments of 82, 58 and 41 bp were found in the digested products of the 20 chimpanzees examined, and there were no individual differences. The digested products from three green monkeys with Mva-1 gave three bands of 69, 58 and 54 bp. Thus, chimpanzee and green monkey showed a different band pattern from human and the other primates after Mva-1 digestion of the amplified products.

DNA sequencing

In every sequence of the 181 bp DNA fragment from humans and all the primates examined, there were commonly found one Mva-1 site at the positions 57–61 and one Hha-1 site at the positions 33–36 (Fig. 3).

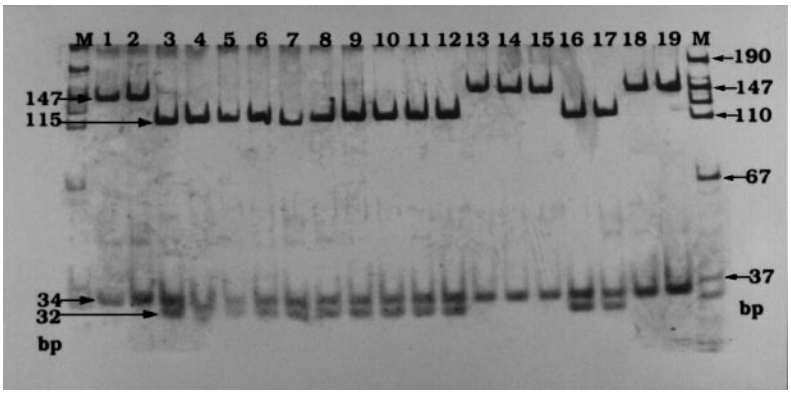


Fig. 1 Electrophoretic patterns of Hha-1 digested human and primate DNA after PCR-amplification with the human specific primers 3f and 3r. Human, chimpanzee and green monkey showed different patterns from those of the other primates. Lanes 1 and 2, chimpanzee; Lanes 3 and 4, gibbon; Lanes 5 and 6, baboon; Lanes 7 and 8, Japanese monkey; Lanes 9 and 10, crab-eating monkey; Lanes 11 and 12, rhesus monkey; Lanes 13–15, green monkey; Lanes 16 and 17, tufted capuchin monkey; Lanes 18 and 19, human; Lane M, DNA molecular size marker; bp, base pairs

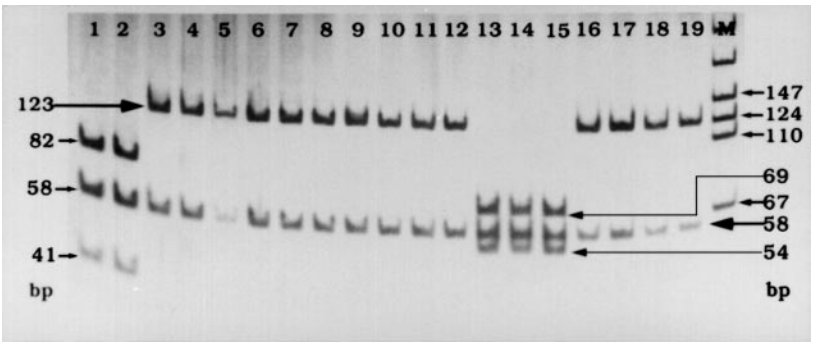
In the sequences of human 181 bp DNA fragment of AA-, OO- and AO-genotypes, position 96 was cytosine (C), and 142 was guanine (G). In the sequence of the BB-genotype, position 96 was thymine (T), and 142 was adenine (A). The sequences of BO- and AB-genotypes showed that position 96 and 142 were "N", meaning no definite reading by the computer used, and their sequencing graphs showed two peaks of C and T, or A and G (Table 2).

Sequencing results of the 181 bp DNA fragment from chimpanzees revealed two point mutations at positions 28 and 141, G→C and C→T, respectively, and the latter mutation generated a new Mva-1 site. In green monkeys, the positions 115, 118, and 143 of the 181 bp DNA fragment showed the point mutations A→G, C→T, and G→C, respectively, and the point mutation at position 115 produced the second Mva-I site. In the sequence of the other primates examined, position 68 was commonly point mutated as T→C and newly formed the second Hha-1 site. Furthermore, a total of 14 point mutations at different sites were found in the primates examined i.e. two point mutations in an individual of pongidae, three in an individual of old world monkey, and seven in an individual of new world monkey. These findings are shown in Table 2.

Fig. 2 Electrophoretic patterns of Mva-1 digested human and primate DNA after PCR-amplification with the human specific primers 3f and 3r. Chimpanzee and green monkey showed different patterns from those of human and the other primates. Lanes 1 and 2, chimpanzee; Lanes 3 and 4, gibbon; Lanes 5 and 6, baboon; Lanes 7 and 8, Japanese monkey; Lanes 9 and 10, crab-eating monkey; Lanes 11 and 12, rhesus monkey; Lanes 13–15, green monkey; Lanes 16 and 17, tufted capuchin monkey; Lanes 18 and 19, human; Lane M, DNA molecular size marker; bp, base pairs

Discussion

The most popular methods used for species identification are immunological methods, protein electrophoresis and isoelectric focusing, although they have also some problems such as the complicated extraction procedures and the difficulty in interpreting protein gel patterns (Soteriou et al. 1995). DNA analysis has been also applied to species identification (Tajima et al. 1989; Naito et al. 1992; Guglich et al. 1993, 1994; Stucki et al. 1993; Oorschot et al. 1994; Soteriou et al. 1995; Sparkes et al. 1996). From the view point of difference in the Alu and Myo loci, human DNA could be discriminated from mouse, rat, dog and deer DNA, but not from Japanese monkey or chimpanzee DNA, since there was hardly any difference in the Alu and Myo repeated sequences between human and Japanese monkey or chimpanzee (Tajima et al. 1990). The electrophoretic band pattern of the PCR-amplified products at rRNA or SON locus allowed human DNA to be discriminated from Japanese monkey, green monkey, dog, cat, pig, mouse, rat, chicken, frog and fish, but could not



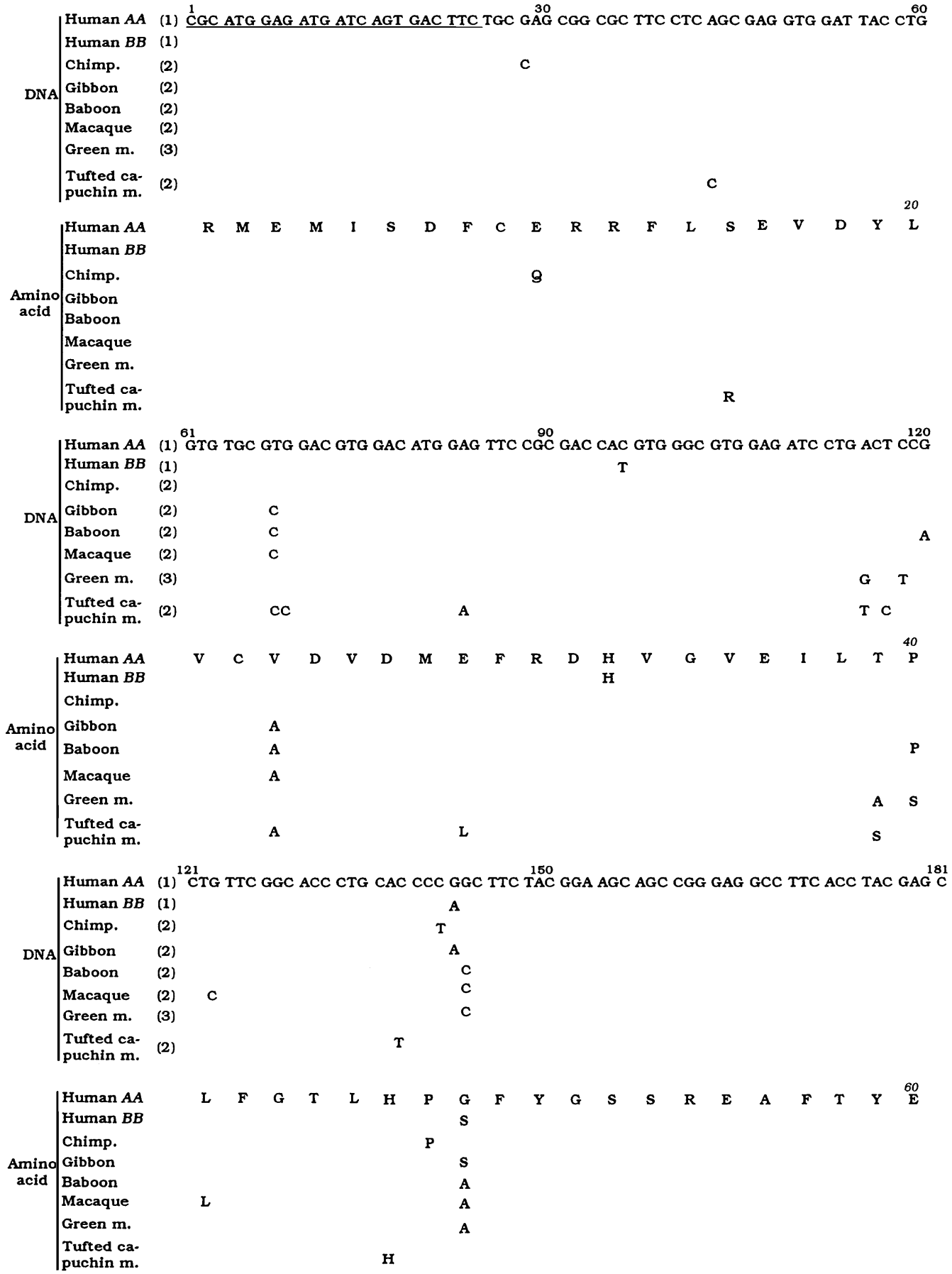

DNA
Amino acid
Human AA
Human BB
Chimp.
Gibbon
Baboon
Macaque
Green m.
Tufted capuchin m.
1 30 60
CGC ATG GAG ATG ATC AGT GAC TTC TGC GAG CGG CGC TTC CTC AGC GAG GTG GAT TAC CTG
20
R M E M I S D F C E R R F L S E V D Y L
61 90 120
GTG TGC GTG GAC GTG GAC ATG GAG TTC CGC GAC CAC GTG GGC GTG GAG ATC CTG ACT CCG
40
V C V D V D M E F R D H V G V E I L T P
121 150 181
CTG TTC GGC ACC CTG CAC CCC GGC TTC TAC GGA AGC AGC CGG GAG GCC TTC ACC TAC GAG C
60
L F G T L H P G F Y G S S R E A F T Y E

Table 2 Point mutations of human (BB) and primates in 181-bp DNA fragment at human ABO locus cDNA (562nd ~ 742nd nucleotide)

Species	No. of nucleotide in cDNA of human AA genotype														
	28	43	68	69	82	96	115	117	118	120	123	138	141	142	143
Human															
AA, AO, OO	G(E)	A(S)	T(V)	G(V)	G(E)	C(H)	A(T)	T(T)	C(P)	G(P)	G(L)	C(H)	C(P)	G(G)	G(G)
Human BB						T(H)								A(S)	
Chimpanzee	C(Q)												T(P)		
Gibbon			C(A)											A(S)	
Baboon			C(A)							A(P)					C(A)
Japanese monkey			C(A)								C(L)				C(A)
Crab-eating monkey			C(A)								C(L)				C(A)
Rhesus monkey			C(A)								C(L)				C(A)
Green monkey							G(A)		T(S)						C(A)
Tufted capuchin monkey		C(R)	C(A)	C(A)	A(L)		T(S)	C(S)				T(H)			

( ): Amino acid deduced from mutated code of the DNA sequence

be discriminated from chimpanzee (Naito et al. 1992; Soteriou et al. 1995).

In the present study, through the digestion of the 181 bp DNA fragment at the ABO blood group locus with Mva-1 and Hha-1, human DNA could be simply, clearly and specifically discriminated from the primates examined, especially from chimpanzee. By the first digestion of PCR-amplified product with Hha-1, DNA specimens of humans, chimpanzees and green monkeys were discriminated from those of the other primates. By the second digestion of the identical PCR-amplified products with Mva-1, human specimens were discriminated from chimpanzees and green monkeys.

Experimentally, even 10 pg DNA from fresh whole blood was sufficient as the template for successful amplification of the expected 181 bp fragment, using 3f+3r primer pair under the same reaction conditions (data not shown). Furthermore, the authors have already applied the 3f+3r primer pair to ABO genotyping of forensic samples, and it became obvious that 50~100 pg of DNA extracted from forensic materials such as old bloodstains (e.g. stains aged 5, 10 and 20 years), formalin-fixed, paraffin embedded human tissues and human mummies (Lin 1995; Lin et al. 1996) could be correctly amplified. This means that this novel PCR-RFLP method can be applied to species identification with forensic materials as well.

Additionally, this method has the advantage that these PCR-amplified products can also be employed in parallel for ABO genotyping by Msp-I digestion (Sasaki et al. 1994; Lin et al. 1996). Thus, species identification and ABO genotyping can be performed on the same PCR amplified products.

In the human 181 bp DNA fragment of the glycosyltransferase gene, the point mutations were found at positions 96 and 142, and corresponded well with a previous study (Yamamoto et al. 1990). In the sequences of the primates examined, two Mva-I recognition sites and one Hha-I site were found in chimpanzees and green monkey, one Mva-I site and one Hha-I site was detected in human, and one Mva-I site and two Hha-I sites in the other primates as well, thus corresponding well with the PCR-RFLP data.

The concept of primate evolution (Socha and Ruffie 1983) has been extensively supported by the analysis of primate mitochondrial DNA (Hayasaka et al. 1988; Hasegawa et al. 1990; Ruvolo et al. 1991). In our study, the number of point mutations was specific for the different genus of primates, as two point mutations were found in the sequences of pongidae, three in the old world monkey, and seven in the new world monkey. Additionally, among three different kinds of macaques, common point mutations were detected as well, thus indicating that the number of point mutations was closely correlated with the phylogeny of primates. This corresponded well with the generally accepted concept of primate evolution.

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◀ **Fig. 3** Direct DNA sequencing results for the 181-bp fragment of human and the primates and their deduced amino acid sequence. Only the differences from human A gene are shown for each sequence. Chimp.: chimpanzee. Macaque: including Japanese monkey, crab-eating monkey and rhesus monkey. m.: monkey. ( ): showing the number of individual examined. Underlined 24 nucleotides (the 1 to the 24) correspond with one of the primers (3f) for direct DNA sequencing

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