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Multiplex amplified product-length polymorphism analysis for rapid detection of human mitochondrial DNA variations

A number of mutations in coding and noncoding regions of mitochondrial DNA (mtDNA) have previously been studied. In the present study, we simultaneously typed six mutation sites in the coding region by use of amplified product-length polymorphism (APLP) analysis. The mtDNA variations of 2471 individuals from 20 populations of Japanese, Korean, Chinese, and German were examined and classified into 18 haplotypes. Two of these haplotypes, B1 (estimated ancestral haplotype) and C1, were distributed among all populations tested. However, the haplotypes A1, A2, B2, B3, and C2 were mostly restricted to the Mongoloid populations, whereas haplotypes B5 and C5 appeared almost exclusively in the German population. Phylogenetic analysis by the neighbor-joining method revealed that the Japanese populations were more closely related to each other than to the other East Asian populations surveyed. The multiplex APLP method is suitable for large-scale screening studies of mtDNA variability because it is both rapid and economical.

Keywords: Mitochondrial DNA / Single nucleotide polymorphisms / Amplified product-length polymorphism / Polymerase chain reaction / Population study EL 4536

1 Introduction

The occurrence of a large number of mitochondrial DNA (mtDNA) polymorphisms likely reflect the high mutation rate of mtDNA, which is thought to be caused mainly by high levels of reactive oxygen species present in the mitochondrion [1]. The mtDNA mutations observed in both noncoding control and coding regions are being widely used to characterize human evolution and for identification [2–4]. For the former purpose, the nucleotide (nt) substitution in the coding region seem to be more suitable for study, because the high mutation rate of mtDNA in the noncoding control region results in complicated substitutions that may make phylogenetic analysis difficult. Several methodological approaches are available for detection of single nucleotide polymorphisms (SNPs), which include direct sequencing, RFLP, SSCP, and the use of allele-specific primers and sequence-specific oligonucleotide probes. The RFLP analysis is one of the most widely used techniques for detecting known point mutations in the coding region of mtDNA [2, 5]. In this method, some ambiguities remain with respect to the actual mutation site. Multiplex solid-phase fluorescent minisequencing [6] and the immobilized sequence-specific oligonucleotide probe-based assay [7] are also applied to the analysis of mtDNA polymorphisms. However, these techniques are expensive and time-consuming. Recently, we developed the simple amplified product-length polymorphism (APLP) method for the typing of SNPs [8, 9]. The principle of this method is based on an attachment of a noncomplementary sequence to the 5'-end of one of two allele-specific primers. The use of such primers permits the amplication of two size-different products distinguishing two alleles.

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Abbreviations: APLP, amplified product-length polymorphism, **9bp**, 9-bp repeats in the noncoding cytochrome oxidase II/tRNA^{Lys} intergenic region; **nt**, nucleotide; **SNP**, single nucleotide polymorphism

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Here, we describe the multiplex APLP technique using such primer sets for rapid detection of several polymorphic sites of mtDNA at the same time. A total of six polymorphic sites of mtDNA were analyzed (Table 1). These sites seemed to be the important mutations in East Asian populations. Five of them are SNPs of the coding regions (nt3010, nt4386, nt5178, nt8794, and nt10398) and the other, a 9-bp repeat variation in the noncoding cytochrome oxidase II/tRNA^{Lys} intergenic region (9bp) [2, 10–12].

Table 1. Detected sites of human mtDNA

Polymorphic sites	Locus	Nucleotide change	Amino acid change
3010	16S rRNA	G → A	–
4386	tRNA^{Gln}	T → C	–
5178	ND2	C → A	Leu → Met
8794	ATP6	C → T	His → Tyr
8272–8289	9bp	9-bp deletion	–
10398	ND3	A → G	Thr → Ala

2 Materials and methods

2.1 Preparation of DNA templates

The 2471 blood samples from healthy individuals were obtained from East Asian populations including 16 Japanese, 2 Korean, and 1 Chinese (Fig. 1), along with a German population (Munich) which was previously analyzed for alcohol dehydrogenase 2 and aldehyde dehydrogenase 2 [9]. Genomic DNA was prepared from each blood sample according to standard protocols.

2.2 Amplification of mtDNA

The oligonucleotide primers for the APLP analysis [8] were designed from the nucleotide sequence of mtDNA [13] and used at concentrations shown in Table 2. PCR amplifications were carried out in a total volume of 20 μL containing 2–20 ng genomic DNA, 1 X PCR buffer, 120 μM of each dNTP, optimal concentrations of each primer (shown in Table 2), and 0.5 units of HotStar *Taq* polymerase (Qiagen, Hilden, Germany) in a DNA Thermal Cycler 9700 (Applied Biosystems, Foster City, CA, USA). After an initial 15 min denaturation at 95°C, 33 amplification cycles were carried out, each consisting of 10 s at 94°C, 10 s at 52°C, and 5 s at 72°C, followed by a final extension step of 1 min at 72°C. When any of six loci failed to amplify, the locus was judged by PCR with the corresponding primer set.

2.3 Separation conditions

A 3-μL aliquot of the PCR product was separated by electrophoresis in a 6.5 cm native polyacrylamide gel (10%T, 5%C) containing 375 mM Tris-HCl buffer (pH 8.9)

Table 2. Oligonucleotide primers used for PCR

Site	Primer	Sequence (5'–3')	Amount (pmol/20 μL)	Product size (genotype)
3010	3010G	aTTGGATCAGGACtTCCCG	1.6	75 bp (G type)
	3010A	gctacaTGGATCAGGACAaCCCA	2.0	79 bp (A type)
	3010R	GATCACGTAGGACTTTAATCG	2.0	
4386	4386C	aTCTCCGTGCCACCTAC	1.2	85 bp (C type)
	4386T	agttgTTCTCCGTGCCAgCTAT	1.2	90 bp (T type)
	4386R	attgCAACATTTTCGGGGTATG	1.2	
5178	5178C	gTCGCACCTGAAgCAAGC	1.7	96 bp (C type)
	5178A	tgatcaaCGCACCTGAAACAAGA	1.7	101 bp (A type)
	5178R	attGCAAAAAGCaGGTTAGCG	1.7	
8794	8794C	aCCTCGGACTtCTGCCTC	2.5	107 bp (C type)
	8794T	attggaCCTCGGACTCtTGCCTT	2.5	112 bp (T type)
	8794R	aCAGCGAAAGCCTATAATCAC	2.5	
9bp	9BPF	acaGGAGCAtACCACAGTTTC	2.0	122 bp (1; deletion)
	9BPR	CTAAGTTAGCTTTACAGTGGG	2.0	131 bp (2; normal)
				140 bp (3; insertion)
10398	10398G	CTACAAgAAGGATTAGACTGRG	5.9	145 bp (G type)
	10398A	atcactCTACAAAgAGGATTAGACTGAA	3.9	151 bp (A type)
	10398R	ctAGAAGTGAGATGGTAAATGC	3.9	

The noncomplementary nucleotides are written in small letters.

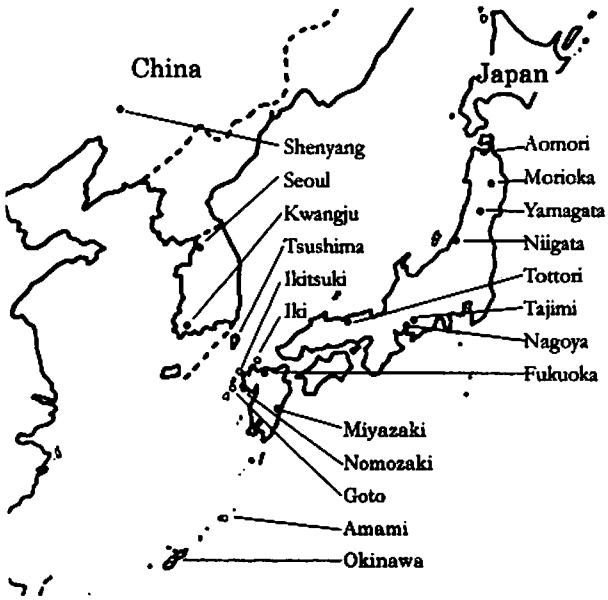

Figure 1. Geographic distribution of East Asian populations analyzed for mtDNA variation. Open circles indicate the small island.

with running buffer (25 mM Tris, 192 mM glycine; pH 8.3). DNA bands were detected fluorographically after staining with SYBR Gold (Molecular Probes, Rockland, ME, USA).

2.4 Direct sequencing of PCR products

PCR products from the 9-bp region that differed in migration were subjected to direct DNA sequencing by use of the Abi Prism 310 sequencer and BigDye Terminator Cycle Sequencing FS Kit (Applied Biosystems).

2.5 Phylogenetic analysis

Cavalli-Sforza's chord distance was calculated by using the haplotype frequencies of each population [14]. Distance matrices were clustered by the neighbor-joining procedure [15]. These analyses were performed with the computer program PHYLIP 3.5c (Felsenstein, J., University of Washington, WA, USA) and TreeView [16].

3 Results and discussion

To develop a convenient method for the determination of haplotypes of a large number of DNA samples, we prepared six primer sets for the amplification of products ranging from 75 to 151 bp in size and mixed them in a single tube. The APLP assay was validated by testing 50 human mtDNA samples that had been previously

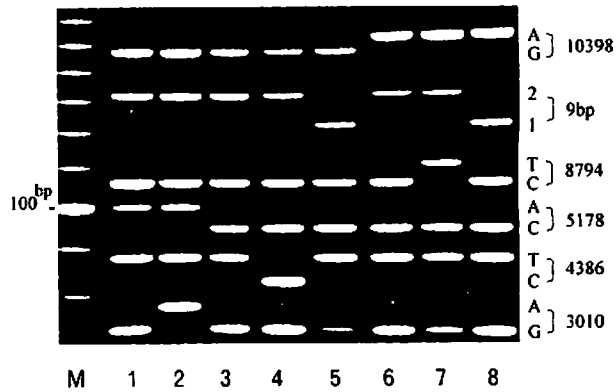

Figure 2. APLP band patterns of mtDNA haplotypes. Lane 1, A1; 2, A2; 3, B1; 4, B2; 5, B3; 6, C1; 7, C2; 8, C3; M, 10-bp DNA ladder marker (Life Technologies).

characterized by sequencing. The results of the APLP and sequencing analysis coincided. This method is very simple, because it only requires PCR amplification in a single tube and PAGE using a nondenaturing gel in combination with SYBR Gold staining. The multiplex APLP band patterns of human mtDNA haplotypes after separation on a polyacrylamide gel are shown in Fig. 2. Each allele was correctly typed by comparing the PCR products with the known point mutation.

mtDNA polymorphisms are now widely used as a maternally inherited marker to elucidate human evolution and migrations. By RFLP analysis, mtDNA was previously classified into three types (A–C) by two mutation sites of nts 5178 and 10398, and the frequency of the haplogroup A was significantly higher in centenarians than in younger Japanese individuals [10, 11]. In the present study, these three haplogroups (A, B, and C) were further classified into 18 haplotypes (A1-3, B1-8, and C1-7) based on four additional mutation sites (Table 3). The frequencies of these haplotypes in Asian and German populations are shown in Table 4. Over 99% of the mtDNA were composed of 10 mtDNA haplotypes (A1, A2, B1, B2, B3, B5, C1, C2, C3, and C5). A phylogenetic network for the evolutionary relationship of mtDNA haplotypes is shown in Fig. 3. Haplotypes B1 (estimated ancestral haplotype) and C1 were distributed among all populations. However, haplotypes A1, A2, B2, B3, and C2 are mostly restricted to Mongoloid populations, and B5 and C5 appeared almost exclusively in the German population. Haplogroup A was present in all Mongoloid populations, but not in the German one. Haplotypes A1 and A2 were widely distributed in Asian groups. In the Japanese populations, the A2 frequency ranged from 26 to 44%, while the Chinese had the lowest frequency (17%). Conversely, Chinese possessed the highest A1 frequency (12%). Though haplotype B2 was observed in all Japanese populations at

Table 3. Haplotype-specific polymorphic sites in mtDNAs

Haplogroup and haplotype		Polymorphic site					
		3010	4386	5178	8794	9 bp[a)]	10398
A:	A1	G	T	A	C	2	G
	A2	A	T	A	C	2	G
	A3	A	T	A	C	1	G
B:	B1	G	T	C	C	2	G
	B2	G	C	C	C	2	G
	B3	G	T	C	C	1	G
	B4	A	C	C	C	2	G
	B5	A	T	C	C	2	G
	B6	A	T	C	C	3	G
	B7	G	T	C	C	2a	G
	B8	G	T	C	C	2b	G
C:	C1	G	T	C	C	2	A
	C2	G	T	C	T	2	A
	C3	G	T	C	C	1	A
	C4	G	C	C	C	2	A
	C5	A	T	C	C	2	A
	C6	G	T	C	T	3	A
	C7	G	T	C	C	3	A

a) Designations in this column indicate the following: 1,deletion of the 9-bp repeat; 2, two copies of the 9-bp repeat; 3, three copies of 9-bp repeat; 2a, 4-bp insertion (heteroplasmic); 2b, 3-bp deletion (heteroplasmic).

the high frequency, especially in Okinawa (21.7%), its frequency was low in the two combined Korean populations (3.1%) and zero in the Chinese and German ones. We surmised that B2 existed first in South Japan in prehistorical times, increased in frequency by genetic drift, and subsequently diffused to North Japan and Korea.

The frequency of the 9-bp deletion (CCCCCTCTA) in Japanese populations varied from 19% in Tajimi to 7% in Tsushima. The values are nearly equal to those of previous studies [17, 18]. The 9-bp deletion is characteristic of individuals who belong to haplotypes B3 and C3. Moreover, five individuals with the irregular band were clarified by sequencing. Three of them had a triplication of the 9-bp segment. In addition, an autogenous formation of heteroduplexes was observed in the other two samples; one was a heteroplasmic approx. 4-bp insertion (CCCCCCCCCCCTACCCCCTCTA) and the other, a heteroplasmic approx. 3-bp deletion (CCCCCCCCCCCT CTA). Similar changes have been reported previously [19–21]. We confirmed that the mutation giving length variation in the 9-bp repeat sequence in the COII-tRNA^{Lys} intergenic region has occurred several times.

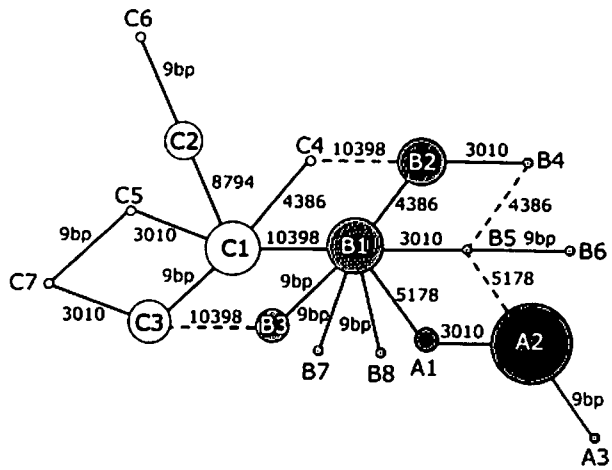

Figure 3. Phylogenetic network of mtDNA haplotypes. The most likely root is haplotype B1, whereas the Cambridge reference sequence [13] corresponds to haplotype C1. Circle areas are roughly proportional to haplotype frequencies of Japanese. Interrupted lines show less likely relationships.

A phylogenetic tree was constructed by use of Cavalli-Sforza's chord distance and the neighbor-joining method, as shown in Fig. 4. This tree reveals that the Japanese populations have stronger genetic affinity with each other than with the other East Asian populations surveyed. The clustering in the unrooted network was observed in the order of German, North Chinese, Korean, and Japanese. The relationship between German and Okinawa was the remotest. Very interestingly, the phylogenetic tree obtained from the present simple experiment considerably resembled the trees from enormous data obtained with conventional genetic markers [22] and the noncoding region of mtDNA [18]. Furthermore, we are planning to use the APLP method for the analysis of SNPs on X and Y chromosome, because it has turned out to be very powerful for mtDNA analysis.

4 Concluding remarks

Prior to the use of APLP methods, it is necessary to determine the specificity and optimal concentrations of oligonucleotide primers and annealing temperature for PCR. Once these conditions have been optimized, reproducible results are obtained. PCR-APLP is a fast, easy, and sensitive method to screen known point mutation sites of mtDNA. Moreover, it only requires PCR and native gel electrophoresis, and dispenses with expensive fluorescence- or biotin-labeled primers, restriction endonucleases, and expensive equipment. Thus, PCR-APLP is very effective for the analysis of variations in mtDNA.

Table 4. Distribution of mtDNA haplotypes

Population (*n*)	Haplotype																	
	A1	A2	A3	B1	B2	B3	B4	B5	B6	B7	B8	C1	C2	C3	C4	C5	C6	C7
German																		
Munich (152)	0	0	0	10	0	0	0	12	0	0	0	102	0	2	0	26	0	0
Chinese																		
Shenyang (114)	14	19	0	35	0	6	0	0	0	0	0	15	11	8	4	1	1	0
Korean																		
Seoul (109)	11	26	0	32	3	2	0	0	0	0	0	12	11	11	1	0	0	0
Kwangju (117)	4	30	0	29	4	7	0	1	0	1	0	20	8	12	1	0	0	0
Japanese																		
Aomori (110)	5	39	0	26	8	1	1	0	0	0	0	14	8	8	0	0	0	0
Morioka (119)	4	39	0	23	7	9	0	0	0	0	0	18	6	13	0	0	0	0
Yamagata (164)	6	58	0	38	13	11	0	0	0	0	0	16	9	13	0	0	0	0
Niigata (144)	4	59	0	28	11	4	2	0	0	0	0	12	9	15	0	0	0	0
Tajimi (117)	7	38	0	21	8	10	1	0	0	0	1	16	3	12	0	0	0	0
Nagoya (107)	4	38	0	22	8	4	0	0	0	0	0	15	5	10	1	0	0	0
Tottori (116)	4	34	0	26	13	10	1	0	0	0	0	10	8	10	0	0	0	0
Fukuoka (150)	6	46	1	36	19	2	0	0	0	0	0	19	7	13	1	0	0	0
Miyazaki (130)	2	48	0	28	13	6	2	0	0	0	0	13	6	12	0	0	0	0
Iki (84)	3	25	0	19	10	7	0	0	0	0	0	6	8	6	0	0	0	0
Tsushima (106)	12	35	0	23	6	3	0	0	0	0	0	13	10	4	0	0	0	0
Nomozaki (99)	0	26	0	20	9	3	0	0	0	0	0	20	6	15	0	0	0	0
Ikitsuki (130)	10	47	0	12	20	3	0	0	1	0	0	24	4	8	1	0	0	0
Goto (126)	0	55	0	13	9	9	0	0	0	0	0	22	13	5	0	0	0	0
Amami (148)	3	56	0	23	24	6	0	0	0	0	0	12	13	10	0	0	0	1
Okinawa (129)	0	50	0	16	28	3	2	0	0	0	0	11	7	12	0	0	0	0

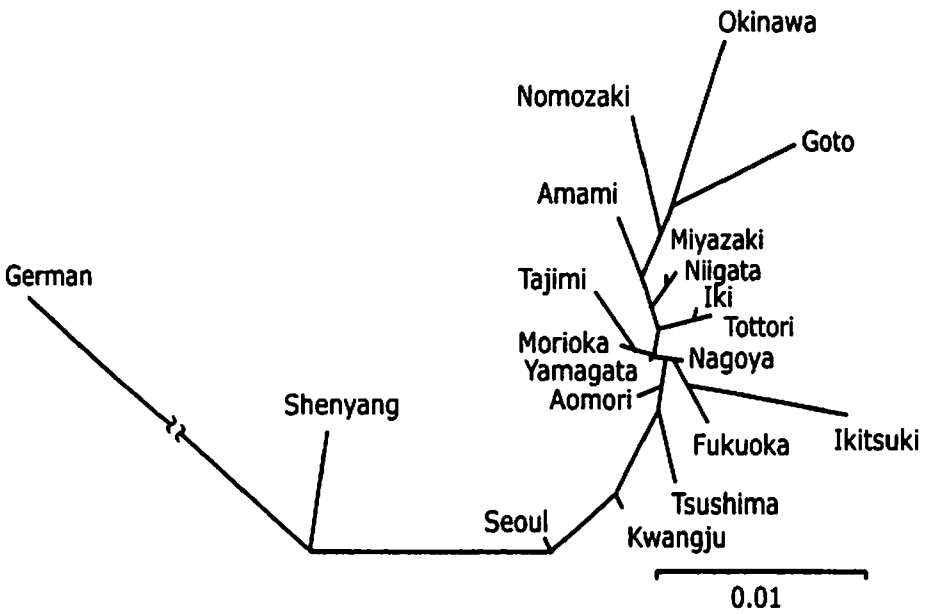

Figure 4. Neighbor-joining tree showing phylogenetic affinity between 20 populations based on Cavalli-Sforza's chord genetic distance. The distances are proportional to the branch length.

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