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Distribution of Length Variation of the mtDNA 9-bp Motif in the Intergenic *COII/tRNA^{Lys}* Region in East Asian Populations

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Length variations in human mitochondrial DNA (mtDNA) offer useful markers in the study of female aspects of human population history. One such length variation is a 9-bp deletion in the small noncoding segment located between the COII and lysine tRNA genes (*COII/tRNA^{Lys}* intergenic region) which usually contain two tandemly arranged copies of a 9-bp sequence (ccccctcta) in human mtDNA. The mtDNA 9-bp deletion and polymorphic variants of expanded 9-bp repeat motif in the intergenic *COII/tRNA^{Lys}* region have been found at varying frequencies among different human ethnic groups. We have examined the length variation of the mtDNA *COII/tRNA^{Lys}* intergenic region from a total of 813 individuals in east Asian populations. The occurrence of the 9-bp deletion was found to be relatively homogeneous in northeast Asian populations (Chinese, 14.2%; Japanese, 14.3%; Koreans, 15.5%), with the exception of Mongolians (5.1%). In contrast, Indonesians (25.0%) and Vietnamese (23.2%) of the southeast Asian populations appeared to have relatively high frequencies of the 9-bp deletion. We identified the existence of a new expanded 9-bp repeat motif which likely resulted from a slipped mispairing insertion of six more cytosines in the intergenic *COII/tRNA^{Lys}* region. It was present at low frequencies in the Korean (2/349) and Japanese populations (2/147). Based on the results of this study, the Korean population may reflect a close genetic affinity with the Japanese and Chinese populations than the others surveyed east Asian populations.

Genetic studies on the variations of classic genetic markers of protein and nuclear DNA show that Koreans tend to have a close genetic relationship with Mongolians among northeast Asians (Goedde et al., 1987; Saha and Tay, 1992; Hong et al., 1993; Nei and Roychoudhury, 1993). In contrast, genetic surveys of mitochondrial DNA (mtDNA) and Y chromosomal DNA polymorphisms indicate that Koreans are not closely related to Mongolians, but closely allied with Japanese and Chinese among several east Asian populations (Harihara et al., 1988; Ballinger et al., 1992; Horai et al., 1996; Hong et al., 1998; Kim et al., 1998; Shin et al., 1998). The discordance between classic genetic markers and mtDNA/Y chromosomal DNA results remains somewhat puzzling in genetic studies of the Korean population.

mtDNA has widely been used as a genetic tool to study evolutionary relationships among different human ethnic groups. Since mtDNA is known to have particular properties of inheritance (e.g., maternal inheritance, absence of recombination and high mutation rates), its variation resulting from the sequential addition of mutational changes along radiating maternal lineages can give us valuable information for a better understanding of human population history (Brown et al., 1979; Giles et al., 1980; Olivo et al., 1983). The current analyses of variation in human mtDNA have mainly been focused on nucleotide substitution (Horai and Hayasaka, 1990; Vigilant et al., 1991; Horai et al., 1995, 1996; Comas et al., 1996; Melton et al., 1998; Schurr et al., 1999) and length variation (Wrischnik et al., 1987; Redd et al., 1995; Soodyall et al., 1996; Hong et al., 1998) to quantify genetic relationships among human populations.

Cann and Wilson (1983) inferred several length changes (deletions and insertions) in human mtDNA by restriction enzyme analysis. Wrischnik et al. (1987) demonstrated that one such inferred deletion was loss of one of two adjacent copies of a 9-bp sequence (cccctcta) in the small noncoding segment located between the COII and lysine tRNA genes (*COII/tRNA^{Lys}* intergenic region). A triplication of the 9-bp repeat and a

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T-to-C transition with subsequent slipped mispairing insertion of three or four more cytosines have been reported in humans (Wrischnik et al., 1987; Lum and Cann, 1998). Recently, Watkins et al. (1999) report the presence of four different variants of the expanded 9-bp repeat motif in populations of south India. The 9-bp deletion in the intergenic *COII/tRNA^{Lys}* region has been reported at varying frequencies in populations from Asia, Polynesia, the New World and sub-Saharan Africa (Horai et al., 1987, 1996; Stoneking and Wilson, 1989; Vigilant, 1990; Ward et al., 1991, 1993; Ballinger et al., 1992; Harihara et al., 1992; Torroni et al., 1992, 1994; Horai, 1993; Melton et al., 1995; Redd et al., 1995; Soodyall et al., 1996). There is a geographic cline of the 9-bp deletion frequency across Pacific Islander populations, as well as from mainland Asia to the Malaysian peninsula (Horai et al., 1987; Hertzberg et al., 1989; Stoneking and Wilson, 1989; Horai, 1991; Ballinger et al., 1992; Harihara et al., 1992; Lum et al., 1994; Redd et al., 1995). Therefore, the clinal pattern suggests that the 9-bp deletion can be used for studies of maternal lineages and population history in east Asians.

Earlier surveys, based on the frequencies and the distribution of the 9-bp deletion implied that the deletion occurred once in Asia (Wrischnik et al., 1987; Hertzberg et al., 1989; Horai et al., 1996). However, other findings have suggested multiple origins of the 9-bp deletion in human populations (Schurr et al., 1990; Ballinger et al., 1992; Torroni et al., 1994; Redd et al., 1995; Watkins et al., 1999). The 9-bp deletion was subsequently found in high frequencies in African Pygmies (Vigilant, 1990; Chen et al., 1995). Soodyall et al. (1996) also reported that the 9-bp deletion arose independently in sub-Saharan Africa and Asia. The 9-bp deletion is thus not entirely exclusive to Asians and may even have multiple origins in humans. On the other hand, in the case of east Asia, the D-loop sequence variation indicated that the 9-bp deletion event may have occurred only once in the ancestry of east Asians (Horai et al., 1996). The 9-bp deletion marker could be useful for investigating genetic relationships and for complementing genetically based studies of other markers in east Asians. To better understand the genetic relationships and the distribution of the mtDNA 9-bp deletion in east Asians, we examined the length variation of the *COII/tRNA^{Lys}* intergenic region in several east Asian populations. Here, we report results on the frequency of the mtDNA 9-bp deletion and the existence of a new expanded variant in the intergenic *COII/tRNA^{Lys}* region from east Asian populations.

Materials and Methods

Subjects

We analyzed a total of 813 unrelated individuals from six east Asian populations (Chinese, Japanese, Koreans, Mongolians, Indonesians and Vietnamese). DNA samples were obtained from volunteers living in Korea (south): 349 Koreans, 3 Han Chinese, 15 Japanese, 40 Indonesians (Java) and 8 Vietnamese (Most of these east Asians usually come to Korea on temporary work permits). We also isolated DNA samples from 55 Vietnamese volunteer donors living in Hanoi. In addition, the DNA samples provided by other investigators are as follows: 39 Han Chinese and 39 Mongolians (Buryats), by C. Tyler-Smith; 100 Japanese (Ibaraki) and 118 Chinese (Beijing), by S. Harihara; 9 Han Chinese and 6 Vietnamese, by D. Labuda; 32 Japanese (Oita) by Y. Tamaki.

DNA extraction

mtDNAs were prepared from whole blood by the standard method (Sambrook et al., 1989). Many samples of total DNA were also extracted from buccal cells according to the procedure of Richards et al. (1993).

Polymerase chain reaction (PCR) and Electrophoresis

The length variation in the intergenic *COII/tRNA^{Lys}* region was detected by PCR amplification using flanking primers as described in Wrischnik et al. (1987): forward primer, 8196-5′-ACAGTTTCATGCCCATCGTC-3′-8215; reverse primer, 8316-5′-ATGCTAAGTTAGCT TTACAG-3′-8297. The normal type of the intergenic *COII/tRNA^{Lys}* region containing two tandemly arranged copies of a 9-bp sequence resulted in a 121 bp PCR product amplified by the flanking primers. In contrast, the 9-bp deletion type in this region can be detected by this set of primers as a size of the 112 bp fragment. The PCR reaction was performed in a total volume of 50 µl containing 100 ng of total DNA, 20 pM each primer, 200 µM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 U *AmpliTaq* DNA polymerase (Perkin-Elmer). PCR cycling conditions were 94℃ for 10 min, and then 40 cycles at 94℃ for 1 min, 55℃ for 30 sec, 72℃ for 1 min, and final extension at 72℃ for 10 min. The PCR products derived from the intergenic *COII/tRNA^{Lys}* region were separated by using 12% polyacrylamide gel electrophoresis (PAGE) for 12 h at 120 V. Following PAGE, the DNA fragments were visualized using ethidium bromide staining and ultraviolet light.

DNA sequencing

We have confirmed the expanded repeat motif of 6-bp insertion in the intergenic *COII/tRNA^{Lys}* region by DNA sequencing analysis. Each PCR product of the normal type, 9-bp deletion and 6-bp expanded variant, was then further cloned by ligation into pCR^{TM}II vector plasmids using the TA cloning kit (Invitrogen). Sequencing was accomplished by the chain termination method (Sanger et al., 1977) using the T7 reverse primer (5′-TAATACGACTCACTATAGGG-3′) and the Sequenase kit (U.S. Biochemical) according to the supplier's protocol.

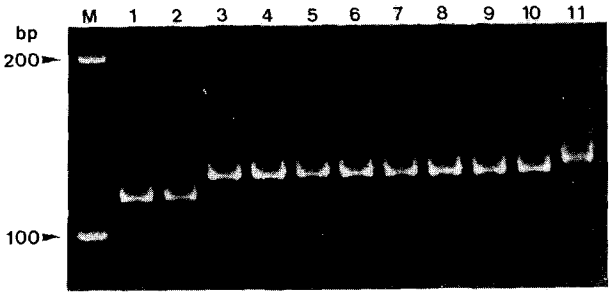

Fig. 1. Electrophoretic analysis of three allelic classes in mtDNA intergenic *COII/tRNA*^{Lys} region produced by PCR amplification. M, molecular size marker (100 bp ladder); 1-2, 112 bp PCR products containing a deletion of one of the 9-bp repeats; 3-10, 121 bp PCR products containing both copies of the 9-bp repeat; 11, 127 bp PCR product carrying an expanded 9-bp repeat motif with six more cytosines.

Results and Discussion

Based on the result of PCR amplification, three types of PCR products of 121 bp, 112 bp and 127 bp fragments were found for a normal type (two copies), 9-bp deletion and a new expanded variant in the intergenic *COII/tRNA*^{Lys} region, respectively (Fig. 1). DNA sequencing analysis indicated that the variant with the expanded 9-bp repeat motif probably resulted from a slipped mispairing insertion of six more cytosines (Fig. 2). In an earlier survey, a rare expanded variant in the intergenic *COII/tRNA*^{Lys} region have been reported in humans, which differs from the normal type by two mutations in the first copy of the 9-bp sequence: the mutations were raised by a T-to-C transition followed by a slipped mispairing insertion of four more cytosines in this region (Wrischnik et al., 1987). Recently, polymorphic variants of the expanded 9-bp repeat motif with three or four more cytosines were found at low frequencies in South Indian populations (Watkins et al., 1999). In contrast, the expanded variant detected in this survey most likely resulted from a slipped mispairing insertion of six more cytosines in the intergenic *COII/tRNA*^{Lys} region. This new additional variant was found

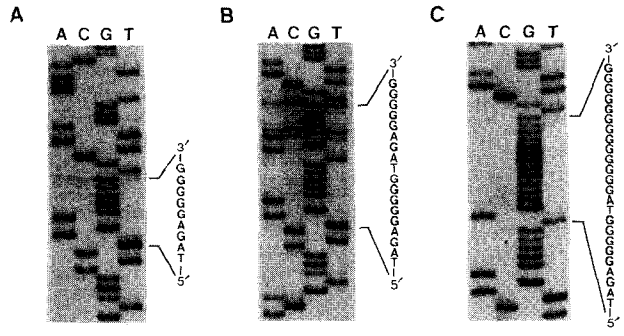

Fig. 2. Nucleotide sequence segments of mtDNA intergenic *COII/tRNA*^{Lys} region in three individuals from Korea. Each sequence represents the complementary strand sequenced with the reverse primer. A, One copy of the 9-bp sequence (5′ CCCCCTCTA 3′). B, Two copies of the 9-bp sequence (wild type). C, An expanded variant which was likely raised by a T-to-C transition followed by a slipped mispairing insertion of six more cytosines in this region.

Table 1. Distribution of the frequencies of 9-bp deletion motif in the intergenic *COII/tRNA*^{Lys} region in several east Asian populations

Population (n)	No. (%) of		
	Normal type	9-bp deletion	6-bp insertion
Northeast Asians			
Chinese (169)	145 (85.8)	24 (14.2)	0
Japanese (147)	124 (84.4)	21 (14.3)	2 (1.4)
Korean (349)	293 (84.0)	54 (15.5)	2 (0.6)
Mongolian (39)	37 (94.9)	2 (5.1)	0
Southeast Asians			
Indonesian (40)	30 (75.0)	10 (25.0)	0
Vietnamese (69)	53 (76.8)	16 (23.2)	0

in only Korean (2/349) and Japanese samples (2/147) (Table 1).

Somewhat regional differences in the distribution pattern of mtDNA 9-bp deletion frequencies were observed among east Asian populations (Table 1). The northeast Asian populations (Chinese, 14.2%; Japanese, 14.3%; Koreans, 15.5%) reflected a homogeneous distribution pattern of the 9-bp deletion frequencies, with the exception of Mongolians (5.1%). In contrast, Indonesians (25.0%) and Vietnamese (23.2%) of the southeast Asian populations represented relatively high frequencies of the 9-bp deletion when compared with other northeast Asians surveyed here. This result is consistent with the earlier surveys that showed a general increase in the frequency of the 9-bp deletion from Japan to mainland Asia to the Malaysian peninsula (Horai et al., 1987; Hertzberg et al., 1989; Stoneking and Wilson, 1989; Horai, 1991; Ballinger et al., 1992; Harihara et al., 1992; Chen et al., 1995). The 9-bp deletion frequency in our sample of Koreans (15.5%) is almost the same as that of the previous result of Koreans (16.0%) reported by Hong et al. (1998). Hong et al. (1998) also pointed out the homogeneous distribution pattern of the 9-bp deletion frequencies from those found in northeast Asians including the Koreans, Japanese and Mongolians. Most northeast Asians studied here shared a common ancestral population (Nei and Roychoudhury, 1993; Horai et al., 1996; Hong et al., 1998; Kim et al., 1998; Shin et al., 1998). However each population of northeast and southeast Asians may not be necessarily similar. Because 9-bp deletion frequencies appeared to be relatively homogeneous in most northeast Asian populations, their mtDNA are not discriminated. For example, the occurrence of homogeneous distribution pattern of the 9-bp deletion indicates that very few mtDNA were differentiated in Chinese, Japanese and Korean populations (14.2-15.5%) (Table 1). Therefore we need to use additional mtDNA markers (e.g., D-loop sequence variation) and populations from diverse regions in Asia to differentiate more mtDNA in these populations.

The 9-bp deletion frequency of Mongolians (5.1%) is lower than those of Koreans, Japanese and Chinese (Table 1). This result suggests that Koreans are not so closely related with Mongolians, but closely allied with Japanese and Chinese among east Asian populations.

Table 2. Cavalli-Sforza's genetic distance for the intergenic *COII/tRNA^{Lys}* region in six east Asian populations. Cavalli-Sforza's cord genetic distance (4*D*) in the Table body was based on allele frequency in the intergenic *COII/tRNA^{Lys}* region

Population	Chinese	Korean	Japanese	Mongolian	Indonesian	Vietnamese
Chinese						
Korean	0.0141					
Japanese	0.0064	0.0019				
Mongolian	0.0251	0.0403	0.0376			
Indonesian	0.0188	0.0316	0.0198	0.0869		
Vietnamese	0.0135	0.0265	0.0153	0.0750	0.0004	

Based on the result of Cavalli-Sforza's Chord genetic distance (4*D*) (Cavalli-Sforza and Bodmer, 1971), it revealed a close genetic affinity between the Japanese and Korean than the others surveyed east Asian populations (Table 2). There is also evidence for a migration of Yayoi people from their original places via China and the Korean peninsula to Japan starting around 2,300 years ago (Chard, 1974; Hanihara, 1991; Hammer and Horai, 1995; Horai et al., 1996; Omoto and Saitou, 1997). The Yayoi migration would lead us to expect a common genetic affinity in contemporary populations from Korea and Japan. The mtDNA D-loop variation detected by Horai et al. (1996) supported that a great quantity of maternal lineages were introduced into Japan by immigrants from the Korean peninsula after the Yayoi period. Our earlier surveys on the variation of Y chromosomal DNA haplotypes also revealed that Koreans and Japanese are more closely related than those of Mongolians (Kim et al., 1998; Shin et al., 1998). Further genetic surveys are required to verify this pattern of genetic relationship with sufficient samples from various Mongolian tribes which are known to vary greatly even along tribal lines.

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