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PAPER

CRIMINALISTICS

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A New Strategy for the Discrimination of Mitochondrial DNA Haplogroups in Han Population

ABSTRACT: Mitochondrial DNA (mtDNA) haplogroup discrimination is interesting not only for phylogenetic and clinical but also for forensic studies. We discriminated the mtDNA haplogroups of 570 healthy unrelated Han people from Zhejiang Province, Southeast China, by comprehensive analysis mutations of the hypervariable segments-I sequence and diagnostic polymorphisms in mtDNA coding region using real-time polymerase chain reaction (RT-PCR), which was compared with the widely used PCR and restriction fragment length polymorphism (PCR-RFLP) method. The results showed that in superhaplogroup M, haplogroup D was the most common haplotype within this assay to 24.6%, and in the other superhaplotype N, haplogroup B and F were the most common groups. Samples re-identified by PCR-RFLP showed the consistent results that were got with RT-PCR. In conclusion, the RT-PCR strategy appears to be an accurate, reproducible, and sensitive technique for the discrimination of mtDNA haplogroups, especially for mass screenings quickly and economically.

KEYWORDS: forensic science, mtDNA haplogroup, single nucleotide polymorphisms, hypervariable segments-I, real-time polymerase chain reaction, polymerase chain reaction–restriction fragment length polymorphism

Mitochondrial DNA (mtDNA) has been widely analyzed in human evolution/population genetic studies (1–3) and in molecular medicine to correlate pathological mutations to a variety of human diseases (4). Haplogroups, which have been determined using single nucleotide polymorphisms (SNPs) of mtDNA, are major monophyletic clades in the mtDNA tree (5). In the forensic field, analysis of mtDNA haplogroups is particularly important in human identification because of its unique characteristics of maternal inheritance, high copy number per cell, high mutation rate, and absence of recombination.

The analysis of the genetic variation in the hypervariable segments (HVS) I/II of the control region is the most common approach to discriminate mtDNA haplogroups in the forensic field. But the two hypervariable segments (HVS-I and HVS-II) alone cannot support a very reliable estimate of the mtDNA phylogeny (6), and coding region information is indispensable for phylogenetic analysis of mtDNA. Different applications were recently developed to discriminate the mtDNA haplogroups more accurately. These include the technique of direct sequencing, restriction fragment length polymorphism (RFLP) (7), the SNaPshot assay (8), denaturing high-performance liquid chromatography (9), a temporal temperature gradient gel electrophoresis strategy (10), etc. Haplogrouping mtDNA by sequence analysis is not practical, particularly in forensic and ancient samples. Some of these methods appear to be not convincing when determining low proportion of heteroplasmy and require a dedicated instrument not always available in general medical genetic laboratories. A fast and economic assay of mtDNA haplogroup identification will save time and money and is suitable for mass screenings (11).

Recent publications (12–14) investigated the distribution of the major east Asian haplogroups in different population samples by RFLP and/or control region sequences. Specific mutations discriminating many haplogroups have been characterized, but the most detailed molecular dissection of mtDNA haplogroups of Han population was performed by Kong et al., etc. (14). The results are consistent, showing Han members from the two superhaplogroups of M and N. The most commonly observed Han haplogroup was the B4. Haplogroups A, F, and D were also common. However, the techniques and the selected markers were still time-consuming and/or expensive strategies. Here, we investigate a new strategy to discriminate the mtDNA haplogroups of Han population quickly and accurately for mass screenings and compare it with the polymerase chain reaction (PCR)-RFLP method.

Materials and Methods

This study was approved by the Institutional Ethics Committee of Zhejiang University. The mtDNA haplogroup was determined in 570 healthy presumably unrelated Han people from Zhejiang Province, Southeast China. The SNPs were chosen to discriminate between the main haplogroups present in Han population and their subclades (14). We designed a motif of specific haplogroup decision tree (Fig. 1).

Samples

Genomic DNA was extracted from whole blood samples of the study participants using the whole DNA extraction kit (Sangon,

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Shanghai, China). The concentration of DNA was measured by spectrophotometers with 260 nm wavelength ultraviolet light. All DNA samples were stored at −20°C.

PCR and Sequence

The sequence of the HVS-I from position 15 996 to 16 498 (relative to the revised Cambridge reference sequence [rCRS]) (15) was amplified and sequenced. The primers used for PCR were F-CTCCACCATTAGCACCCAAAGC and R-CCTGAAGTAGGAACCAGATG (16). The sequence of mtDNA from position 8196 to 8316 was also amplified to evaluate a 9-bp deletion, which was used to detect haplogroup B. The primers were F-ACAGTTTCATGCCCATCGTC and R-ATGCTAAGTTAGCTTTACAG (16).

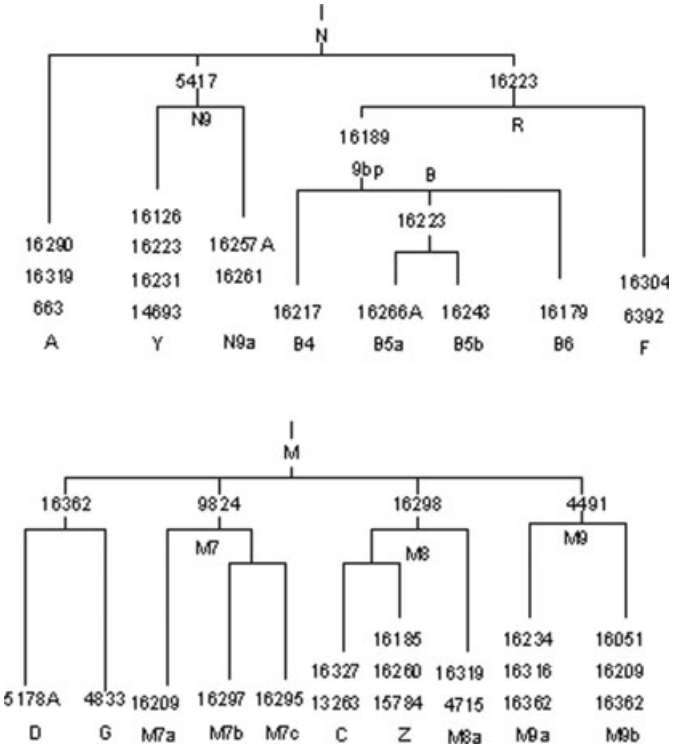

FIG. 1—*Decision tree for haplogroup assignment.*

Real-time PCR

According to the preliminary typing determined by HVS-I sequence analysis, we tested the specific polymorphisms used to define haplogroups in the mtDNA coding region. Eleven FAM-labeled oligonucleotide probes (Genecore, Shanghai, China) were designed to examine the diagnostic polymorphisms and are listed in Table 1. We performed PCR with fluorescence-labeled hybridization probes using a Master Mix kit (ABI, Foster City, CA) on Mx3005P Real-time QPCR system (Stratagene, San Diego, CA) in Real-time Allele Discrimination-SNP's mode. Because the mutations we detected were haploid, we designed FAM-labeled fluorescence sign as goal sign and HEX-labeled fluorescence sign as a dummy sign defined zero.

PCR amplification was carried out in a total volume of 50 μL. Final concentrations for standard PCR reagents were 2 μL DNA sample, 0.5 μL primers (10 nM), 3 μL $MgCl_2$ (25 mM), 5 μL PCR buffer (×10), 1 μL Taq polymerase (5 U/μL;), and 1 μL deoxynucleotide triphosphates (dNTPs) (all reagents applied Sangon). Thermal cycling for PCR was carried out as follows: initial denaturation for 2 min at 94°C, 40 cycles of 95°C for 40 sec, 54°C for 60 sec, 72°C for 60 sec, final extension at 72°C for 5 min, and storage at 4°C. The PCR products were purified and sequenced by Sangon.

PCR amplification was carried out in a total volume of 25 μL. Final concentrations for standard PCR reagents were 2 μL DNA sample (8 ng DNA), 1.125 μL primers (20 nM), 0.625 μL FAM-labeled oligonucleotide probes (20 nM), and 12.5 μL PCR Mix buffer. Thermal cycling for PCR of 5178A probe was carried out as follows: initial denaturation for 2 min at 50°C followed by 15 min at 95°C, 40 cycles of 95°C for 30 sec, 60°C for 45 sec,

TABLE 1—*The DNA primers and probes used to detect polymorphisms in the mitochondrial DNA coding region.**

Haplogroups (DSNP)	Primers	Probes
F (6392)	F: CACCTAGCAGGTGTCTCCTCTATCT R: GGGCGTTTGGTATTGGGTTA	FAM-CCATCAA<u>C</u>TTCATCACAAC
N9a (5147)	F:GATGAATAATAGCAGTTCTACCGTACAAC R: AGCTTGTTTCAGGTGCGAGATAG	FAM-CAGCACCAC<u>A</u>ACCCT
Y (14693)	F: AACCCACACTCAACAGAAACAAAG R: GAGGTCGATGAATGAGTGGTTAATT	FAM-CTACA<u>G</u>CCACGACCAAT
A (663)	F: GCTCACATCACCCCATAAACAAATA R: GTGGTGATTTAGAGGGTGAACTCAC	FAM-TGGTCCT<u>G</u>GCCTTTC
D (5178A)	F: TCCAGCACCACGACCCTACTA R: GGAGGGTGGATGGAATTAAGG	FAM-CCTGAAACAAGA<u>T</u>AACATGAC
G (4833)	F: CCTTTCACTTCTGAGTCCCAGA R: GGGGCTAGTTTTTGTCATGTGAG	FAM-CAAGGC<u>G</u>CCCCTCT
M7 (9824)	F: CCCTTCACCATTTCCGACG R: TGAAGCAGATAGTGAGGAAAGTTGA	FAM-ACGGACTTT<u>A</u>CGTCAT
C (13263)	F: CATCAAAAAAATCGTAGCCTTCTC R: ACAGATGTGCAGGAATGCTAGGT	FAM-CAAGTCA<u>G</u>CTAGGACTC
Z (15784)	F: CGCAGACCTCCTCATTCTAACC R: TTAGGATTGTTGTGAAGTATAGTACGGAT	FAM-AGCTACCC<u>C</u>TTTACCATCA
M8a (4715)	F: AACCGCATCCATAATCCTTCTAAT R:TTAATGATGAGTATTGATTGGTAGTATTGG	FAM-TCCGG<u>G</u>CAATGAA
M9 (4491)	F: GCCCATACCCCGAAAATGT R: GTGATGAGTGTGCCTGCAAAGA	FAM-CCCAACCC<u>A</u>TCATCTA

*The position of base alteration in de generate oligonucleotide probes is underlined.
DSNP, diagnostic single nucleotide polymorphism; F, forward; R, reverse.

72°C for 60 sec, and storage at 4°C. Thermal cycling for PCR of other probes was carried out as follows: initial denaturation for 2 min at 50°C followed by 10 min at 95°C, 50 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec, and storage at 4°C. Finally, comprehensive analysis of the HVS-I sequences and diagnostic polymorphisms in the mtDNA coding region identified the haplogroups (Fig. 1).

PCR-RFLP

Five samples were randomly selected from each mtDNA haplogroups A, D, M7, and M9, for which discrimination in the mutation sites of coding region could also be detected by endonuclease digestion. The samples were re-identified by PCR-RFLP, which has been described in detail previously (16). The maps of endonuclease digestion are shown in Fig. 2.

Results

Using a strategy of comprehensive analysis of the HVS-I sequences and diagnostic polymorphisms in the mtDNA coding region by real-time PCR, 551 of 570 samples from Han population were assigned to a particular haplogroup, all of which belong to the two superhaplogroups of M or N. The other 19 samples were assigned as "others," which belong to uncommon haplogroups. A total of 19 different haplotypes were found; 47.7% of all samples belonged to superhaplogroup M, and haplogroup D was the most common haplotype within this assay to 24.6%. In the other superhaplotype N, haplogroups B and F were the most common groups. According to the mutations of HVS-I, haplogroup B could be divided into B4 (10.9%), B5 (5.1%), and B6 (1.8%). The frequency of different haplogroups can be seen in Table 2.

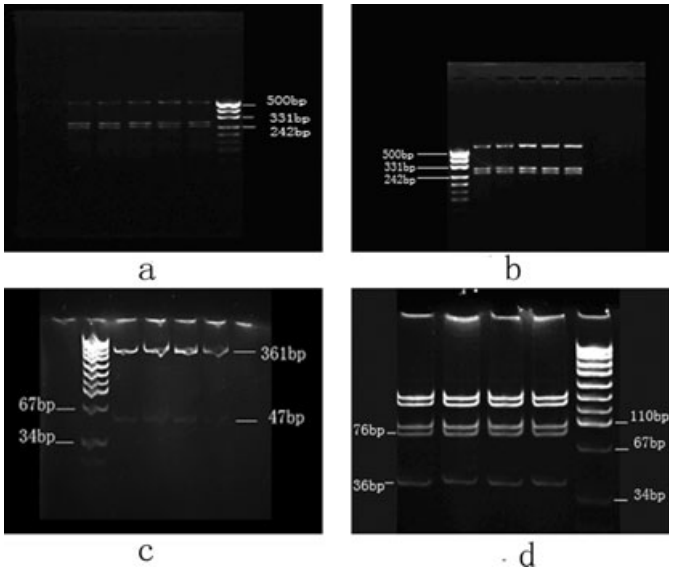

FIG. 2—*The maps of endonuclease digestion. (a) The map of* Hae*III endonuclease digestion for 663A/G which defines haplogroup A. Two characteristic fragments were shown (290 and 257 bp length). (b) The map of AluI endonuclease digestion for 5178C/A which defines haplogroup D. Two characteristic fragments were shown (312 and 284 bp length). (c) The map of* Hin*fI endonuclease digestion for 9824T/C which defines haplogroup M7. Two characteristic fragments were shown (361 and 47 bp length). (d) The map of* Hae*III endonuclease digestion for 4491T/C which defines haplogroup M9. A series of fragments were shown including two characteristic fragments (76 and 36 bp length).*

Five samples were randomly selected from each mtDNA haplogroup A, D, M7, and M9. The mutation sites in the coding region for discriminating these haplogroups can also be detected by endonuclease digestion. The samples were re-identified by PCR-RFLP, and consistent results were achieved with real-time PCR.

Discussion

Mitochondrial DNA haplogroup discrimination is interesting not only for phylogenetic and clinical but also for forensic studies. Several assays have been developed to quantify mtDNA point mutations and discriminate haplogroups. Sequencing the whole mtDNA seems the most accurate method, but time consuming and too expensive for discrimination of a large number of samples (15). Sequence analysis of the genetic variation in the hypervariable segments HVS-I/II of the control region might elevate the efficiency of discrimination, for the frequencies of the hotspots observed in HVS are significantly higher than in the coding region (17). In cases where direct information from the coding region is not available, one can at least anticipate the haplogroup according to mutations of HVS-I. Specifically, we can discriminate the haplogroup status of most east Asian HVS-I sequences via the Han database (16). For instance, haplogroup A usually has an HVS-I motif 16223T–16290T–16319A (18). Nevertheless, attempts at estimating a phylogeny solely from HVS-I/II without any reference to coding region sites would go astray, in particular, if the mutations of HVS-I/II to assign haplogroup were hotspots (19). For instance, mutations of HVS-I to assign haplogroup D were 16223 and 16362, which have high mutation frequencies, and it was difficult to distinguish haplogroup G from D. Coding region information is indispensable for phylogenetic analysis of mtDNA. For haplogroup D, 5178A is a characteristic mutation, which can discriminate haplogroup D and G. In east Asian populations, the random match probability of HVS is estimated to be about 2% for HVS-I, about 4% for HVS-II (20,21). When sequencing of HVS-I and haplogrouping of the coding region (22) are combined, the resultant value in the east Asian population is improved to about 1.5%.

PCR-RFLP was the most commonly employed method used to detect specific mutations of coding region. A phylogenetic analysis by RFLP typing of specific mutations detecting the major Han

TABLE 2—*The frequencies of the main subhaplogroups of Han population.*

Haplogroup	Number (%)
M7b	33 (5.8)
M7c	16 (2.8)
M7	51 (8.9)
M8a	13 (2.2)
CZ	31 (5.4)
M8	45 (7.9)
M9a	16 (2.8)
M9D	17 (3.0)
G	19 (3.3)
D	140 (24.6)
A	43 (7.5)
N9a	19 (3.3)
N9	25 (4.4)
B4	62 (10.9)
B5	29 (5.1)
B6	10 (1.8)
B	101 (17.7)
F	101 (17.7)
R9	110 (19.3)
Others	19 (3.3)

mtDNA haplogroups was performed on the Han samples by Yao et al. (16) This approach is still quite time consuming and expensive. Furthermore, an incomplete endonuclease digestion might hamper analysis if a single site of cleavage is chosen (23). Detection of coding region mutations can also be hampered by endonuclease digestion if the endonuclease cleaves the DNA sequence at a coding region mutation site. In a Han phylogenetic analysis according to Yao et al. (16), only six mutation sites could be detected by endonuclease digestion.

Compared with PCR-RFLP, we found the real-time PCR method that uses a FAM-labeled oligonucleotide probe to detect mutations of the coding region is a rapid method, which can be used widely. Oligonucleotide probes can be used for SNP detection or mutation analysis in a PCR where a separate probe is designed for an allele and is labeled with a different fluorophore (e.g., with FAM). However, in these assays, it can be challenging to optimize conditions to prevent the probes from annealing nonspecifically to the wrong allele. In general, enhanced specificity for SNP analysis is achieved by using a new type of oligonucleotide probe known as a minor groove binder (MGB) probe. The MGB probes are similar to the standard TaqMan probes, but they include the addition of a minor groove-binding moiety on the 3′ end that acts to stabilize annealing to the template. The stabilizing effect that the MGB group has on the Tm of the probe allows for the use a much shorter probe (down to ~13 bp). The shorter probe sequence is more susceptible to the destabilizing effects of single bp mismatches, which makes these probes better than standard TaqMan probes for applications that require discrimination of targets with high sequence homology. In our study, we used this kind of probe. Primer and probe design was viewed as the most challenging step. For optimal performance, the region spanned by the primers (measured from the 5′ end of each primer) should be between 70–150 bp in length for probe-based chemistries. The probes were designed with a Tm 8–10°C higher than Tm of the primers. According to the conditions above, we designed the 11 probes to detect the mutation sites of the coding region (Table 2). Real-time PCR also provides an optimized quality control (see Fig. 3), which reduces false negatives or positives by monitoring the progress of the amplification reaction. In our study, five samples were randomly selected from each mtDNA haplogroup A, D, M7, and M9. The coding region mutation sites for discrimination of these haplogroups could also be detected by endonuclease digestion. The samples were re-identified by PCR-RFLP, and the consistent results were achieved with real-time PCR.

Comprehensive analysis of the HVS-I sequences and diagnostic polymorphisms in mtDNA coding region by real-time PCR is sensitive and specific, and it has three main advantages: (i) it best uses the HVS-I mutation information to increase the efficiency of discrimination, which saves time and money. With PCR-RFLP, we should spend more than 10 h for a complete endonuclease digestion, which is followed by electrophoresis. If there were a large number of samples, electrophoresis and reading results would also need much time. However, with RT-PCR, we need less time for reaction—about 1 or 2 h—and can read results directly without electrophoresis. The cost of one real-time PCR was about 1 dollar, which was about one-third of that of RFLP; (ii) RT-PCR is a reaction requiring very little optimization. It can detect mutation sites more accurately than endonuclease digestion, if suitable primers and probes are designed; and (iii) furthermore, it is more facile to analyze the samples with low amounts of DNA. We have diluted DNA to test the sensitivity of RT-PCR and found that it can show positive results even with 4–6 ng DNA sample. Another advantage is its high-throughput potential. When more and more subhaplogroups are defined, a "probes bank" could be established, which would make it possible for quick and economical mass screenings in clinical and forensic medicine.

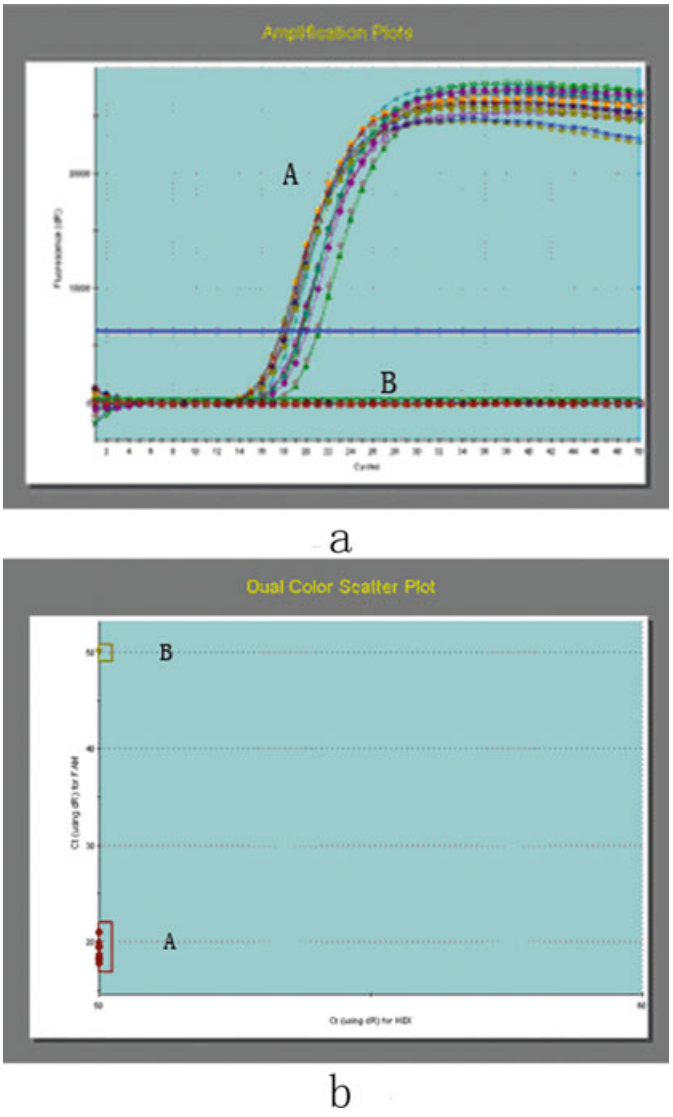

FIG. 3—*The sketch map of real-time PCR in real-time allele discrimination-single nucleotide polymorphism's mode, which could provide an optimized quality control by monitoring the progress of PCR. (a) The curve map of the amplification reaction (A: the "S" curves of FAM-labeled hybridization reaction, which showed positive results; B: the flat curves including HEX-labeled dummy hybridization reaction and blank control). (b) The dot map of the amplification reaction, which proved the accuracy and sensitive of the assay (A: the dots of FAM-labeled hybridization reaction showed about 20* C_t *value; B: the dots of HEX-labeled dummy hybridization reaction showed about 50* C_t *value).*

Using a strategy of the comprehensive analysis of the HVS-I sequences and diagnostic polymorphisms in mtDNA coding region by real-time PCR, we achieved consistent results with the published frequencies of mtDNA haplogroups (16), thus proving the sensitivity and accuracy of this strategy.

Conclusions

We have developed a new strategy for screening and quantification of mtDNA mutations and discrimination mtDNA haplogroups of Han population. This method appears to have high accuracy and

sensitivity and correlates well with more traditional assays used in mtDNA haplogroup discrimination. Although it is necessary to further expand the analysis of variants, it will be possible to utilize this strategy to detect any pathogenic mtDNA mutation and discriminate mtDNA haplogroups. Because of the feasibility of this system and the need for only small amounts of DNA, we can anticipate its use in studies of mtDNA-associated disorders, individual/species identification, and maternity testing in forensic medicine. We will expand this strategy to an even larger multiplex ''probes bank'' to distinguish between all major branches of the mtDNA phylogeny, making it possible for quick and economical mass screenings.

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References

1. Torroni A, Lott MT, Cabell MF, Chen YS, Lavergne L, Wallace DC. mtDNA and the origin of Caucasians: identification of ancient Caucasian-specific haplogroups, one of which is prone to a recurrent somatic duplication in the D-loop region. Am J Hum Genet 1994;55:760–76.
2. Krings M, Stone A, Schmitz RW, Krainitzki H, Stoneking M, Paabo S. Neanderthal DNA sequences and the origin of modern humans. Cell 1997;90:19–30.
3. Ingman M, Kaessmann H, Paabo S, Gyllensten U. Mitochondrial genome variation and the origin of modern humans. Nature 2000;408:708–13.
4. Taylor RW, Turnbull DM. Mitochondrial DNA mutations in human disease. Nat Rev Genet 2005;6:389–402.
5. Torroni A, Richards M, Macaulay V, Forster P, Villems R, Norby S, et al. mtDNA haplogroups and frequency patterns in Europe. Am J Hum Genet 2000;66:1173–7.
6. Bandelt H-J, Macaulay V, Richards M. Median networks: speedy construction and greedy reduction, one simulation, and two case studies from human mtDNA. Mol Phylogenet Evol 2000;16:8–28.
7. Claudio MB, Juan PL, Patricia MM, Maria VR, Pilar PG, Guillermo G. A simple method for domestic animal identification in Argentina using PCR-RFLP analysis of cytochrome b gene. Legal Med 2004;6:246–51.
8. Quintans B, Alvarez-Iglesias V, Salas A, Phillips C, Lareu MV, Carracedo A. Typing of mitochondrial DNA coding region SNPs of forensic and anthropological interest using SNaPshot minisequencing. Forensic Sci Int 2004;140:251–7.
9. Biggin A, Henke R, Bennetts B, Thorburn DR. Christodoulou J, Mutation screening of the mitochondrial genome using denaturing high-performance liquid chromatography. Mol Genet Metab 2005;84:61–74.
10. Wong LJ, Chen TJ, Tan DJ. Detection of mitochondrial DNA mutations using temporal temperature gradient gel electrophoresis. Electrophoresis 2004;25:2602–10.
11. Szibor R, Plate I, Schmitter H, Wittig H, Krause D. Forensic mass screening using mtDNA. Int J Legal Med 2006;120:372–6.
12. Tanaka M, Cabrera VM, Gonza′lez AM, Larruga JM, Takeyasu T, Fuku N, et al. Mitochondrial genome variation in eastern Asia and the peopling of Japan. Genome Res 2004;14:1832–50.
13. Allard MW, Wilson MR, Monson KL, Budowle B. Control region sequences for East Asian individuals in the Scientific Working Group on DNA Analysis Methods forensic mtDNA data set. Legal Med 2004;6:11–24.
14. Kong QP, Bandelt HJ, Sun C. Updating the East Asian mtDNA phylogeny: a prerequisite for the identification of pathogenic mutations. Hum Mol Genet 2006;15:2076–86.
15. Andrews RM, Kubacka I, Chinnery PF. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet 1999;23:147.
16. Yao YG, Kong QP, Bandelt HJ, Toomas K, Zhang YP. Phylogeographic differentiation of mitochondrial DNA in Han Chinese. Am J Hum Genet 2002;70:635–51.
17. Gilbert MTP, Hansen AJ, Willerslev E, Rudbeck L, Barnes I, Lynnerup N, et al. Characterization of genetic miscoding lesions caused by postmortem damage. Am J Hum Genet 2003;72:48–61.
18. Bandelt HJ, Lahermo P, Richards M, Macaulay V. Detecting errors in mtDNA data by phylogenetic analysis. Int J Legal Med 2001;115:64–9.
19. Richards M, Corte-Real H, Forster P, Macaulay V, Wilkinson-Herbots H, Demaine A, et al. Paleolithic and Neolithic lineages in the European mitochondrial gene pool. Am J Hum Genet 1996;59:185–203.
20. Nishimaki Y, Sato K, Fang L, Ma M, Hasekura H, Boettcher B. Sequence polymorphism in the mtDNA HV1 region in Japanese and Chinese. Legal Med 1999;1:238–49.
21. Imaizumi K, Parsons TJ, Yoshino M, Holland MM. A new database of mitochondrial DNA hypervariable regions I and II sequences from 162 Japanese individuals. Int J Legal Med 2002;116:68–73.
22. Umetsu K, Tanaka M, Yuasa I, Adachi N, Miyoshi A, Kashimura K, et al. Improved multiplex amplified product-length polymorphism (APLP) analysis for rapid and detailed identification of East Asian mitochondrial DNA haplogroups. Electrophoresis 2005;26:91–8.
23. Taylor RW, Taylor GA, Morris CM, Edwardson JM, Turnbull DM. Diagnosis of mitochondrial disease: assessment of mitochondrial DNA heteroplasmy in blood. Biochem Biophys Res Commun 1998;251:883–7.

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