TECHNICAL ANNEX

1. Isolation of Genomic DNA

Venous blood (2 ml) was collected from each subject into a tube containing 50 mM EDTA; and genomic DNA was isolated from the total blood samples by the standard phenol-chloroform method, or by use of a DNA isolation kit Dr GenTLE (Takara, Shiga, Japan) or an automated DNA extraction system MagExtractor MFX-2000 (Toyobo, Osaka, Japan).

2. Amplification of DNA

The entire mitochondrial genome was amplified as 6 fragments by the first PCR, and then 60 overlapping segments were amplified by the second PCR, essentially as described previously (Tanaka et al., Methods in Enzymology, 1996).

First PCR

The entire mitochondrial genome was amplified as 6 fragments (A to F), each approximately 3.0 kb in length, by a symmetric PCR method with the primer pairs (L and H primers) shown in Table 1. The oligonucleotide primers, synthesized and purified by gel filtration, were obtained from Bio-Synthesis (Lewisville, Texas). The sequences of primers L and H are shown in Tables 3 and 4, respectively. PCR amplification was carried out in a final reaction volume of 40 µl, containing 200 ng human genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM concentration of each dNTP, 0.5 or 1.0 µM concentration of each primer, and 1 unit of *Taq* DNA polymerase (Takara, Shiga, Japan). The PCR conditions used were the following: an initial denaturation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 52-62°C for 15 s, and extension at 72°C for 3 min, with a final extension of 10 min at 72°C. Table 2 shows the primer concentration and annealing temperature used for amplification of each fragment. The amplified fragments were analyzed by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide.

Second PCR

The first PCR DNA templates for the sequence analysis of the entire mitochondrial genome were amplified as 60 overlapping segments (1 to 60), each of approximately 600-1000 bp, by a symmetric PCR method with the primer pairs (FL and H primers) shown in Table 1. The sequence of primers H and FL are shown in Tables 4 and 5, respectively. The FL primer was a 38-mer oligonucleotide, consisting of an 18-base sequence of an universal forward sequencing primer (-21M13, 5'-TGTAAACGCGGCCAGT-3') connected on its 5' side to the 3' side of a 20-base L-strand-specific sequence (Table 5). PCR amplifications were carried out in a final reaction volume of 20 µl, containing 200 ng of the first PCR product, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM concentration of each dNTP, 1.0 µM concentration of each primer, and 0.5 units of *Taq* DNA polymerase

(Takara, Shiga, Japan). The PCR conditions used were the same as for the first PCR except that the annealing temperature used was 60°C. These second PCR products were purified by use of MultiScreen-PCR Plates (Millipore, Bedford, MA), and then the qualities of DNA templates were examined by electrophoresis on a 1.2% agarose gel after staining with ethidium bromide by use of a Ready-To-Run Separation Unit (Amersham Pharmacia Biotech AB, San Francisco, CA).

3. Sequence analysis and identification of mtSNP

Sequence reactions were performed by use of the second PCR template, -21M13 forward primer, and a BigDye Terminator Cycle Sequence Ready Reaction Kit version 1.0, 3.0, or 3.1 (Applied Biosystems, Foster City, CA). The following PCR conditions were used: an initial denaturation step at 96°C for 5 min, followed by 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 62°C for 4 min. After the sequence reaction, excess dye terminators were removed by gel filtration on a MultiScreen-PCR HV Plate (Millipore). The purified DNA samples were dried and suspended in the template suppression reagent (TSR) or formamide from Applied Biosystems. The dissolved DNA samples were heated at 95°C for 2 min for denaturation, and then immediately cooled on ice. Sequences were analyzed with an automated DNA sequencer Prism 310 or 377 from Applied Biosystems. Sequence analysis was performed by use of Sequencing Analysis Program version 4.1 software (Applied Biosystems). Complete sequences were aligned, assembled, and compared with the program Sequencher 4.1 (Gene Codes, Ann Arbor, MI). For verification, visual inspection of each candidate mtSNP was carried out. At least 2 overlapping DNA templates amplified with different primer pairs were used for identification of each mtSNP.

4. Reverse sequencing of 5 segments

In some cases, the light-strand sequences could not be determined on the 3' side of long stretches of C due to T>C transitions at positions 310, 961, and 16189 or due to the poly-C sequences at 568-573 and 5895-5899. For such cases, the heavy-strand (reverse) sequences were determined for 5 segments (02, 03, 21, 58, and 60). For this purpose, the primers H81, H528, H807, and H6158 listed in Table 6 were used for sequencing the second PCR products with a BigDye terminator cycle sequence ready reaction kit version 3.1 from Applied Biosystems. The segment 03 was amplified from the first PCR product as the template by use of the FL700 and the T7H1162 primers. The T7H1162 primer was a 40-mer oligonucleotide, which was a 20-base sequence of a T7 promoter (5'-TAATACGACTCACTATAGGG-3') connected on its 5' side to the 3' side of a 20-base H-strand-specific sequence (5'-CACCGCCAGGTCCTTTGAGT-3'). The sequence reaction was performed at 37°C for 1 hr by use of the second PCR product (FL700-T7H1162) as the template with a CUGA7 sequencing kit (Nippon Genetech, Tokyo, Japan) designed for the ABI Prism 310 genetic analyzer. After removal of excess dye

terminators by gel filtration on a Centri-Sep spin column (Applied Biosystems), the sample was applied to the ABI Prism 310 genetic analyzer.

5. Comparison with the revised Cambridge reference sequence (rCRS):

Each of the mtDNA sequences was compared with the original Cambridge sequence (Anderson et al., Nature, 1981) and the revised Cambridge reference sequence (rCRS) (Andrews et al., Nat Genet, 1999).

6. Subject groups

Centenarian group (TC and GC): This group consists of 96 centenarians over 100 years of age who were registered by the Department of Gerontology, Keio University School of Medicine, in Tokyo (TC group: n=85, 60 females and 25 males) or by Gifu International Institute of Biotechnology (GC group: n=11, 6 females and 5 males).

Parkinson's disease group (PD): The PD group consists of 96 patients with Parkinson's disease (mean age of 62.1 ± 8.9 years; range, 39-81 years) who were diagnosed at the Department of Neurology, Juntendo University School of Medicine, in Tokyo (53 females and 43 males).

Alzheimer's disease group (KA): The KA group comprises 96 patients with Alzheimer's disease (mean age of 76.5 ± 9.7 years; range, 47-93) who were diagnosed at the Department of Neurology, Nihon Medical School, in Saitama (n=96, 76 females and 20 males).

Obesity group (ON): The ON group consists of 96 young obese male subjects (mean age of 21±2 years; range, 18-25 years) with a body mass index (BMI) of 30.1±2.5, who were registered at the Research Center of Health, Physical Fitness and Sports, Nagoya University, in Nagoya.

Healthy non-obese group (HN): The HN group consists of 96 young healthy non-obese male subjects (mean age of 20±3 years; range, 18-25) with a body mass index (BMI) of 20.2±2.3, who were registered at the same location as the obese group.

Diabetes group (ND) in Nagoya: The ND group consists of 96 patients with type-2 diabetes mellitus (mean age of 58±5 years; range, 43-65), who were registered at the same location as the obese group (n=96, 42 females and 54 males).

Diabetes group (JD) in Tokyo: The TD group comprises 96 type-2 diabetes patients with severe vascular involvement (mean age of 65±10 years; range, 43-92), who were registered at the Department of Internal Medicine, Juntendo University School of Medicine, in Tokyo (n=96, 48 females and 48 males).

Data from Ingman et al. (Ingm): The complete mtDNA sequences of 53 humans of diverse origins were reported by Ingman et al. (Ingman M, Kaessmann H, Paabo S, Gyllensten U, Mitochondrial genome variation and the origin of modern humans. Nature 408: 708-713, 2000). For further information, please visit:

http://www.genpat.uu.se/mtDB/, http://www.genpat.uu.se/anthropology/ and http://www.actionbioscience.org/evolution/ingman.html.

7. Contributors

Masashi Tanaka (GiiB)
Noriyuki Fuku (GiiB, JST)
Raita Hirose (GiiB, JST)
Takeshi Takeyasu (GiiB, Nagoya University)
Li-Jun Guo (GiiB, Nagoya University)
Osamu Ogawa (Juntendo University)
Miyuki Kurata (GiiB)
Yoshiji Yamada (GiiB)
Masayo Shamoto (GiiB, JST, NILS)
Harm-Jan W. Borgeld (GiiB, Institute of Applied Biochemistry)
Kunio Yagi (GiiB, Institute of Applied Biochemistry)

8. Collaborators

Yoshiharu Oshida (Nagoya University)
Yuzo Sato (Nagoya University)
Yasumichi Arai (Keio University)
Nobuyoshi Hirose (Keio University)
Nobutaka Hattori (Juntendo University)
Yoshikuni Mizuno (Juntendo University)
Shigeo Ohta (Nihon Medical School)
Takeshi Nomiyama (Juntendo University)
Hajime Matsunaga (Juntendo University)
Yasushi Tanaka (Juntendo University)
Ryuzo Kawamori (Juntendo University)
Wakako Maruyama (NILS)
Hiroshi Shimokata (NILS)
Jian-Sheng Gong (GiiB, NILS)
Jin Zhang (GiiB, Caltech)

9. Advisors

Yasuo Kagawa (Kagawa Nutrition University, Jichi Medical School)

Nobuo Tanaka (Tokyo Institute of Technology)

Kazuo Umetsu (Yamagata University)

Yuichi Goto (National Center of Neurology and Psychiatry)

Naruya Saito (National Institute of Genetics)

Table 1. Primer pairs for first PCR amplification of 6 fragments and for second PCR amplification of 60 segments of the mitochondrial genome

Fragment segment	First PCR			Second PCR			Fragment segment	First PCR			Second PCR		
	L1 Primer	H1 Primer	Length (bp)	FL2 Primer	H2 Primer	Length (bp)		L1 Primer	H1 Primer	Length (bp)	FL2 Primer	H2 Primer	Length (bp)
Fragment A	L77	H3538	3481				Fragment D	L8281	H11571	3310			
1				FL100	H742	680	31				FL8345	H9133	826
2				FL398	H1014	654	32				FL8635	H9235	638
3				FL700	H1696	1034	33				FL8913	H9483	608
4				FL995	H1591	634	34				FL9213	H10137	962
5				FL1254	H1828	612	35				FL9484	H10137	691
6				FL1485	H2060	613	36				FL9747	H10629	920
7				FL1779	H2385	644	37				FL10028	H10629	639
8				FL2045	H2669	662	38				FL10297	H10913	654
9				FL2332	H3082	788	39				FL10616	H11179	601
10				FL2616	H3538	960	40				FL10910	H11519	647
Fragment B	L2815	H6158	3358				Fragment E	L10796	H14378	3062			
11				FL2864	H3538	712	41				FL11183	H11791	646
12				FL3119	H3876	795	42				FL11467	H12000	571
13				FL3455	H4222	805	43				FL11766	H12451	723
14				FL3712	H4552	878	44				FL12057	H12818	799
15				FL4008	H4552	582	45				FL12357	H12921	602
16				FL4250	H4854	642	46				FL12600	H13492	930
17				FL4529	H5528	1037	47				FL12889	H13492	641
18				FL4827	H5528	739	48				FL13188	H13767	617
19				FL5110	H5759	687	49				FL13479	H14102	661
20				FL5362	H6009	685	50				FL13721	H14321	638
Fragment C	L5545	H9133	3571				Fragment F	L13901	H609	3297			
21				FL5602	H6158	594	51				FL13986	H14754	806
22				FL5890	H6454	602	52				FL14279	H14909	668
23				FL6186	H6757	609	53				FL14559	H15162	641
24				FL6452	H7005	591	54				FL14837	H15340	541
25				FL6716	H7613	935	55				FL15126	H15755	667
26				FL6957	H7613	694	56				FL15405	H16016	649
27				FL7245	H7758	551	57				FL15696	H81	992
28				FL7497	H8144	685	58				FL15948	H81	740
29				FL7762	H8395	671	59				FL16221	H342	728
30				FL8053	H9133	1118	60				FL16504	H581	684

Table 2. First PCR conditions differing by fragment

Fragment	A	B	C	D	E	F
Concentration of primers	0.5 μM	0.5 μM	0.5 μM	0.5 μM	0.5 μM	1.0 μM
Annealing temperature	58ºC	60ºC	54ºC	62ºC	56ºC	52ºC

Table 3. L primers for amplification of light-strand of mitochondrial DNA

Primer	Position		Sequence (5'-3')
	From	to	
L77	77	96	ACGCGATAGCATTGCGAGAC
L2815	2815	2834	GGGCGACCTCGGAGCAGAAC
L5545	5545	5564	ACAGCTAAGGACTGCAAAAC
L8281	8281	8300	CCCCCTCTAGAGCCCACTGT
L10796	10796	10815	CCACTGACATGACTTTCCAA
L13901	13901	13920	TCTCCAACATACTCGGATTC

Table 4. H primers for amplification of heavy-strand of mitochondrial DNA

Primer	Position		Sequence (5'-3')
	From	to	
H81	81	100	CAGCGTCTCGCAATGCTATC
H342	342	361	TTTTTGGGGTTTGGCAGAGA
H581	581	600	TTTGAGGAGGTAAGCTACAT
H609	609	628	GCCCGTCTAAACATTTTCAG
H742	742	761	TTGATGCTTGTTCCTTTTGA
H1014	1014	1033	AGCCACTTTCGTAGTCTATT
H1696	1696	1715	GGTTGTCTGGTAGTAAGGTG
H1591	1591	1610	TACACTCTGGTTCGTCCAAG
H1828	1828	1847	AGGTATAGGGGTTAGTCCTT
H2060	2060	2079	GGGGATTTAGAGGGTTCTGT
H2385	2385	2404	ACTTGTTGGTTGATTGTAGA
H2669	2669	2688	GGCAGGTCAATTTCACTGGT
H3082	3082	3101	TAGAAACCGACCTGGATTAC
H3538	3538	3557	AGAAGAGCGATGGTGAGAGC
H3876	3876	3895	GGGTTCGGTTGGTCTCTGCT
H4222	4222	4241	GAGATTGTAATGGGTATGGA
H4552	4552	4571	TTCTAGGCCTACTCAGGTAA
H4854	4854	4873	TTTTGTCATGTGAGAAGAAG
H5528	5528	5547	TTGAAGGCTCTTGGTCTGTA
H5759	5759	5778	TCAAACCTGCCGGGGCTTCT
H6009	6009	6028	CCCAGCTCGGCTCGAATAAG
H6158	6158	6177	TATCGGGGGCACCGATTATT
H6454	6454	6473	GATTAGGACGGATCAGACGA
H6757	6757	6776	ATGGTGTGCTCACACGATAA
H7005	7005	7024	ACAACGTAGTACGTGTCGTG
H7613	7613	7632	ATAGGGGAAGTAGCGTCTTG
H7758	7758	7777	GACGGTTTCTATTTCCTGAG
H8144	8144	8163	TAGTATACCCCCGGTCGTGT
H8395	8395	8414	GTATGGGGGTAATTATGGTG
H9133	9133	9152	ATTAAGGCGACAGCGATTTC
H9235	9235	9254	TCATGGGCTGGGTTTTACTA
H9483	9483	9502	CAGAAAAATCCTGCGAAGAA
H10137	10137	10156	TTTTCTATGTAGCCGTTGAG
H10629	10629	10648	GGCACAATATTGGCTAAGAG
H10913	10913	10932	GAGGAAAAGGTTGGGGAACA
H11179	11179	11198	TGCCTGCGTTCAGGCGTTCT
H11519	11519	11538	GGGTAGGCTATGTGTTTTGT
H11571	11571	11590	TAGGCAGATGGAGCTTGTTA
H11791	11791	11810	GAGTTTGAAGTCCTTGAGAG
H12000	12000	12019	GGTGAGTGAGCCCCATTGTG
H12451	12451	12470	ATAAAGGTGGATGCGACAAT
H12818	12818	12837	TGCTGTGTTGGCATCTGCTC
H12921	12921	12940	CTATTTGTTGTGGGTCTCAT
H13492	13492	13511	TTGGAGTAGAAACCTGTGAG
H13767	13767	13786	GGGGGATTGTTGTTTGGAAG
H14102	14102	14121	GAGTGGGAAGAAGAAAGAGA
H14321	14321	14340	GTGGTGGTTGTGGTAAACTT
H14378	14378	14397	TTAGTGGGGTTAGCGATGGA
H14754	14754	14773	GGGGTTAATTTTGCGTATTG
H14909	14909	14928	GTTGAGGCGTCTGGTGAGTA
H15162	15162	15181	TACTGTGGCCCCTCAGAATG
H15340	15340	15359	ATCCCGTTTCGTGCAAGAAT
H15755	15755	15774	ACTGGTTGTCCTCCGATTCA
H16016	16016	16035	CCCATGAAAGAACAGAGAAT

Table 5. FL primers for amplification of light-strand of mitochondrial DNA

Primer Name	Position		Sequence (5'-3')
	From	to	
FL100	100	119	TGTAAAACGACGGCCAGTGGAGCCGGAGCACCCTATGT
FL398	398	417	TGTAAAACGACGGCCAGTTTTTATCTTTTGGCGGTATG
FL700	700	719	TGTAAAACGACGGCCAGTAGCATCCCCGTTCCAGTGAG
FL995	995	1014	TGTAAAACGACGGCCAGTAAAACTCCAGTTGACACAAA
FL1254	1254	1273	TGTAAAACGACGGCCAGTCTATATACCGCCATCTTCAG
FL1485	1485	1504	TGTAAAACGACGGCCAGTGCCCGTCACCCTCCTCAAGT
FL1779	1779	1798	TGTAAAACGACGGCCAGTATAGTACCGCAAGGGAAAGA
FL2045	2045	2064	TGTAAAACGACGGCCAGTACTTTAAATTTGCCCACAGA
FL2332	2332	2351	TGTAAAACGACGGCCAGTCGCATAAGCCTGCGTCAGAT
FL2616	2616	2635	TGTAAAACGACGGCCAGTAATAGGGACCTGTATGAATG
FL2864	2864	2883	TGTAAAACGACGGCCAGTTCACCAGTCAAAGCGAACTA
FL3119	3119	3138	TGTAAAACGACGGCCAGTCCCTGTACGAAAGGACAAGA
FL3455	3455	3474	TGTAAAACGACGGCCAGTCTGACGCCATAAAACTCTTC
FL3712	3712	3731	TGTAAAACGACGGCCAGTGTAGCCCAAACAATCTCATA
FL4008	4008	4027	TGTAAAACGACGGCCAGTAAACACCCTCACCACTACAA
FL4250	4250	4269	TGTAAAACGACGGCCAGTCCCCTCAAACCTAAGAAATA
FL4529	4529	4548	TGTAAAACGACGGCCAGTAGCGCTAAGCTCGCACTGAT
FL4827	4827	4846	TGTAAAACGACGGCCAGTCAAGGCACCCCTCTGACATC
FL5110	5110	5129	TGTAAAACGACGGCCAGTCCGCATTCCTACTACTCAAC
FL5362	5362	5381	TGTAAAACGACGGCCAGTACTCCACCTCAATCACACTA
FL5602	5602	5621	TGTAAAACGACGGCCAGTCCCACTCTGCATCAACTGAA
FL5890	5890	5909	TGTAAAACGACGGCCAGTCCTCACCCCCACTGATGTTC
FL6186	6186	6205	TGTAAAACGACGGCCAGTCCCCGCATAAACAACATAAG
FL6452	6452	6471	TGTAAAACGACGGCCAGTCTTCGTCTGATCCGTCCTAA
FL6716	6716	6735	TGTAAAACGACGGCCAGTAGGTATGGTCTGAGCTATGA
FL6957	6957	6976	TGTAAAACGACGGCCAGTGGCCTGACTGGCATTGTATT
FL7245	7245	7264	TGTAAAACGACGGCCAGTACCACATGAAACATCCTATC
FL7497	7497	7516	TGTAAAACGACGGCCAGTGGCCTCCATGACTTTTTCAA
FL7762	7762	7781	TGTAAAACGACGGCCAGTGGAAATAGAAACCGTCTGAA
FL8053	8053	8072	TGTAAAACGACGGCCAGTACAAGACGTCTTGCACTCAT
FL8345	8345	8364	TGTAAAACGACGGCCAGTCCAACACCTCTTTACAGTGA
FL8635	8635	8654	TGTAAAACGACGGCCAGTCTCATCAACAACCGACTAAT
FL8913	8913	8932	TGTAAAACGACGGCCAGTACCACAAGGCACACCTACAC
FL9213	9213	9232	TGTAAAACGACGGCCAGTCACCAATCACATGCCTATCA
FL9484	9484	9503	TGTAAAACGACGGCCAGTTCTTCGCAGGATTTTTCTGA
FL9747	9747	9766	TGTAAAACGACGGCCAGTTACTTCGAGTCTCCCTTCAC
FL10028	10028	10047	TGTAAAACGACGGCCAGTAACTAGTTTTGACAACATTC
FL10297	10297	10316	TGTAAAACGACGGCCAGTAAACAACTAACCTGCCACTA
FL10616	10616	10635	TGTAAAACGACGGCCAGTCAACACCCACTCCCTCTTAG
FL10910	10910	10929	TGTAAAACGACGGCCAGTAGCTGTTCCCCAACCTTTTC
FL11183	11183	11202	TGTAAAACGACGGCCAGTCGCCTGAACGCAGGCACATA
FL11467	11467	11486	TGTAAAACGACGGCCAGTAAAACTAGGCGGCTATGGTA
FL11766	11766	11785	TGTAAAACGACGGCCAGTGCACTCACAGTCGCATCATA
FL12057	12057	12076	TGTAAAACGACGGCCAGTAAAACACCCTCATGTTCATA
FL12357	12357	12376	TGTAAAACGACGGCCAGTAACCACCCTAACCCTGACTT
FL12600	12600	12619	TGTAAAACGACGGCCAGTATTCATCCCTGTAGCATTGT
FL12889	12889	12908	TGTAAAACGACGGCCAGTGCCTTAGCATGATTTATCCT
FL13188	13188	13207	TGTAAAACGACGGCCAGTCACTCTGTTCGCAGCAGTCT
FL13479	13479	13498	TGTAAAACGACGGCCAGTAGGAATACCTTTCCTCACAG
FL13721	13721	13740	TGTAAAACGACGGCCAGTTATTCGCAGGATTTCTCATT
FL13986	13986	14005	TGTAAAACGACGGCCAGTACTCCTCCTAGACCTAACCT
FL14279	14279	14298	TGTAAAACGACGGCCAGTGACCCCTCTCCTTCATAAAT
FL14559	14559	14578	TGTAAAACGACGGCCAGTCGACCACACCGCTAACAATC
FL14837	14837	14856	TGTAAAACGACGGCCAGTTGAAACTTCGGCTCACTCCT
FL15126	15126	15145	TGTAAAACGACGGCCAGTCCTTCATAGGCTATGTCCTC
FL15405	15405	15424	TGTAAAACGACGGCCAGTTCCACCCTTACTACACAATC
FL15696	15696	15715	TGTAAAACGACGGCCAGTTTCGCCCACTAAGCCAATCA
FL15948	15948	15967	TGTAAAACGACGGCCAGTAGGACAAATCAGAGAAAAAG
FL16221	16221	16240	TGTAAAACGACGGCCAGTCCCTCAACTATCACACATCA
FL16504	16504	16523	TGTAAAACGACGGCCAGTGTTCCTACTTCAGGGTCATA

Table 6. H primers for sequence reaction or amplification of heavy-strand of mitochondrial DNA

Segment	Primer	Position		Sequence (5'-3')
		From	to	
02R	H807	807	826	TAATCACTGCTGTTTCCCGT
21R	H6158	6158	6177	TATCGGGGGCACCGATTATT
58R	H81	81	100	CAGCGTCTCGCAATGCTATC
60R	H528	528	547	TTCGGGGTATGGGGTTAGCA