For example, D5 is prevalent in southern areas. D4a is abundant in Chukchi of northeast Siberia, but D4a1 has its highest frequency in the Ryukyuans and clade D4n in the Ainu (Table 2).

Haplogroup M9

It is confirmed that haplogroup M9 is characterized by mutation 4491 (Fig. 1A), as recently proposed (Kong et al. 2003). Subhaplogroup M9a, as redefined by Kong et al. (2003), was identified by positions 153, 3394, 14308, 16234, and 16316 (Yao et al. 2002a). Nevertheless, not all lineages have 153. Although M9 could be RFLP-diagnosed by +1038 NlaIII and +3391 HaeIII polymorphisms, the latter one should be avoided; as 3391 is also present in some D4d1 lineages (Fig. 1B) and thus could produce misclassification. We have grouped lineages with 11963 as M9a1 and those with 153 as M9a2. M9 has a central and eastern Asian geographic distribution, and it reaches its greatest frequency (11%) and diversity (87%) in Tibet. In Japan, in addition to mainland Japanese it has been detected in the indigenous Ainu and Ryukyuans (Horai et al. 1996).

Haplogroup G

This haplogroup was first detected by Ballinger et al. (1992) and later named G by Torroni et al. (1994). It was defined by the presence of the combined RFLP polymorphism +4830 HaeII/ +4831 HhaI. In addition, the basal branch has mutations 709, 5108, and 14569 (Fig. 1; Kivisild et al. 2002). Subhaplogroup G1 was defined by transition 16017 (Schurr et al. 1999) and G2 by mutations 7600 and 16278 (Yao et al. 2002a). Recently, mutations 8200, 15323, and 15497 have been used for G1 status (Kong et al. 2003). This is confirmed with our Japanese sequences; consequently, we have defined G1a by 7867 (Fig. 1A). To avoid repetitions, the G1 group of Schurr et al. (1999) has been provisionally renamed as G5 (Table 2). At least two mutations (5601 and 13563) characterize G2; and five more, G2a (Fig. 1A; Kong et al. 2003). We have defined subclade G2a1 by the presence of 16189 and the derivative G2a1a by the addition of 16227, whereas 16051 and 16150 identify G2a2 lineages. Furthermore, two new subclades, G3 and G4, are also apparent in Japanese (Fig. 1A). Subgroup G5 is dominant in northeastern Siberia, but we have not detected it in our set of Japanese complete sequences. However, G1a1 has its highest frequencies in a cluster embracing Japanese, Ainu, Ryukyuan, and Koreans. On the contrary, G2 is relatively abundant in northern China and central Asia, reaching notable frequencies in the Mansi and in Tuvinians at the respective west and east ends of South Siberia (Table 2).

Haplogroup E

Haplogroup E was first RFLP-defined as having +16389 HinfI and −7598 HhaI by Ballinger et al. (1992), who named it G, and then later it was renamed E by Torroni et al. (1994). As a loss of restriction sites can be produced by different nucleotide mutations within the recognition sequence, since the beginning, some G2 sequences characterized by the 7600 transition were erroneously classified as belonging to haplogroup E. Recently, based on the complete sequences of coding regions, Herrnstadt et al. (2002) defined three Asiatic lineages as E, although only one (sequence 214) seems to be a genuine representative. It possesses transition 7598, which, similar to 7600, is also detectable with HhaI as a site loss; and it also harbors mutations 10834 and 869, which were found by Ballinger et al. (1992) as −10830 HinfI and +868 DdeI in all and some individuals respectively classified as E. However, the inclusion of a Philippine complete sequence (Ingman and Gyllensten 2003) in our global tree clearly demonstrates that the last two mutations might only define a branch of E, as the Philippine sequence lacks both of them. On the contrary, in addition to 7598 and 16390, some of the four E mutations represented in Figure 1A before the branching point might be basic mutations. In Herrnstadt et al. (2002), sequence 169 belongs to Haplogroup M9 because it has all coding-region positions defining this haplogroup; and sequence 287 to M1 because it has 6446 and 6680, the coding-region mutations that define the basic branch of M1 (Fig. 1). It must be mentioned that the ambiguous Korean lineage classified as E/G by Schurr et al. (1999), because it had both the −7598 HhaI characteristic E site and the +4830 HhaI characteristic G site, has been recently found again in a Korean sample (Snäll et al. 2002). All of them are, in fact, members of subhaplogroup G2. It seems that haplogroup E has a southern Asia distribution. Until now it has been detected in the Malay peninsula populations and in the Sabah of Borneo (Ballinger et al. 1992); and it is also present in coastal Papua New Guinea (Stoneking et al. 1990) as well as in some Pacific islands such as Guam (Herrnstadt et al. 2002) and the Philippines (Ingman and Gyllensten 2003). However, until now, it has not been detected in more northern Continental populations or islands such as the Japanese archipelago.

Haplogroup M8

A monophyletic clade (Fig. 1A) groups M8a, C, and Z lineages. Mutations 4715, 15487T, and 16298 have been proposed as diagnostic for this clade (Yao et al. 2002a). The transversion 7196A and the transition 8584 should also be included in its definition (Fig. 1A; Kivisild et al. 2002). However, as the 248d is also shared by all Z and C lineages (Fig. 1A), a basal node defined by this deletion and named CZ has been recently proposed (Kong et al. 2003). Subhaplogroup C was RFLP-defined by Torroni et al. (1992) by +13262 AluI. Yao et al. (2002a) added 248d, 14318, and 16327 as characteristic of C. In addition, positions 3552A, 9545, and 11914 are also diagnostic of this clade (Fig. 1A; Kivisild et al. 2002). The Japanese TC52 has the C1 status and the Buryat 6970 and the Evenky 6979 have the C4 status proposed by Kong et al. (2003). Subhaplogroup Z was defined by Schurr et al. (1999) by the presence of the following noncoding motifs: 16185, 16223, 16224, 16260, and 16298. Recently, it was considered that only 16185 and 16260 mutations should be counted as basic for the group (Yao et al. 2002a). However, in full agreement with the characterization proposed on the basis of complete Chinese Z sequences (Kong et al. 2003), three additional mutations (6752, 9090, and 15784) have been placed on the basal branch of Z (Fig. 1A). We detected four Japanese Z clades that, in addition, shared mutation 152 and another without it. Tentatively, they have been named from Z1 to Z5 (Fig. 1A). Yao et al. (2002a) defined M8a by 14470, 16184, and 16319 transitions. Two more mutations (6179 and 8684) are also characteristic of this subhaplogroup (Kong et al. 2003). In Japanese we have found that 16184 is not harbored by all M8a members. Consequently, lineages with this mutation have M8a2 status and those lacking it M8a1 status (Fig. 1A). The largest diversities for C are in Korea (100%), central Asia (86%), and northern China (78%–74%). Therefore, C can be considered a clade with a Northeast Asian radiation. Representatives of subhaplogroup Z extend from the Saami (Finnilä et al. 2001) and Russians (Malyarchuk and Derenko 2001) of west Eurasia to the people of the eastern peninsula of Kamchatka (Schurr et al. 1999). Its largest diversities are found in Koreans (88%), northern China (73%), and central Asia (67%), compatible with a central–East Asian origin of radiation for this group. Finally, M8a has its highest diversity in Koreans (100%), and southern (100%) and eastern Chinese, including Taiwanese (73%). Thus, southeastern China was a potential focus of radiation of this group. All these subhaplogroups are present in mainland Japanese but neither in Ryukyuans nor in Ainu.

Haplogroup M7

This haplogroup was defined by Bamshad et al. (2001) as having two branches, M7a characterized by 16209 and M7b by 16297

Table 2. Frequency (in Percentage) of Each Haplogroup in Each Group of Populations

Group	JPN	RYU	AIN	Ch1	Ch2	Ch3	Ch4	Ch5	Ch6	CA1	CA2	TWA	MAN	ITE	FIU	ALU	KAM	CHU	TUV	BUR	KOR	TIB	SAK	FIL	IND	SAB
Sample	1312	50	51	213	435	32	72	757	67	204	93	208	98	46	38	56	91	60	36	40	537	65	20	32	40	34
L2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5	–	–	–
M/N	1.3	18	1.96	3.29	1.61	–	4.17	5.55	–	1.96	1.08	2.4	1.02	–	2.63	–	–	–	–	25	5.96	9.23	–	6.25	10	17.6
I/W/N	–	–	–	–	–	–	–	–	–	2.45	–	–	–	–	2.63	–	–	–	–	–	0.37	–	–	–	–	–
A1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.67	–	–	0.19	–	–	–	–	–
A1a	2.13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.54	–	–	–	–	–
A1b	4.57	2	3.92	5.16	7.36	–	5.56	2.77	7.46	6.86	2.15	0.48	3.06	6.52	–	1.79	3.3	–	5.56	5	2.98	6.15	–	–	–	–
A1b1	0.15	–	–	0.47	0.46	–	1.39	0.92	–	–	–	–	–	–	–	–	–	–	–	–	0.74	–	–	–	–	–
A2a	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10	–	–	–	–	–	–	–	–
A2b	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.57	2.2	50	–	–	0.37	3.08	–	–	–	–
N9a	4.57	–	–	3.76	1.38	–	6.94	2.91	–	1.96	–	–	–	–	–	–	–	–	–	–	3.91	–	–	–	2.5	–
N9b	2.13	2	2	–	–	–	–	0.13	–	–	–	–	–	–	–	–	–	–	–	–	0.19	–	–	–	–	–
Y	0.38	–	21.6	3.29	1.38	–	–	0.53	–	0.49	–	0.96	–	4.35	–	7.14	9.89	–	–	–	2.23	–	–	3.13	2.5	2.94
HV	0.91	4	–	0.47	1.84	–	–	1.85	–	13.7	20.5	–	18.4	–	36.79	–	1.1	–	2.78	5	1.12	–	–	3.13	5	–
JT	–	–	–	2.82	1.15	–	–	–	–	5.4	8.61	–	19.4	–	23.65	–	–	–	–	–	–	–	–	–	–	–
UK	–	–	–	–	1.38	–	–	0.39	–	10.3	5.39	–	25.5	–	10.52	–	–	–	2.78	–	–	–	–	–	5	–
R9a	0.08	–	–	1.88	0.69	–	–	1.85	–	–	–	7.69	–	–	–	–	–	–	–	–	–	–	–	3.13	–	2.94
R11	–	–	–	0.94	0.46	12.5	–	1.85	–	0.49	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B	1.3	–	–	0.47	1.84	–	4.17	1.19	–	0.98	1.08	–	–	–	–	–	–	–	–	–	–	–	–	3.13	–	2.94
B4	0.76	2	–	1.88	2.53	9.38	1.39	1.98	5.97	1.96	1.08	–	–	–	–	–	–	–	–	2.5	0.74	1.54	–	–	–	–
B4a	0.84	–	–	2.35	1.61	–	1.39	4.36	–	0.49	1.08	14.9	–	–	–	–	–	–	2.78	–	0.56	–	–	–	–	–
B4a1	0.84	2	–	–	–	–	–	–	–	–	–	0.48	–	–	–	–	–	–	–	–	0.56	–	–	–	–	–
B4a2	–	–	–	0.47	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B4a3	–	–	–	0.47	–	15.6	2.78	1.85	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B4b	0.53	–	–	1.88	1.61	–	6.94	1.72	–	–	–	4.33	–	–	–	–	–	–	11.1	–	0.74	–	–	–	–	–
B4b1	2.13	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.56	–	–	–	–	–
B4c1	1.6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.74	–	–	–	–	–
B4c1b	0.61	–	–	1.88	–	–	–	1.06	–	–	–	4.81	–	–	–	–	–	–	–	–	0.56	–	–	–	–	–
B4c2	0.08	–	–	–	–	–	–	1.19	–	0.49	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B4f	0.3	4	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.38	–	–	–	–	–
B5	–	–	–	–	–	–	–	0.13	–	–	–	0.48	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B5a	–	–	–	1.88	0.69	–	1.39	6.74	5.97	0.98	1.08	6.25	–	–	–	–	–	–	–	–	–	–	–	3.13	–	11.8
B5a1	0.61	–	–	0.47	1.38	–	–	–	–	0.49	1.08	–	–	–	–	–	–	–	–	–	3.54	–	–	–	–	–
B5b	0.3	2	–	–	1.38	–	–	0.4	–	0.49	–	–	–	–	–	–	–	–	–	–	0.19	–	–	12.5	–	–
B5b1	0.99	–	–	–	–	–	–	0.13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B5b2	2.29	–	–	0.47	1.15	–	1.39	0.26	–	0.49	–	–	–	–	–	–	–	–	–	–	0.93	–	–	–	–	–
B5b3	0.08	–	–	–	–	–	–	0.13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F	0.23	–	–	0.94	2.3	–	–	2.25	1.49	1.96	2.15	1.92	–	–	–	–	–	–	–	–	0.37	1.54	–	–	–	2.94
F1a	0.15	–	–	0.47	1.61	15.6	4.17	4.62	1.49	–	1.08	–	–	–	–	–	–	–	–	–	0.74	–	–	12.5	35	2.94
F1a1	1.52	–	–	2.82	1.84	15.6	2.78	7.93	–	1.47	–	7.21	–	–	–	–	–	–	–	–	1.12	–	–	–	5	–
F1b	3.13	2	2	2.82	2.99	–	2.78	1.19	2.99	3.92	–	–	1.02	–	–	–	–	–	8.33	5	2.05	–	–	–	–	–
F1c	–	–	–	1.41	1.15	–	1.39	0.4	–	0.49	–	–	–	–	–	–	–	–	–	–	0.19	1.54	–	–	–	–

(continued)

Table 2. *Continued*

Group / Sample	JPN 1312	RYU 50	AIN 51	Ch1 213	Ch2 435	Ch3 32	Ch4 72	Ch5 757	Ch6 67	CA1 204	CA2 93	TWA 208	MAN 98	ITE 46	FIU 38	ALU 56	KAM 91	CHU 60	TUV 36	BUR 40	KOR 537	TIB 65	SAK 20	FIL 32	IND 40	SAB 34
F2	–	–	–	0.47	0.46	3.13	6.94	0.4	–	–	–	–	–	–	5.26	–	–	–	–	–	–	–	–	–	–	–
F2a	0.15	–	–	0.47	0.92	–	1.39	0.79	7.46	–	–	–	–	–	–	–	–	–	–	–	0.19	–	–	–	–	–
F2a1	0.08	–	–	–	–	–	–	0.66	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.5	–
F3	0.08	–	–	–	0.46	–	–	0.53	–	–	–	–	–	–	–	–	–	–	–	–	0.19	–	–	–	–	–
F4	–	–	–	0.47	–	–	–	0.92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.13	2.5	14.7
F4b	–	–	–	–	–	–	–	0.13	–	–	–	10.1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
M	0.38	2	3.92	–	–	–	–	0.26	–	0.49	–	–	–	–	–	–	–	–	–	–	0.19	–	–	–	–	–
M (PNG)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10	–
M5/D4a/G1	0.46	–	13.7	0.94	0.23	18.8	–	0.26	–	0.49	2.15	–	–	–	–	–	–	3.33	–	2.5	1.68	–	5	–	2.5	2.94
M7a	7.39	12	15.7	–	0.23	–	–	0.53	4.48	–	–	–	–	–	–	–	–	–	–	2.5	3.35	–	–	6.25	–	–
M7a1	0.08	14	–	1.41	1.38	–	1.39	2.25	–	0.98	–	0.48	–	–	–	–	–	–	–	–	–	–	–	3.13	–	–
M7b	–	–	–	0.47	0.46	–	1.39	2.11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
M7b1	0.08	–	–	2.35	2.99	–	–	5.02	–	0.49	–	12	–	–	–	–	–	–	–	–	0.56	–	–	–	2.5	–
M7b2	4.73	8	3.92	0.47	0.69	–	1.39	0.13	–	–	–	–	1.02	–	–	–	–	–	–	–	2.79	–	–	–	–	–
M7c	0.76	2	–	1.88	2.07	–	2.78	2.51	–	1.96	–	4.33	–	–	–	–	–	–	–	2.5	3.72	3.08	–	18.8	2.5	20.6
M8	0.15	–	–	0.47	–	–	–	0.13	–	–	–	–	–	–	–	–	–	1.67	–	–	0.37	–	–	–	–	–
M8a	1.22	–	–	4.23	0.92	–	4.17	1.59	–	1.47	–	–	–	–	–	–	–	–	–	2.5	1.68	–	–	–	–	–
M8a2	–	–	–	–	0.23	–	–	–	–	–	1.08	–	–	13	–	–	20.9	1.67	2.78	–	0.37	–	–	–	–	–
C	–	–	–	1.41	6.67	–	2.78	4.1	7.46	3.92	9.68	–	12.2	–	–	16.1	26.4	5	16.7	10	0.37	1.54	–	–	–	–
C1	0.3	–	–	–	0.46	–	–	–	–	–	1.08	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
C4a	0.08	–	–	0.47	0.92	–	–	–	–	0.98	1.08	–	4.08	–	–	–	–	–	13.9	–	0.37	–	–	–	–	–
C5	0.08	–	–	–	0.23	–	–	–	–	1.96	2.15	–	1.02	–	–	–	–	–	2.78	–	0.74	–	–	–	–	–
Z	1.3	–	–	2.35	2.76	–	4.17	0.53	10.4	0.49	9.68	–	–	6.52	–	–	8.79	–	–	–	1.49	9.23	–	–	–	–
G	0.53	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.37	–	–	–	–	–
G1a1/D	2.13	–	5.88	–	0.23	–	–	0.4	–	–	–	–	–	–	–	–	–	–	–	–	2.05	–	10	–	–	–
G2a	1.68	4	–	1.88	–	–	–	0.26	–	0.49	–	–	–	–	–	–	–	–	2.78	5	1.86	–	–	–	–	–
G2a1	2.52	–	3.92	5.16	0.92	–	1.39	0.66	–	7.35	1.08	–	6.12	–	–	–	–	–	5.56	–	1.49	–	–	–	–	–
G5	–	–	–	–	–	–	–	–	–	–	–	–	–	69.6	–	67.9	27.5	6.67	–	–	–	–	–	–	–	–
M9	–	–	–	–	–	–	2.78	1.59	2.99	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
M9a	2.44	4	1.96	5.63	1.61	–	1.39	1.98	–	2.45	1.08	–	–	–	–	–	–	–	–	5	3.17	10.8	–	–	–	–
M10	1.3	–	–	2.35	1.84	–	–	1.72	–	0.49	1.08	–	–	–	–	–	–	–	–	2.5	4.66	7.69	–	–	–	2.94
M12	0.08	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.19	6.15	–	–	–	–
D	0.08	–	–	0.47	0.46	–	1.39	0.26	–	–	–	–	2.04	–	–	–	–	–	–	–	0.19	–	–	–	–	–
D4	18.9	2	7.84	12.7	20.9	6.25	1.39	6.74	16.4	11.3	18.3	13.5	3.06	–	18.4	–	–	1.67	13.9	17.5	18.4	12.3	–	18.8	7.5	11.8
D4a	7.39	–	1.96	4.23	–	–	1.39	0.92	–	2.45	1.08	0.48	–	–	–	–	–	11.7	–	–	5.77	–	80	–	–	–
D4a1	0.53	4	–	0.94	1.38	–	2.78	1.06	–	–	–	–	–	–	–	–	–	–	–	–	2.42	1.54	–	–	–	–
D4b	2.36	6	–	1.41	1.61	–	1.39	0.92	–	0.98	3.23	–	1.02	–	–	3.57	–	6.67	2.78	2.5	0.93	18.5	–	–	–	–
D4d	2.67	–	–	0.94	0.69	3.13	–	–	–	1.96	1.08	–	–	–	–	–	–	–	2.78	2.5	0.93	–	–	–	–	–
D4k	0.15	–	–	–	1.15	–	1.39	0.13	22.4	–	–	–	–	–	–	–	–	–	–	–	0.19	3.08	–	–	–	–
D4n	0.61	–	3.92	–	0.23	–	1.39	–	2.99	–	–	–	–	–	–	–	–	–	–	–	0.19	–	–	–	2.5	–
D5	3.73	2	–	1.88	3.22	–	4.17	2.64	–	0.98	–	6.25	1.02	–	–	–	–	–	–	2.5	2.98	1.54	–	3.13	2.5	2.94
D5a	1.07	–	3.92	1.41	3.91	–	4.17	1.59	–	0.49	–	–	–	–	–	–	–	–	2.78	–	0.56	1.54	–	–	–	–

transitions. Yao et al. (2002a) assigned mutations 199 and 9824 as basic for M7. However, our phylogenetic tree points to 6455 and 9824 as the basal mutations for this group, whereas 199 is only common to the M7b and M7c subgroups (Fig. 1A), which coincides with the phylogeny proposed by Kivisild et al. (2002). M7 can be RFLP-diagnosed by the lack of the 6451 MboII restriction site. The M7a subgroup can be defined by several coding-region positions (Fig. 1A; Kivisild et al. 2002). The M7b classification remains as proposed in Kivisild et al. (2002); but M7c has, in addition to 146 and 16295, three more coding-region substitutions (4850, 5442, and 12091) in its basal branch (Fig. 1A). At this point, it is worthwhile pointing out that the ambiguously assigned sequence 536 in Herrnstadt et al. (2002) belongs to M7c, as it has the five identifying coding-region mutations distinctive of this subhaplogroup. As for the geographic distribution, M7a1 has its highest frequencies (14%) and diversities (86%) in the Ryukyuans, and it is also very common in the whole of China, with a mean diversity of ~76%. But, curiously, it has not been detected in Koreans or in Ainu, and is rare in mainland Japanese. In a similar way, M7a has its highest diversity in Ryukyuans (83%). Both groups are rather common in the Philippines. Although M7b has its greatest diversity in northern China (75%–62%), its derivative M7b2, has it again in Ryukyuans (100%), Koreans (53%), and mainland Japanese (45%). On the contrary, M7c is absent in Ainu and rare in mainland Japanese but very common in Sabah and the Philippines, although its highest diversity is in the whole of China (76% ± 11%).

Haplogroup M10

This haplogroup has been defined by substitutions 10646 and 16311 (Yao et al. 2002a). In addition, Kong et al. (2003) have found several new mutations in its basal branch that we confirm here (Fig. 1A). Minor modifications are that a new Japanese lineage shares with M10 only the 8793 mutation, and that a new mutation, 13152, seems to be basal for our M10 Japanese lineages. Although its highest frequency is in Tibetans (8%), the largest diversities are found in China. It is present in Koreans and mainland Japanese but has not been detected in either Ainu or Ryukyuans (Table 2).

Haplogroup M11

This haplogroup has been defined by Kong et al. (2003) by seven coding-region mutations (1095, 6531, 7642, 8108, 9950, 11969, and 13074) and four mutations in HVS-II (146, 215, 318, and 326). We confirm the same characterization for our M11 Japanese lineages. A subclade defined by mutation 14340 was found in Chinese (Kong et al. 2003), but it has not been detected in Japanese. In turn, Japanese have a new subclade characterized by mutation 14790. Finally, our data suggest that mutation 15924 is at the root of M11 and the new clade M12.

Haplogroup M12

This haplogroup has been defined in the present study. It harbors a characteristic motif (16145–16188–16189–16223–16381) in its noncoding region and several unique mutations in its coding region (Fig. 1A). Overall, it is a rare haplogroup, being detected only in mainland Japanese, Koreans, and Tibetans, the last-mentioned sample showing its highest frequency (8%) and diversity (50%).

Haplogroup M1

Although not present in eastern Asia, this haplogroup has been included in the phylogenetic tree of macrohaplogroup M to ascertain its hierarchical level with respect to other M clades. It was first detected in Ethiopia (Quintana-Murci et al. 1999) and defined by four transitions in the HVSI region (16129, 16189, 16249, and 16311). After this, M1 was also detected in the Mediterranean basin including Jordan (Maca-Meyer et al. 2001). Several mutations in the coding region are distinctive of this haplogroup (Fig. 1A). Its RFLP diagnosis is possible by an MnlI site loss at position 12401.

Subdivisions Within Macrohaplogroup N

Representatives of two major superhaplogroup N migratory branches are present in Japan. Two main clades, that directly sprout from the basal N trunk (A and N9), have a prevailing northern Asia dispersion, whereas the other two (B and F), having a southern radiation focus, belong to the derivative R clade, characterized by the loss of 16223 and 12705 mutations. Although not detected in Japan, to compare their hierarchical levels with those of the Asian branches, we have included the rCRS sequence and a N1b sequence (Kivisild et al. 1999) as representatives of the western Eurasian R and N clades, respectively.

Haplogroup A

This haplogroup was defined by an HaeIII site gain at 663 (Torroni et al. 1992). It was subdivided on the basis of HVSI motifs in A1 (16223–16290–16319) and A2 (16111–16223–16290–16319) by Forster et al. (1996). In our Japanese sample, we have detected several A1 representatives characterized by two substitutions (8563, 11536). Two of these lineages (ON67 and ND218) have been ascribed to the A1a subgroup that is defined by 4655, 11647, and 16187 substitutions. Two additional A1 Japanese clusters (A1b and A1c) have also been phylogenetically defined (Fig. 2). The A2 subgroup is represented in the tree by a Chukchi (6971) and two (KA21 and ON125) Japanese lineages, all sharing the 16362 mutation. As the Chukchi harbors the 16111 and 16265 mutations, it has been labeled as an A2a representative, as tentatively proposed by Saillard et al. (2000), having four additional mutations (152, 153, 8027, and 12007) in its basal branch. Owing to their phylogenetic position, three more Japanese lineages (ND28, TC48, and J42) should be classified as representatives of three new A subhaplogroups, respectively named A3, A4, and A5 (Fig. 2). Geographically, whereas A1 has a wide northern and central Asian distribution, subclade A1a is confined to Korea and mainland Japan. The greatest diversity for A1 is in central Asia (79%). In Japan it is present in both mainland and indigenous populations. Subhaplogroup A2 is mainly present in northeast Siberia including the Kamchatka peninsula, although a lineage has also been detected in Tibet. The main diversity (30%) and frequency (60%) for this subhaplogroup are in the Chukchi.

Subhaplogroups Y, N9a, and N9b

Haplogroup N9 characterized by the 5417 substitution (Yao et al. 2002a) phylogenetically comprises three subhaplogroups. Subhaplogroup N9a was mentioned as another N subcluster with a distinctive HVSI motif (16223, 16257A, 16261) by Richards et al. (2000). It appears named as N9a in Yao et al. (2002a), who added as basal substitutions 150 and 5231. Recently, Kong et al. (2003) added mutations 12358 and 12372 at the basal branch of N9a, which is according to our Japanese phylogeny (Fig. 2). A Japanese N9a1 lineage (TC2) shares mutations 4386, 12007, 16111, and 16129 with the Chinese lineage GD7834 of Kong et al. (2003). Three more N9a Japanese clusters sharing 16172 as their basal mutation have been considered distinct N9a2 branches (Fig. 2). Subhaplogroup Y was first identified by a set of HVSI polymorphisms (16126, 16189, 16231, 16266, 16519), an HaeIII site loss at 8391 and MboI and DdeI site gains at 7933 and 10394, respectively (Schurr et al. 1999). However, according to the classification of Kong et al. (2003), all these mutations define the Y1a1 branch specifically. Our Japanese (Fig. 2) and the Chinese (Kong et al. 2003) phylogenies characterize Y by seven mutations (8392, 10398, 14178, 14693, 16126, and 16231 gains and a 16223 loss).

The branch Y1 would be identified by mutations 3834 and 16266, and the Y1a subcluster by 7933 (Fig. 2; Kong et al. 2003). In Japan we have found a new subclade (Y1b) characterized by four mutations (146, 10097, 15221, 15460). Furthermore, a new branch (Y2) with the same phylogenetic consideration as Y1, and distinguished by six basal mutations must be aggregated to the Y phylogeny (Fig. 2). Finally, we have detected a sister branch of Y in Japan. This new lineage, named N9b, shares two basal mutations (5147 and 16519) with Y and is further characterized by four (10607, 11016, 13183, 14893) additional mutations in its basal branch. All N9b1 representatives seem to have the 16189 mutation, and three branches of this trunk (a, b, and c) have been provisionally defined (Fig. 2). The geographic distribution of subhaplogroup Y is predominantly in Northeast Asia. The highest frequency (22%) is in the Ainu, although only one lineage accounts for this frequency. The greatest diversities are in northern China (80%), and this group is also very diverse in the Nivkhs from northeast Siberia (Torroni et al. 1993a). As for N9a, it has a great diversity in the whole of China (83%) and Korea (79%). In Japan, only mainland Japanese have N9a representatives. Finally, N9b is very scarce, being detected in southern China and Korea. Surprisingly, it is most abundant in the Japanese including the indigenous Ryukyans and Ainu.

Haplogroup F

This haplogroup was first defined as group A by Ballinger et al. (1992), and later renamed as F by Torroni et al. (1994). This group was characterized by the lack of HincII and HpaI sites at 12406. According to the newly proposed nomenclature (Kivisild et al. 2002; Kong et al. 2003), 12406 is now one of the six mutations that specifically define subhaplogroup F1. Recently, haplogroup F has been phylogenetically included as a subcluster of haplogroup R9 (Yao et al. 2002a). Besides F1, two new subgroups (F2 and F3) have been defined by Kong et al. (2003). We have found a new subcluster, named F4 (Fig. 2), that is characterized by three coding-region mutations (5263, 12630, 15670). This group has a particularly high incidence in Southeast Asia (Ballinger et al. 1992), but only subhaplogroup F1b is well represented in the Japanese, including the indigenous Ainu and Ryukyuan. The highest diversities for this subgroup are in eastern China including Taiwan (100%).

Haplogroup B

Renamed as B after Torroni et al. (1992), this haplogroup was identified by the presence of a 9-bp deletion in the COII/tRNA^{Lys} intergenic region of mtDNA. This polymorphism was first detected in Asia by RFLP analysis (Cann and Wilson 1983). It was used to classify Japanese on the basis of the presence/absence of this deletion (Horai and Matsunaga 1986). Even in Asia, the monophyletic status of this cluster has been repeatedly questioned (Ballinger et al. 1992; Yao et al. 2000b); but although the 9-bp deletion has a high recurrence, it seems that together with transition 16189 it defines fairly well a monophyletic cluster, at least in eastern Asia. Recently, a sister clade of B, keeping the 16189 mutation but lacking the 9-bp deletion, has been detected in China, being designated as R11 (Kong et al. 2003). Asian subhaplogroups of B have been named as B4, identified by the 16217 mutation and B5, characterized by 10398 and 16140 mutations (Yao et al. 2002a). It has been deduced from analysis of complete sequences that transitions 709, 8584, and 9950 are also in the basal branch defining B5 (Fig. 2; Kong et al. 2003). Lower-level subdivisions have also been proposed. Three subclades (B4a, B4b, and B4c) were defined within B4 (Kong et al. 2003). At the same phylogenetic level are our Japanese branches named B4d, B4e, and B4f; and several new secondary clusters have also been detected in Japan within B4a, B4b, and B4c (Fig. 2). It is worthwhile to mention that those lineages harboring 16189, 16217, 16247, and 16261, also known as the Polynesian motif (Soodyall et al. 1995), belong to a branch of B4a, having in addition to 16247, 146, 6719, 12239, 14022, and 15746 as basic mutations. The B5 cluster was also subdivided in B5a and B5b on the basis of the HVSI mutations 16266A and 16243, respectively (Yao et al. 2002a), and reinforced with several additional positions after the analysis of complete Chinese (Kong et al. 2003) and Japanese (Fig. 2) sequences. Within B5b, new subdivisions are necessary to accurately classify the Japanese sequences (Fig. 2). Finally, on the basis of characteristic HVSI motifs, we had tentatively defined as B4a3 those lineages with 16189, 16217, 16261, and 16292 transitions. However, the phylogenetic position of a Chinese complete sequence (GD7812) belonging to this HVSI group (Kong et al. 2003) shows that a future redefinition of B4a might be necessary. The geographic distribution of haplogroup B is very complex. As expected from its age, the ancestral motif is widely distributed in Asia excluding Koryacks and other Siberians. The likewise old subhaplogroup B4 has mainly a central–eastern Asian distribution with diversities near 100% from central Asia to Japan. B4a shows a similar distribution as B4, having branches prevalent in Ryukyuans, Lahu of Yunnan, and aborigine Taiwanese (Table 2). In a similar vein, some branches of B4c are more abundant in southern areas (B4c2), whereas others (B4c1) are mainly detected in Korea and Japan, with derivatives in Taiwan (B4c1b). On the other hand, subhaplogroup B5a has its greatest diversity in southern–eastern China (89%), including Taiwan aborigines (67%), but its B5a1 derivative shows the greatest diversity in northern China (71%), being present in mainland Japanese. In turn, subhaplogroup B5b has its major diversity in Korea (83%) and also reached the Philippines (50%). Curiously, the B5b1 derivative shows its highest diversity (67%) and frequency (1%) in mainland Japanese.

Lineage Sorting and Population Pooling

A total of 110 clades with different phylogenetic range have been proposed on the basis of the pool of the eastern Asian complete sequences (Figs. 1A,B and 2). Of these subdivisions (Table 2), 83 have been used to classify all Asian partial sequences analyzed in this study. As a test of accuracy in the sorting of partial sequences into haplogroups, we classified our 672 Japanese complete sequences by using only their HVSI motifs and found that 34 of them (5%) had an ambiguous status or were misclassified. The main sources of errors were those sequences that differed from CRS in only one or two mutations. For instance, the 16223 mutation was found in M and N backgrounds. The 16189, 16223 motif can be D6 or N9b. Within M, sorting into D or G was one of the main sources of ambiguity. Some 16223, 16325, 16362 lineages were D4 and some G1. The motif 16114A, 16223, 16362, classified as D4, was in reality G3. Sometimes further subdivision within a haplogroup is rather difficult; for example, there are 16189, 16223, 16362 representatives in D4 and in D5. Because of recurrency and isolation, it can be expected that this uncertainty level increases with geographic distance. For instance, we have found that several 16129, 16223 Japanese lineages belong to D4, but to infer from this that southern Asian sequences with the same HVSI motif are also D4 would be inappropriate. From a total of 4713 sequences analyzed, 9.2% had an ambiguous status. In spite of this percentage there are enough sequences left to carry out population analysis with statistical confidence.

In a first approach, Japanese, Ainu, and Ryukyuan samples were compared with the rest of Asian samples shown in Table 3 by means of F_{ST}. The closest affinities of mainland Japanese were to three population groups. The first include Korean and Han from Shandong (mean *P*-value = 0.29 ± 0.06), the second Han from Liaoning and Xinjiang, and the Tu ethnic minority

(0.20 ± 0.06), and the third Han from Xi'an and the Sali, a branch of the Yi ethnic group (0.15 ± 0.06). Ryukyuans and Ainu behave as outliers with significant differences with all the samples. Population groups resulting from the F_{ST} and CLUSTER analysis are defined in Table 3. Although mainland Japanese from Aichi were significantly different from other mainland Japanese because of their high frequency of haplogroup B, they were merged with them as JPN for comparisons with other areas. Control of the conglomerate number expected in CLUSTER analysis allows for a hierarchical grouping of populations. With two conglomerates, the first distinguished isolate was the aboriginal Sakai from Thailand (Fucharoen et al. 2001). This group was unique among other Thai people owing to its lack of lineages with the 9-bp deletion that characterizes haplogroup B, and to the high frequency of the authors' C6 cluster (included in our D4a). The lack of any representative of macrohaplogroup N in a population anthropologically considered one of the oldest groups in Thailand, if not caused by genetic drift, is compatible with the hypothesis that derivatives of macrohaplogroup N had, in southern Asia, a different route from macrohaplogroup M (Maca-Meyer et al. 2001). Also striking is the presence in Sakai of an unequivocal representative (16223–16274–16278–16294–16309) of the sub-Saharan African L2a haplogroup (Torroni et al. 2001), which again is compatible with the physical characteristics of this Negrito group. Although the suggestion that the first spreading out of Africa of modern humans could have carried some L2 lineages in addition to the L3 ancestors (Watson et al. 1997) is a tempting explanation, a recent admixture is more in consonance with the phylogenetic proximity of this lineage to the present African ones. The next outsiders were the majority of the Siberian isolates, which could not be pooled because of big differences in the frequency of distinctive haplogroups (Table 2). This considerable differentiation was already emphasized (Schurr et al. 1999), with strong genetic drift being its most probable cause. Subsequent isolates belong to some Chinese minorities such as those of Lisu and Nu, Lahu, and Taiwanese aborigines. Unexpectedly, other Chinese minorities (Bai, Sali, and Tu) were left in Han Chinese northern clusters. The Bai belong to the Sino-Tibetan Tibeto-Burman ethnic linguistic group and have been strongly influenced by Han. The Sali are a minority within the Yi ethnic group whose most probable ancestors were the Qiang from northwest China. Finally, the Tu, although belonging to the Mongolian branch of the Altaic Family, show their main genetic affinities to the Han from Xi'an ($P = 0.95$), Xinjiang ($P = 0.89$), and Shanghai ($P = 0.79$), all of them clustered in the Ch2 group. On the other hand, Thais, Vietnamese, and Cambodians joined with southern Chinese. As already observed (Chunjie et al. 2000; Yao et al. 2002a), the Han Chinese do not comprise a homogeneous group. With the exception of cluster Ch4, that includes samples from Hubei and Guandong (Table 3), they appear geographically differentiated. The two central Asian groups detected mainly differ in their frequencies for A1b, Z, and G2a. With less than 14 conglomerates, the Japanese, including Ainu and Ryukyuans, were part of a big group formed by Korean, Buryat, Tibetans, and northern Chinese. Ainu was the first differentiated Japanese sample. Ryukyuans separated later, when mainland Japanese and Koreans still comprised a single group. The lack of homogeneity between Ainu and Ryukyuans was pointed out by Horai et al. (1996), who questioned that they shared a recent common ancestor. The main differences between them were attributed to two dominant clusters (C1 and C16, corresponding to our Y and M5/D4a/G1, respectively) present in Ainu but absent in Ryukuyans, and two Ryukyuan dominant clusters (C3 and C13, belonging to our R and M, respectively) absent in Ainu. In addition, applying the present haplogroup nomenclature to the same data, the high frequency of M7a1 and D4a1/D4b in Ryukyuans, but their absence in Ainu, stands out. The MDS plot (Fig. 3A), based on F_{ST} haplogroup frequency distances between final groups (data not shown), only partially reflects the sequential process described above, as only Sakai and Siberians are well differentiated from the rest. On the contrary, relationships obtained from haplotype matches (Fig. 3B) show populations highly structured by geography with the only exceptions being the Ainu and Tuvinian isolates.

The Peopling of Japan

To further know the relative affinities of the Japanese between themselves and with the different Asian groups formed, the data obtained from the global approaches based on haplogroup frequency distances and on sequence match identities are presented in Table 4. Both values are moderately correlated in the comparisons involving the mainland Japanese ($r = -0.479$; two-tail probability 0.012) but not at all in those involving aborigine Ryukyuans ($r = -0.310$; two-tail probability 0.115) and Ainu ($r = 0.087$; two-tail probability 0.667). This result can be explained by assuming that these aboriginal people have suffered important genetic drift effects with substantial changes in haplogroup frequencies and lineage losses or, less probably, that these populations have been isolated long enough to have accumulated new variation. Results based on haplogroup frequencies by far relate mainland Japanese to Koreans followed by northern Chinese. Ryukyuans present the smallest distances to Buryats from South Siberia, followed in short by southern Chinese. In turn, the Ainu have their closest affinities with mainland Japanese, Koreans, and northern Chinese. As regards sequence matches, mainland Japanese also joins first to Koreans and second to Buryats. Aborigine Ryukyuans are closest to Buryats and then to Koreans. Finally, Ainu show comparatively less shared sequences, their greater affinities being toward Chukchi and Koryaks of Kamchatka. This global picture is congruent with an important influence on mainland Japanese from northern Asian populations through Korea, that the Ryukyuans had a dual northern and southern Asian background previous to the new northern influences acquired by admixture with mainland Japanese, and that the Ainu represent the most isolated group in Japan in spite of the genetic input received from Kamchatka. Also noticeable is the great distance and low identity values obtained for the Ainu–Ryukyuan pair compared with those obtained in their respective comparison to mainland Japanese, which is another hint of its notable maternal isolation.

The distance and identity statistics used above are based on frequencies of haplogroups and haplotypes, respectively; however, frequencies are more affected by genetic drift than the number of different haplotypes present in a population. To measure the relative affinities of Japanese populations between them and to Continental Asia in a frequency-independent way, we chose a haplotype-sharing approach calculating the relative contribution of lineages shared with other areas to the number of different haplotypes present in each Japanese population. In these comparisons all other Asians were merged. Table 5 shows the results of this analysis. Note that despite the difference in sample size the haplotype frequency in mainland Japanese and Ainu is ~50%, whereas in Ryukyuans it is 84%; which means that, if there was not a bias in the sampling process, in spite of its small size, the Ainu sample seems to be representative of that population. However, it would be desirable to enlarge that of the Ryukyuans (Helgason et al. 2000). Haplotypes present only in a given population account for 13% in Ainu but ~50% in mainland Japanese (60%) and Ryukyuans (45%). This finding once more points to the existence of important drift effects in Ainu. Mainland Japanese exclusively share with Ryukyuans and Ainu only 3% and 2%, respectively, of its lineages, which could reach 6% and 3% if those

Table 3. Asian Populations Used in This Study

Population	Locality	Ethnic group	Group	Sample	HVRI	HVRII	Other[a]	References
Japan	Tokyo	Japanese	JPN	373	16024–16569	1–648	649–16023	This work
Japan	Nagoya	Japanese	JPN	299	16024–16569	1–648	649–16023	This work
	Japan	Japanese	JPN	20	1600–16413	—		Bamshad et al. 2001
				19	—	71–270		Jorde et al. 1995
Japan	Tokyo	Japanese	JPN	162	16051–16365	73–340		Imaizumi et al. 2002
Japan	Tokyo	Japanese	JPN	150	16030–16481			Nishimake et al. 1999
Japan	Tokyo	Japanese	JPN	13	16024–16569	1–648	RFLPs	Abe et al. 1998
Japan	Miyazaki	Japanese	JPN	100	15998–16400	30–407		Seo et al. 1998
Japan	Tottori	Japanese	JPN	89	16026–16396			Oota et al. 2002
Japan	Shizuoka	Japanese	JPN	62	16129–16569	1–41		Horai et al. 1996
Japan	Aichi	Japanese	JPN	.50	16040–16375	20–430		Koyama et al. 2002
Japan	Okinawa	Ryukyuan	RYU	50	16129–16569	1–41		Horai et al. 1996
Japan	Hokkaido	Ainu	AIN	51	16129–16569	1–41		Horai et al. 1996
Korea		Korean	KOR	306	16020–16400	1–70		Lee et al. 1997
Korea		Korean	KOR	4	16024–16370			Torroni et al. 1993a,b
Korea		Korean	KOR	60	16024–16365	73–340		Pfeiffer et al. 1998
Korea		Korean	KOR	2	16000–16413	—		Bamshad et al. 2001
Korea					—	71–270		Jorde et al. 1995
Korea		Korean	KOR	64	16129–16569	1–41		Horai et al. 1996
Korea		Korean	KOR	3	16128–16408			Horai and Hayasaka 1990
Korea		Korean	KOR	98	16075–16362	73–315	14747–15887	Lee et al. 2002
China	Liaoning	Han	Ch1	51	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Shandong	Han	Ch1	50	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Yunnan	Bai	Ch1	31	16001–16495			Yao et al. 2002b
China	Changsha	Han	Ch1	82	16026–16396			Oota et al. 2002
China	Xinjiang	Han	Ch2	47	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Yunnan	Sali	Ch2	31	16001–16495			Yao et al. 2002b
China	Qinghai	Tu	Ch2	35	16001–16495			Yao et al. 2002b
China	Xi'an	Han	Ch2	84	16026–16396			Oota et al. 2002
China	Shanghai	Han	Ch2	120	13030–16481			Nishimake et al. 1999
Mongolia		Mongolian	Ch2	103	16020–16400		RFLPs	Kolman et al. 1996
Mongolia		Mongolian	Ch2	15	16001–16495			Yao et al. 2002b
China	Yunnan	Lahu	Ch3	32	16048–16569	1–49		Qian et al. 2001
China	Hubei	Han	Ch4	42	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Guangdong	Han	Ch4	30	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Yunnan	Han	Ch5	43	16001–16497	30–47	10171–10659 and RFLPs	Yao et al. 2002a
China	Taiwan		Ch5	6	16024–16370			Torroni et al. 1993a,b
China	Taiwan		Ch5	3	15999–16413			Bamshad et al. 2001
China	Taiwan		Ch5	9	16065–16375			Sykes et al. 1995
China	Taiwan		Ch5	66	16129–16569	1–41		Horai et al. 1996
China	Taiwan	Han	Ch5	155	15997–16569	1–407		Tsai et al. 2001
China	Yunnan	Dai	Ch5	21	16048–16569	1–49		Qian et al. 2001
China	Yunnan	Wa	Ch5	22	16048–16569	1–49		Qian et al. 2001
China	Yunnan	Dai	Ch5	38	16001–16495			Yao et al. 2002b
China	Guangxi	Zhuang	Ch5	83	16001–16495			Yao et al. 2002b
China	South China	Han	Ch5	28	16024–16399			Betty et al. 1996
Thailand			Ch5	32	16001–16495			Yao et al. 2002b
Thailand		See ref.	Ch5	121	16048–16569	1–41		Fucharoen et al. 2001
Thailand	See ref.	Native	Ch5	74	16048–16569	1–41		Fucharoen et al. 2001
Vietnam			Ch5	35	16026–16396			Oota et al. 2002
Vietnam			Ch5	9	15999–16413	—		Bamshad et al. 2001
					—	71–270		Jorde et al. 1995
Cambodia			Ch5	12	15999–16413	—		Bamshad et al. 2001
					—	71–270		Jorde et al. 1995
China	Yunnan	Lisu	Ch6	37	16001–16495			Yao et al. 2002b
China	Yunnan	Nu	Ch6	30	16001–16495			Yao et al. 2002b
China	Taiwan	Native	TWA	28	15997–16400	30–407		Melton et al. 1998
China	Taiwan	Native	TWA	180	16048–16569	1–41		Tajima et al. 2003
Central Asia		Uygur	CA1	46	16001–16495			Yao et al. 2000a
Kazagstan		Kazakh	CA1	55	15997–16400			Comas et al. 1998
Kirgizistan	Talas	Kirghiz	CA1	48	15997–16400			Comas et al. 1998
Kazagstan		Uygur	CA1	55	15997–16400			Comas et al. 1998
Central Asia		Kazak	CA2	30	16001–16495			Yao et al. 2000a
Kirgizistan	Sary-Tash	Kirghiz	CA2	46	15997–16400			Comas et al. 1998
Siberia	See ref.	Altai	CA2	17	16024–16383			Shields et al. 1993
Tibet		Tibetan	TIB	1	16024–16370			Torroni et al. 1993a,b
Tibet		Tibetan	TIB	40	16001–16495			Yao et al. 2000b
Tibet		Tibetan	TIB	24	16048–16569	1–41		Qian et al. 2001
Russia	East Ural	Mansi	MAN	98	16039–16519	64–295	RFLPs	Derbeneva et al. 2002a

(continued)

Table 3. *Continued*

Population	Locality	Ethnic group	Group	Sample	HVRI	HVRII	Other[a]	References
Siberia		Finno-Ugrian	FIU	38	13021–16505			Voevoda Accession nos. AF214068–AF214105
South Siberia		Tuvinian	TUV	36	16000–16400		RFLPs	Derenko et al. 2000
South Siberia		Buryat	BUR	40	16000–16400		RFLPs	Derenko et al. 2000
Siberia		Chukchi	CHU	60	16001–16405			Voevoda et al. 1994
Siberia	Aluitor	Koryak	ALU	56	16000–16525			Schurr et al. 1999
Siberia	Karagin	Koryak	KAM	37	16000–16525			Schurr et al. 1999
Siberia	Palan	Koryak	KAM	54	16000–16525			Schurr et al. 1999
Siberia	Kovran	Itel men	ITE	46	16000–16525			Schurr et al. 1999
Philippine			FIL	32	16065–16375			Sykes et al. 1995
Thailand	Trang	Sakai	SAK	20	16048–16569	1–41		Fucharoen et al. 2001
Malaysia			IND	6	15999–16413	—		Bamshad et al. 2001
					—	71–270		Jorde et al. 1995
Indonesia			IND	34	16024–16400	31–407		Redd and Stoneking 1999
Borneo		Sabah	SAB	34	16065–16375			Sykes et al. 1995

[a]RFLPs and additional sequences.

also shared with Continental Asian populations are added. In comparison they shared 21% of its lineages with other Asians. On the contrary, Ryukyuans and Ainu share about 50% of their lineages with mainland Japanese and only 10% and 21%, respectively, with Continental populations, which may reflect other independent Asian influences on Japan. With respect to those lineages exclusively shared by Japanese and Continental Asian populations, it is worth mentioning that, again, Korea is the main contributor, participating in ~50% of the haplotype sharing with mainland Japanese (55%), as much as with Ryukyuans (50%) and Ainu (50%). However, differences exist in the provenance of the rest of the shared lineages. Whereas in Ainu (northern China and Siberia) and in Ryukyuans (northern China and central Asia) they are from northern areas, the second region contributing to mainland Japanese is southern China (17.5%), followed, at the same level (12.5%), by northern China and central Asia. In addition, there exists a minor percentage of exclusive sharing with Indonesia (2.5%). On the other hand, all the matches with Siberia and Tibet are also shared with other populations. From these results, it can be deduced that the ancient Japanese inhabitants came from northern Asia and that southern areas affected the Japanese by later immigration. Nevertheless, it must be borne in mind that older influences could be undetectable by lineage sharing. With respect to the haplogroup affiliation of those lineages that Ainu and Ryukyuans exclusively shared with no Japanese samples, new differences appear between them. Ainu share derived lineages of haplogroups A, G, M9, and D5, all of them compatible with a rather recent Siberian influence. In contrast, those shared by Ryukyuans are basical M lineages, more congruent with an older radiation from southern China. These dual influences are also detected when the haplogroup affiliation of the Ainu and Ryukyuan unique lineages is studied. First, the percentage of lineages belonging to macrohaplogroup N is larger in Ainu (50%) than in Ryukyuans (15%) and from a different provenance, as those in Ainu are from haplogroups N, N9b, and Y, whereas those of Ryukyuans belong to the southern haplogroups F and B. The remaining 50% of the Ainu lineages equitably belong to different M haplogroups (M, M7c, G1, and D5a), but in Ryukyuans the remainder are mainly concentrated in M7a (41%) and M7b2 (18%), two groups that have their greatest Asian diversities precisely in Ryukyuans. Although an indigenous focus of radiation cannot be discarded, it is more conservative to suppose that the most probable origin of these lineages is again southern China. Thus, Ainu and Ryukyuans are not only largely isolated populations, but they most probably had different maternal origins.

Although no matches are involved, the geographic distribution of haplogroup frequency and diversities for some groups present in Japan and in other distinct Asian areas are also relevant to trace these older connections. For instance, haplogroups M9, M10, M12, D4b, and F1c have correlated geographic frequencies with a peak in an area that comprises Tibet (Table 2). Curiously, one of these haplogroups (M12) is today absent in China but present in Korea and Japan.

DISCUSSION

Although the recent out-of-Africa origin for all modern humans (Cann et al. 1987) is being widely supported (Takahata et al.

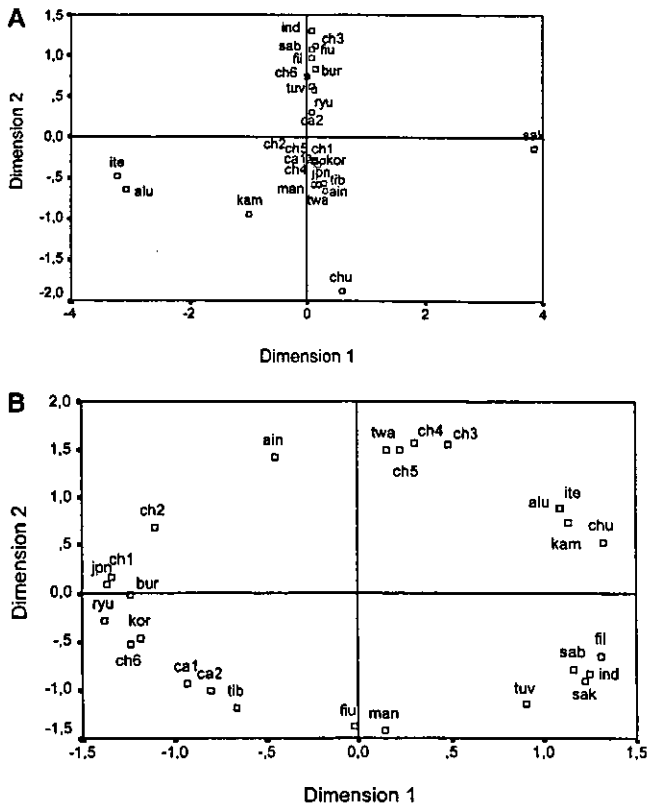

Figure 3 MDS plots based on (*A*) F_{ST} and (*B*) D match distances. Population groups are as detailed in Table 3.

Table 4. Frequency-Based F_{ST} and Sequence Match Identities (in Percentage) Between Japanese Samples and With Other Asian Populations

	JPN		RYU		AIN	
	F_{ST}	Matches	F_{ST}	Matches	F_{ST}	Matches
RYU	0.04	0.41				
AIN	0.04	0.33	0.05	0.04		
KOR	0.00	1.10	0.04	0.57	0.04	0.25
CH1	0.01	0.59	0.04	0.11	0.04	0.18
CH2	0.01	0.51	0.05	0.19	0.05	0.21
CH3	0.07	0.01	0.10	0.00	0.08	0.00
CH4	0.03	0.06	0.03	0.00	0.05	0.03
CH5	0.03	0.16	0.03	0.09	0.05	0.08
CH6	0.04	0.01	0.08	0.00	0.08	0.09
TWA	0.04	0.23	0.07	0.08	0.08	0.04
TIB	0.04	0.36	0.04	0.18	0.08	0.06
CA1	0.02	0.58	0.04	0.25	0.05	0.16
CA2	0.04	0.73	0.07	0.20	0.08	0.19
ITE	0.29	0.00	0.39	0.00	0.40	0.26
FIU	0.06	0.50	0.08	0.32	0.10	0.10
MAN	0.06	0.24	0.06	0.24	0.08	0.04
ALU	0.29	0.01	0.39	0.00	0.39	0.46
KAM	0.14	0.01	0.16	0.00	0.15	0.45
CHU	0.17	0.01	0.21	0.00	0.22	0.00
TUV	0.03	0.09	0.07	0.17	0.07	0.05
BUR	0.03	0.97	0.02	2.75	0.07	0.15
FIL	0.03	0.11	0.05	0.13	0.06	0.00
IND	0.09	0.04	0.09	0.00	0.11	0.00
SAK	0.29	0.00	0.44	0.00	0.43	0.00
SAB	0.06	0.09	0.05	0.29	0.08	0.12

2001), the most probable time and routes chosen by these earliest migrants to reach eastern Asia is an open issue. In the following discussion we weigh the different alternatives proposed in light of the phylogenetic tree obtained from complete mtDNA sequences. One of the first questions raised was whether there was more than one out-of-Africa dispersion. All the mtDNA lineages detected in Old World populations belong to one of two M and N macrohaplogroups with only secondary representatives in Africa. The proposed radiation ages for both, 30,000 to 58,000 years ago and 43,000 to 53,000 years ago, respectively (Maca-Meyer et al. 2001), give a temporal frame compatible with only one main dispersion or two successive dispersions, in which case the M precursor is the most probable candidate for the older exit. Even if the one dispersion option is chosen, more than one geographical route to eastern Asia is possible. In fact, a northern Continental route through the Near East and western-central Asia and a southern coastal route through the Arabian and Indian peninsulas have been proposed (Cavalli-Sforza et al. 1994; Kivisild et al. 1999). The geographical distribution of these two macrohaplogroups, with lack of ancient M representatives and the presence of deep N lineages in western Asia, and the abundance of basal M lineages in India and southwestern Asia and concomitant lack of equivalent-age N clades, gave rise to the hypothesis that N represents the main footprint of the northern Continental expansion, whereas M is the equivalent footprint for the southern coastal expansion. The presence of N and M lineages in alternative areas has been explained to have been the result of secondary migrations (Maca-Meyer et al. 2001). However, another plausible explanation is that both M and N reached southern Asia at the same time, quickly expanding to Papua New Guinea (PNG) during maximal glacial ages when the permafrost boundary precluded a northern human occupation. During postglacial ages, subsequent migrations northward carried derivatives of both macrohaplogroups to northern Asia (Forster et al. 2001). Nevertheless, under this second hypothesis, the presence of basal N clusters should be expected in India, southern Asia, and PNG; but this is not the case. All N representatives in India belong to R, a clade derived from N by the loss of 16223 and 12705 mutations (Fig. 2). In addition, the bulk of these Indian lineages belong to western Caucasian haplogroups that, most probably, reached India as the result of secondary immigrations, as has already been proposed (Kivisild et al. 1999; Bamshad et al. 2001). Similarly, the N representatives in southern Asia belong to haplogroups F and B, two sister clades also derived from R (Fig. 2). Furthermore, when totally sequenced PNG N lineages (Ingman et al. 2000; Ingman and Gyllensten 2003) are added to the N phylogenetic tree (data not shown), they form three monophyletic clades that have their roots in the derived R trunk. On the contrary, the geographically northern Asian clades A, N9a, N9b, and Y (Fig. 2) and the western Eurasian clades W, N1b, I, and X all split from the basal N root (Maca-Meyer et al. 2001), although A, N9a, N9b, and Y radiations were delayed congruent with subsequent northern Asian expansions. Therefore, at present, mtDNA data are compatible with the supposition that the northern route, harboring mainly N precursors, met climatic difficulties and when they finally reached Southeast Asia, the M representatives, brought by the southern route, had already colonized the area. This southern expansion of N derivatives has, as a lower temporal boundary, the coalescence ages of F, B, and PNG R haplogroups being ~46,000 ± 10,000 years ago. However, when recently published (Ingman et al. 2000; Ingman and Gyllensten 2003) Australian N lineages are taken into account, it seems evident that the real situation could be far more complex than the one migration–one lineage hypothesis. Australian N lineages directly sprout from the basal trunk (data not shown). They most probably differentiated in that continent, supporting the idea that ancestral N lineages reached Australia but not PNG, although the undemonstrable possibility of lineage extinctions and subsequent recolonization events in PNG can be an argument. Both hypotheses have difficulties to explain the presence of ancient N lineages in Australia. If the two, M and N lineages, were brought with the southern coastal dispersion, the lack of primitive N in India, southern Asia, and PNG has to be explained by the subsequent loss of all N lineages carried to Australia; if the northern Continental route of N is favored, the loss of N representatives in all populations formed in route to Australia has also to be explained. Recently, an N lineage has been detected in Chenchus, a southern Indian tribal group (Kivisild et al. 2003). From the information published, it can be deduced that this lineage only shares mutation 1719 with the western Eurasian Nb1/I and X clades. More extensive studies of populations in southern India

Table 5. Distribution of Unique and Shared Haplotypes in Japanese Populations

	Japanese populations		
	JPN	RYU	AIN
Sample	1318	50	51
Haplotypes	626	42	24
Haplotype frequency	0.48	0.84	0.47
Singleton + Unique	377 (0.60)	19 (0.45)	3 (0.13)
Shared	249 (0.40)	23 (0.55)	21 (0.87)
JPN	137 (0.22)	20 (0.48)	13 (0.54)
RYU	20 (0.03)	1 (0.02)	1 (0.04)
AIN	13 (0.02)	1 (0.02)	5 (0.21)
Other*	130 (0.21)	4 (0.10)	5 (0.21)

*Other Asians.

and southern and central Asia would add empirical support to any of these theories.

Concerning macrohaplogroup M, it has already been commented that the star radiation of all the main Indian and southeast Asian M clades strongly suggests that this wide geographic colonization could have happened in a relatively short time (Maca-Meyer et al. 2001). This star radiation includes the Australian and PNG M complete sequences recently published (Ingman et al. 2000; Ingman and Gyllensten 2003). However, for those clades and subclades with later northward expansions, long radiation delays are observed. For instance, whereas M7 and M8 have coalescence ages ~35,000 to 45,000 years ago, other groups such as G, D4, M7a, or M7c have coalescence ages ~15,000 to 30,000 years ago, more in frame with those calculated for A, Y, and N9 derivates, which, although belonging to macrohaplogroup N, share with them a central–northern Asian geographic distribution (see Supplemental material). It seems that the simultaneous lineage bursts ~60,000 to 70,000 years ago from Africa (Maca-Meyer et al. 2001), ~30,000 to 55,000 years ago for macrohaplogroups M and N, and ~15,000 to 30,000 years ago for clusters with prominent central–northern Asian radiations were related to main climatic changes. The role of selection in these expansions is an open question (Elson et al. 2004; Ruiz-Pesini et al. 2004).

The application of global pairwise-distance and detailed phylogeographic methods to the peopling of Japan shows that both approaches have different grasps but together demonstrate that the actual Japanese population is the result of a complex demographic history, from which the different theories proposed to explain it only emphasize partial aspects. Global distances and detailed haplotype comparisons confirm that Ainu and Ryukyuans are heterogeneous populations (Horai et al. 1996) and that both are well differentiated from the mainland Japanese. In spite of this, they have common peculiarities such as having the highest frequencies in Asia for M7a, M7b2, and N9b, shared with mainland Japanese. Furthermore, for both, their closest relatives are northern populations. At first sight, these results are against a supposed southern origin for the Paleolithic Japanese, favoring the replacement theory or even that the Paleolithic inhabitants of Japan came from northeastern Asia (Nei 1995). Although based on a single locus, our results are strikingly coincident with the previously proposed northern origin and influences received by the Japanese. In an early study using serum gammaglobulin polymorphisms, it was concluded that the homeland of all Japanese could have been in the Lake Baikal area in Siberia (Matsumoto 1988), which agrees with the close proximity found here between Buryats and Ryukyuans or mainland Japanese. More recently, classical markers (Omoto and Saitou 1997) and mtDNA (Horai et al. 1996) studies demonstrated that the Japanese are most closely related to the Koreans, which is also true in our global analysis. It can be added that a substantial part of this common maternal pool has recent roots, as Korea specifically shares with Ainu, mainland Japanese, and Ryukyuans 10%, 7%, and 5%, respectively, of their haplotypes. This particular affinity is increased with the existence of derived lineages only detected (A1a, B4c1, B4f) or mainly detected (N9b, B4a1, B4b1, G1a, M7b2, M12) in Japanese and Koreans. This Korean influence has been attributed to the archeologically well-documented Continental immigration to Japan during the Yayoi period (Horai et al. 1996). However, specific haplotype matches with other areas increases the geographic range of these recent influences. Thus, mainland Japanese share part of their haplotypes exclusively with South China (2.5%), North China (1.5%), Central Asia (1.5%), and Indonesia (0.3%); and, also, Ryukyuans have specific affinities with North China (2.4%) and Central Asia (2.4%). The recent Siberian input on the Ainu has also been stressed (Schurr et al. 1999). At least, another independent migratory wave from central Asia also affected mainland Japanese. It was first detected by the peculiar distribution of the Y-chromosome marker YAP+, and seems to have originated in an area including Tibet (Su et al. 2000). Haplogroup M12 is its mitochondrial counterpart. As with the Y-chromosome marker, its punctual presence in Tibet and eastern Asia might be explained as the result of subsequent migrations in the Continent that erased the route followed by the people harboring these markers. In addition, there are clues, at least in Ryukyuans, that a substantial part of their maternal pool had an ancient southern Asian provenance. This fraction is represented by the M, M7a, and M7a1 basic lineages (31%), which the Ryukyuans do not share with northern populations. This southern signal is, in part, congruent with the southern Asian origin for the Paleolithic Japanese proposed by the dual structure model (Hanihara 1991). Furthermore, the fact that the highest diversities for M7a, M7a1, and M7b2 have been found in Ryukyuans and for N9b and B5b2 in Japan raises the possibility that this area was within a focus of migratory radiations to northern and southern isles and even to the mainland from Paleolithic to recent times. The significant latitudinal clines detected in Japan for some genetic markers (Orito et al. 2001; Takeshita et al. 2001) could also be explained as the result of southern and northern influences on Japanese. Finally, some mtDNA results obtained from ancient Jomon remains (Horai et al. 1991; Shinoda and Kanai 1999; K.-I. Shinoda, unpubl.) are congruent with a genetically diverse background for the Paleolithic Japanese population (Horai et al. 1996). A tentative comparison of Jomon with present-day Japanese populations based on shared lineages (data not shown) significantly relates Jomon first to the indigenous Ainu and then to Ryukyuans and last to mainland Japanese. In summary, Japan could have received several northern and southern Asian maternal inputs since Paleolithic times, with notable northern Asian immigrations through Korea in the late Neolithic and more specific gene flows from western Asia, Siberia, and southern islands.

METHODS

Samples

Complete mtDNA sequences were obtained from a total of 672 unrelated Japanese including 373 from Tokyo and 299 from the Nagoya area. All subjects gave their written consent to participate in this study, which was approved by the Ethical Committees of the Gifu International Institute of Biotechnology and collaborative institutions. The sources of 11 additional complete sequences used to build the final phylogenetic trees are in Table 1. For the analysis of the peopling of Japan, we used a total of 1438 Japanese and 3275 central and eastern Asian HVI sequences, as detailed in Table 3.

Isolation and Amplification of DNA

Total DNA was extracted from the blood with either Dr. Gen TLE (Takara) or MagExtractor System MFX-2000 (Toyobo). The entire mitochondrial genome was amplified as six fragments (~3000–3400 bp) by the first PCR and 60 overlapping segments (~600–1000 bp) by the second PCR. The primer pairs and their nucleotide sequences were described previously (Tanaka et al. 1996). The conditions for the first and second PCR were the same: an initial denaturation step for 5 min at 94°C, followed by 40 cycles of denaturation for 15 sec at 94°C, annealing for 15 sec at 60°C, and extension for 3 min at 72°C, with a final extension for 10 min at 72°C. The amplified fragments were analyzed by electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. These second PCR products were purified by use of the MultiScreen-PCR Plates (Millipore). The quality of DNA templates was 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).

Sequence Analysis of Mitochondrial DNA

Sequence reactions were carried out with a BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems). After excess dye terminators had been removed with MultiScreen-HV plates (Millipore) packed with Sephadex G50 superfine (Pharmacia), the purified DNA samples were precipitated with ethanol, dried, and suspended in the template suppression reagent (TSR) or formamide from Applied Biosystems. The dissolved DNA samples were heated for 2 min at 95°C for denaturation, then immediately cooled on ice. Sequences were analyzed with automated DNA sequencers 377 and 310 by use of Sequencing Analysis Program version 4.1 (Applied Biosystems). A computer program, Sequencher version 4.1 (Gene Codes Co.), was used to indicate possible single nucleotide polymorphism (SNP) loci. For verification, visual inspection of each candidate SNP was carried out. At least two overlapping DNA templates amplified with different primer pairs were used for identification of each SNP. Mitochondrial SNPs (mtSNPs) were identified by comparison with the revised Cambridge sequence (rCRS) reported by Andrews et al. (1999).

Phylogenetic Analysis of Complete Coding-Region mtDNA Sequences

In this present study, nucleotide positions were numbered as in the Cambridge Reference Sequence (CRS; Anderson et al. 1981), nucleotide substitutions were expressed as differences from the revised CRS (Andrews et al. 1999), transitions were denoted only by their nucleotide positions, and transversions were designated by their nucleotide positions followed by the changed base. A total of 942 complete coding-region mtDNA sequences, including our 672 Japanese; one additional Japanese (GenBank accession no. AB055387); 53 worldwide sequences (Ingman et al. 2000); 42 worldwide sequences (Maca-Meyer et al. 2001); two Finnish sequences having Asian relatives (Finnilä et al. 2001); 17 Asian sequences without concrete geographic assignation (Herrnstadt et al. 2002); 37 sequences from the Bering area (Derbeneva et al. 2002b); 70 Asian, New Guinean, and Australian sequences (Ingman and Gyllensten 2003); and 48 Chinese sequences (Kong et al. 2003) were aligned with the rCRS by CLUSTAL V software, and the coding region was used to construct a phylogenetic network (Bandelt et al. 1999) rooted with a chimpanzee sequence (GenBank accession no. D38113) as implemented in the Network 3.1 program (Fluxus Engineering; http://www.fluxus-engineering.com). The noncoding positions were added by hand using molecular weighted parsimony criteria (Bandelt et al. 2000). The phylogenetic relationships obtained were also confirmed by means of a neighbor-joining tree (1000× bootstrapped; Saitou and Nei 1987), built using MEGA2 (Kumar et al. 2001). From this network (see Supplemental material) we chose 102 Japanese and nine Asiatic sequences that represented the main clusters and subclusters within the two macrohaplogroups M and N that colonized Asia. To define these groups we followed the most generalized cladistic nomenclature actually used to classify mtDNA lineages (Richards et al. 1998). For the haplogroups previously detected, we maintained the same notation as their authors proposed (Richards et al. 2000; Bamshad et al. 2001; Kivisild et al. 2002; Yao et al. 2002a; Kong et al. 2003). Those haplogroups introduced here for the first time were named according to their phylogenetic range deduced from the tree of complete sequences.

Haplogroup Assorting of Published Partial mtDNA Sequences

The unambiguously classified complete mtDNA sequences were used as an initial pool that was hierarchically enlarged by the successive addition of those published partial mtDNA sequences with the largest coding information, ending with those for which information on only control-region sequences for both mtDNA hypervariable segments or just one (HVS-I and/or HVS-II) was available, always following sequence matches or, as default, sequence-relatedness criteria. Some of those partial sequences that could be assigned to more than one haplogroup were tentatively assorted in the most probable one deduced from their geographic origin and the relative haplogroup distribution.

Pooling Small Size Samples and Rare Clades

To avoid small sample sizes and rare alleles in population comparisons, samples with <20 individuals were pooled with others from the same geographic and ethnic group. Within populations, individuals belonging to rare clades were pooled with those classified in the nearest branch. Pairwise sample distances were calculated as linearized F_{ST} distances as implemented in the ARLEQUIN program (Schneider et al. 2000), taking mtDNA as one locus with as many alleles as the different subhaplogroups considered.

Quantitative Affinities of Japanese Samples

Relative affinities of Japanese samples to the other Asiatic populations were assessed by linearized F_{ST} distances, using subhaplogroup frequencies, and haplotype matches' distances (D) estimated simply as $D = 1 - \Sigma(x_i y_i)$, x_i and y_i being the frequency of haplotype i in the two compared populations. To be statistically robust, these analyses require large sample sizes, thus further pooling was necessary. Previous studies in the area prevented us from pooling populations by geographic proximity (Schurr et al. 1999) and/or ethno-linguistic relationship (Comas et al. 1998; Chunjie et al. 2000; Yao et al. 2002a). For this reason, a genetic affinity criterion was chosen. Two approaches were used. In the first, all samples with no significant F_{ST} distances between them and with a similar behavior to the rest of the samples studied, were grouped. In the second, pooling was carried out by means of the CLUSTER algorithm implemented in the SPSS ver 9 package. We followed an iterative method specifying the number of conglomerates from 2 to 30. Different groupings were tested by AMOVA, and that with the least assigned variance within areas was chosen. The data were graphically represented by multidimensional scaling (MDS) plots (Kruskal and Wish 1978) using SPSS.

Qualitative Affinities of Japanese Samples

Particular sharing of subhaplogroups and particular haplotype matches of Japanese samples with concrete Continental areas were phylogeographically analyzed by taking into account the relative genetic diversities of the clades involved in the different areas, measured as relative haplotypic frequencies, and their minimum estimates of coalescence ages based on mean divergence among lineages for the coding region (Saillard et al. 2000). A constant evolutionary rate of 1.7×10^{-8} per site per year (Ingman et al. 2000) was used.

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Alcohol dehydrogenase 2 variant is associated with cerebral infarction and lacunae

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Abstract—The authors examined the association of the alcohol dehydrogenase 2 (ADH2) genotype with vascular events in community-dwelling Japanese (1,102 men/1,093 women). The allele *ADH2*2* encodes an isozyme with a higher level of activity than ADH2*1. Here, the authors show that the *ADH2*1* carriage is associated with high prevalence of cerebral infarction and lacunae in men. Multiple regression analyses confirmed that the risk of lacunae and cerebral infarction was increased by the *ADH2*1* allele.

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Alcohol dehydrogenase (ADH) is one of the key enzymes in alcohol metabolism. *ADH2* and *ADH3* have alleles that encode isoenzymes with distinct enzymatic properties.[1] Among Caucasians, a variant *ADH3* allele is found. On the other hand, among Mongoloids, especially the Japanese, about 85% of individuals are carriers of the β2-subunit encoded by the *ADH2*2* allele, compared to only 5% or less of European and white American populations. The β1 (encoded by *ADH2*1*) and β2 subunits (encoded by *ADH2*2*) differ by only one amino acid residue, Arg-47 in the β1 subunit substituted with His-47 in the β2 subunit. ADH2 functions as a dimer and the β2β2 dimer exhibits about 100 times more catalytic activity than the β1β1 dimer.[1]

We previously reported on the influence of the *ADH2* and aldehyde dehydrogenase 2 genotypes on diabetic vasculopathy in type 2 diabetes.[2] Here we examined whether the *ADH2* genotype would also be associated with vascular events in community-dwelling Japanese and show the association of the *ADH2*1* allele with cerebral infarction.

Materials and methods. A population-based prospective cohort study of aging and age-related diseases was begun in Japan in 1997. All participants (1,126 men and 1,106 women) were independent residents of Aichi prefecture. Residents aged 40 to 79 years old were randomly selected from the register in cooperation with the local government. A total of over 1,000 characteristics, including medication, food and nutrition, bone mineral density, blood and urine analysis, psychological examinations, visual and auditory examinations, physical function tests and physical activities, anthropometry and body composition, and head MRI, were examined (see http://www.nils.go.jp/index-j.html).[3] The study protocol was approved by the Committee on the Ethics of Human Research of National Chubu Hospital and the National Center for Geriatrics and Gerontology. Written informed consent for the entire procedure was obtained from each participant.

Samples of DNA were isolated from peripheral blood cells. Genotypes were determined with a fluorescence-based allele-specific DNA primer-probe assay system (Toyobo Gene Analysis, Tsuruga, Japan). Brain MRI was performed using a 1.5-tesla scanner (Toshiba Visart, Tokyo). The first scanning sequence consisted of a T1-weighted sagittal series centered in the midline to define the orbitomeatal line. The second series of T1-weighted axial images and T2-weighted axial images were oriented parallel to the orbitomeatal line. Fourteen slices were taken at each examination.

A cerebral infarction was defined as a lesion more than 0.3 cm in diameter appearing as a low-signal-intensity area on T1-weighted images that was also visible as a hyperintense lesion on T2-weighted images as described.[3,4] Small lesions (<1.5 cm) were diagnosed as a lacunae. One of the authors (M.F.), a neurologist, who was blinded to the clinical status of the subjects, interpreted all MRI series.

Results. When the subjects were grouped into three according to the genotype of *ADH2*, *ADH2*2/ADH2*2 (ADH2*2/2)*, *ADH2*2/ADH2*1 (ADH2*2/1)*, and *ADH2*1/ADH2*1 (ADH2*1/1)*, the distribution of the *ADH2* genotypes was in Hardy-Weinberg equilibrium. There was no significant difference in characteristics among the three genotypic groups in women (data are not shown). In contrast, in men, the level of total cholesterol (TC) and LDL-cholesterol (LDL-C) significantly differed between the *ADH2*2/2* and *ADH2*1/2* genotypic groups by multiple comparisons (table 1). Although group *ADH2*1/1* did not significantly differ in the levels of TC and LDL-C from the other groups, probably due to an insufficient number in members of group *ADH2*1/1* (5.2%), the *ADH2*1* allele tended to increase the levels of TC and LDL-C. Additionally, alcohol consumption was higher in the *ADH2*1/1* group than the other groups, whereas there was no differ-

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*Table 1 Comparison of clinical characteristics in men among ADH2*2/2, ADH2*2/1, and ADH2*1/1 genotypic groups*

	*ADH2*2/2*	*ADH2*2/1*	*ADH2*1/1*	Genotype: *p* value
No. (%)	689 (61.2)	378 (33.6)	59 (5.2)	NS
Age, y	59.4 ± 0.4	58.8 ± 0.6	58.0 ± 1.4	NS
Alcohol, g/d	28.8 ± 1.4	29.5 ± 1.9	44.5 ± 4.5	2/2 vs 1/1: $p = 0.0049$*
				2/1 vs 1/1: $p = 0.0102$*
Nonsmoker & smoker, %†	21/40/39	22/40/37	24/39/37	NS
Systolic BP, mm Hg‡	120.1 ± 0.8	121.8 ± 1.0	126.1 ± 2.6	NS
Diastolic BP, mm Hg‡	74.9 ± 0.5	76.1 ± 0.6	77.3 ± 1.6	NS
Percent with hypertension§	32.6	37.0	40.7	NS
Height, cm	164.4 ± 0.2	164.7 ± 0.3	164.6 ± 0.8	NS
BMI	23.0 ± 0.1	22.8 ± 0.1	22.9 ± 0.4	NS
T-cho, mg/dL	210.1 ± 1.3	215.7 ± 1.7	217.6 ± 4.3	2/2 vs 2/1: $p = 0.0231$*
LDL, mg/dL	129.7 ± 1.2	135.8 ± 1.7	134.4 ± 4.2	2/2 vs 2/1: $p = 0.0115$*
HDL, mg/dL	57.3 ± 0.6	57.6 ± 0.8	57.4 ± 1.9	NS
TG, mg/dL	134.9 ± 3.7	130.8 ± 5.0	150.2 ± 12.4	NS
Glucose, mg/dL	105.7 ± 0.9	106.1 ± 1.2	103.9 ± 2.9	NS
HbA1c, %	5.32 ± 0.03	5.34 ± 0.04	5.33 ± 0.10	NS
Percent with diabetes	13.3	13.3	13.6	NS
Insulin, μU/mL	8.5 ± 0.2	7.8 ± 0.3	8.7 ± 0.7	NS
Estradiol, pg/mL	28.2 ± 0.4	27.1 ± 0.5	25.9 ± 1.4	NS
F-Testosterone, pg/mL	13.1 ± 0.2	13.3 ± 0.2	13.6 ± 0.5	NS
Brain examination, n (%)	n = 678	n = 367	n = 57	
Lacunal infarction	60 (8.9)	55 (15.0)	8 (14.0)	$p = 0.0085$¶
				2/2 vs 2/1: $p = 0.0025$‖
Cerebral infarction	68 (10.0)	59 (16.1)	9 (15.8)	$p = 0.0129$¶
				2/2 vs 2/1: $p = 0.0043$‖

Values are mean ± SD or n (%).

* *p* Value obtained by the Turkey-Kramer method for multiple comparisons.
† Nonsmoker & smoker = percentage of complete nonsmokers/percentage of past smokers who stopped smoking/percentage of current smokers.
‡ Blood pressure (BP) was analyzed only with subjects not taking oral antihypertension medications.
§ Hypertension was defined as either a systolic blood pressure of over 140 mm Hg or a diastolic blood pressure of over 90 mm Hg, or as receiving antihypertension medication.
¶ *p* Value obtained by the contingency table analysis.
‖ *p* Value by the chi-square analysis between groups *ADH2*2/2* and *ADH2*2/1*.

NS = not significant by multiple comparisons; BMI = body mass index; LDL = low-density lipoprotein; HDL = high-density lipoprotein.

ence in amounts of alcohol consumption between groups *ADH2*2/2* and *ADH2*2/1*.

A total of 1,102 male and 1,093 female subjects were examined by MRI. More striking, in men, higher frequencies of lacunae and cerebral infarction were found in the *ADH2*2/1* group than the *ADH2*2/2* group (see table 1). The frequencies of other abnormal signs on MRI did not differ among the three groups (data are not shown). In women, there was no difference in prevalence of abnormal MRI signs among the three *ADH2* genotypic groups (data not shown).

To confirm the significant difference in the frequencies of lacunae and cerebral infarction according to the *ADH2* genotype, multiple logistic analyses were performed based on 1,102 subjects with an adjustment for aging (table 2). Aging is the most significant risk for lacunae and cerebral infarction. More interestingly, OR and *p* values clearly indicated that the *ADH2*1* allele is a distinct risk for lacunae and cerebral infarction. Even when the effect of alcohol consumption was included, the main conclusion was not altered (see table 2).

Discussion. An influence on lacunae and cerebral infarction by the *ADH* genotype was found only in Japanese men. This discrepancy between genders may be speculated to be due to a difference in alcohol consumption. However, even when the effect of alcohol consumption was included, the main conclusion was not altered. Therefore, the effect by alcohol consumption does not seem responsible for the discrepancy between genders. Instead, ADH2 activity modulated by several hormones may be responsible for the discrepancy. In fact, experiments with ani-

Table 2 Multiple logistic analyses (number of subjects = 1,102)

	OR (95% CI)	*p* Value
Lacunar state in men		
A: Multiple logistic analyses		
ADH2 (carriage of *ADH2*1* allele)	2.16 (1.44–3.25)	0.0002
Age - 10 y	3.46 (2.69–4.45)	<0.0001
B: Multiple logistic analyses including alcohol consumption		
ADH2 (carriage of *ADH2*1* allele)	2.18 (1.49–3.38)	0.0005
Age - 10 y	3.53 (2.68–4.65)	<0.0001
Cerebral infarction in men		
A: Multiple logistic analyses		
ADH2 (carriage of *ADH2*1* allele)	2.06 (1.39–3.06)	0.0003
Age - 10 y	3.44 (2.70–4.37)	<0.0001
B: Multiple logistic analyses including alcohol consumption		
ADH2 (carriage of *ADH2*1* allele)	2.05 (1.35–3.11)	0.0008
Age - 10 y	3.49 (2.70–4.52)	<0.0001

mals indicated that testosterone reduces enzymatic activity in the liver, and that estrogen increases the activity.[5]

ADH catalyzed the first step in the metabolism of ethanol, and in addition, has a wide substrate range, using both aliphatic and aromatic alcohols, aldehydes, sterols, and ω-hydroxy fatty acids. It is worth noting that ADH catalyzes the oxidation of 3,3-dimethylallyl alcohol, the intermediary alcohol of the shunt pathway of mevalonate metabolism, and the branching between the sterol and the shunt pathway could also occur at the level of geranyl pyrophosphate and farnesyl pyrophosphate.[6] Therefore, the genetic variant of *ADH2* may change the flow of the shunt pathway of cholesterol synthesis, thereby causing LDL-C levels to vary between the *ADH2*2/2* and *ADH2*2/1* groups. As for cardiovascular diseases, it was reported that an ADH3 polymorphism is associated with HDL-C levels and myocardial infarction in Caucasians.[7] Thus, our results may provide insight into ethnic differences in the incidence of cerebral or myocardial vascular disease between Mongoloids and Caucasians.

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ASSOCIATION OF CHOLECYSTOKININ-A RECEPTOR GENE POLYMORPHISM WITH ALCOHOL DEPENDENCE IN A JAPANESE POPULATION

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Abstract—Aims: Cholecystokinin (CCK), one of the most abundant neurotransmitter peptides, interacts with dopamine. Dopaminergic neurotransmission between the ventral tegmental area and the limbic forebrain is a critical neurobiological component of alcohol and drug self-administration. CCK modulates dopamine release in the nucleus accumbens via the CCK-A receptor (R). We recently determined the transcriptional start site of the human CCK-AR gene, and detected two sequence changes (–81A/G and –128G/T) in the promoter region. The aims of the present study were to determine the prevalence of the –81A/G and –128G/T polymorphism of the CCK-AR gene between alcoholics and normal control subjects and the occurrences of the polymorphisms in subtypes of alcoholics. **Methods:** The above polymorphisms were examined in 435 alcoholics and 1490 control subjects. We excluded subjects with inactive ALDH2 and employed the subjects with ALDH2*1/2*1 (384 alcoholics and 792 controls). **Results:** The allelic frequency of –81G in the CCK-AR gene polymorphism (–81A/G) was significantly higher in alcoholics than in control subjects. However, there were no differences between the two groups with respect to the frequency of –128G/T. Alcoholic patients with antisocial personality disorder and with first-degree alcoholic relatives were significantly associated with a higher frequency of the –81G allele. In addition, the age of onset of alcohol dependence was significantly earlier in patients with this allele. **Conclusions:** The CCK-AR gene –81A/G polymorphism, especially in the –81G allele, may be associated with intractable alcoholism.

INTRODUCTION

Alcohol-related behaviours and/or alcohol sensitivities are associated with the actions of various neurotransmitters and neuropeptides such as dopamine (Kalivas, 1993; Self and Nestler, 1995). Cholecystokinin (CCK), one of the most abundant neurotransmitter peptides in the brain, is known to interact with dopamine (Crawley, 1991; Marshall *et al.*, 1991; Woodruff *et al.*, 1991; Ladurelle *et al.*, 1994; Hamilton and Freeman, 1995). Thus far, two types of CCK receptors (R) (types A and B) have been cloned (Wank, 1995). Although CCK-BR is widely distributed throughout the central nervous system, CCK-AR is found in specific regions, such as the amygdala, nucleus tractus solitarius, posterior nucleus accumbens, ventral tegmental area, substantia nigra, and raphe nucleus. CCK coexists in the mesolimbic dopamine neurons, and CCK-AR mediates the release of dopamine in the nucleus accumbens (Crawley, 1991; Marshall *et al.*, 1991; Woodruff *et al.*, 1991; Ladurelle *et al.*, 1994; Hamilton and Freeman, 1995; Wank, 1995). The dopaminergic neurotransmission between the ventral tegmental area and the limbic forebrain is a critical neurobiological component of self-administration of alcohol and drugs (Kalivas, 1993; Self and Nestler, 1995).

Recent reports (Blum *et al.*, 1990; Muramatsu *et al.*, 1996) in human subjects showed an association of polymorphisms of the dopamine D2 and/or D4 receptor gene with alcohol dependence, although results have been equivocal. In contrast, Okubo *et al.* (2000) reported that the CCK gene polymorphism does not play a major role in alcohol withdrawal symptoms. Based on our recent finding of two sequence changes in the promoter region (a G to T change in nucleotide –128 and an A to G change in nucleotide –81; GenBank database accession number D85606; Funakoshi *et al.*, 2000), in the present study, we examined the association between CCK-AR gene polymorphisms and alcohol dependence.

Liver mitochondrial aldehyde dehydrogenase-2 (ALDH2) is responsible for metabolizing the acetaldehyde produced from ethanol into acetate. More than 40% of Asians have the inactive form of ALDH2, encoded either as heterozygous ALDH2*1/2*2 or homozygous ALDH2*2 (Higuchi *et al.*, 1995), while the majority of Caucasians possess the active form of ALDH2 (2*1/2*1). A previous report (Murayama *et al.*, 1998) showed that the clinical characteristics of alcoholic patients having inactive ALDH2 differed from those of alcoholic patients with active ALDH2. In this study, we excluded subjects with inactive ALDH2 to avoid the influence of its overwhelming effect as a negative risk factor for alcoholism.

SUBJECTS AND METHODS

Subjects

This study was approved by the ethics committees of the National Alcoholism Center, Kurihama Hospital, of the National Institute of Longevity Sciences (NILS) and of the Tokyo Metropolitan Institute of Gerontology. Written informed consent was obtained from each subject.

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The subjects consisted of 435 (aged 32–74 years) Japanese male alcoholics who had been consecutively hospitalized at Kurihama Hospital. They were diagnosed as having DSM-III-R (American Psychiatric Association, 1987) alcohol dependence, based on the Structured Clinical Interview for DSM-III-R (SCID) assessment (Spitzer *et al.*, 1990).

The age-matched control subjects consisted of 1134 male participants in the NILS Longitudinal Study of Aging (LSA) (Shimokata *et al.*, 2000) and 356 males who were Institute employees. They were free of alcohol dependence, based on the results of the Kurihama Alcoholism Screening Test, the most widely used alcoholism screening test in Japan, which was administered to potential controls before entering into this study.

First, the genotype of the ALDH2 gene was determined by mismatched polymerase chain reaction restriction fragment length polymorphism (PCR–RFLP) method reported previously (Kamino *et al.*, 2000). Then the CCK-AR gene polymorphism was determined in the subjects with ALDH2*1/2*1 (384 alcoholics and 792 controls).

Genotyping procedures

The polymorphism in the promoter region of CCK-AR gene was examined using a mismatched PCR–RFLP method (Funakoshi *et al.*, 2000). In brief, a pair of primers (sense primer = 5′-CATATGTACACATGTGTGTAAAAAGCAGCC-AGAC-3′ and anti-sense primer = 5′-GCCCTTTCCTGGG-CCAGACT-3′), were designed to amplify the 103-bp product, which was subsequently digested with restriction enzyme *Hin*fI, and analysed by 12% polyacrylamide gel electrophoresis. Six genotypes were identified: a wild type (–81A/A, –128G/G); heterozygous mutant types (–81A/G, –128G/G), (–81A/G, –128G/T), (–81G/G, –128G/G), (–81G/G, –128G/T); and a homozygous mutant type (–81G/G, –128T/T).

Clinical data

We used a structured clinical interview for DSM-III-R to diagnose alcohol dependence and antisocial personality disorder (Spitzer *et al.*, 1990). We also used a structured interview to obtain responses to questions on social background as well as history of drinking and alcohol withdrawal. Family histories of alcohol dependence among all biological first-degree relatives were evaluated by using the Family History Research Diagnostic Criteria (Andreasen *et al.*, 1977). Age at onset of alcoholism was defined as the age at which the individual first met the DSM-III-R diagnostic criteria for alcohol dependence.

Statistical analyses

Statistical differences between alcohol-dependent and control subjects were assessed using the chi-squared test. A continuity correction was performed when the frequency of at least one cell was less than 5. An odds ratio (OR) with a 95% confidence interval (CI) was calculated to evaluate the genotype frequencies between groups. Probability differences of $P < 0.05$ were considered statistically significant. To assess the linkage disequilibrium between the two polymorphisms of the CCK-AR gene, we calculated the D value and its significance, using the ASSOCIAT program (downloaded from the website of J. Ott: ftp://linkage.rockefeller.edu/ software/utilities/). All statistical computations were carried out using the Statistical Analysis System package, version 6.12 (SAS Institute, 1998).

RESULTS

The frequency of a wild-type (–81A/A, –128G/G) genotype was lower in alcoholics than in controls, though the difference was not significant ($P = 0.053$) (Table 1). These polymorphisms were in linkage disequilibrium and in Hardy–Weinberg equilibrium. The genotypes of (–81A/A, –128G/T), (–81A/A, –128T/T), and (–81A/G, –128T/T) were not detected. These were not detected in our previous reports, either (Funakoshi *et al.*, 2000; Shimokata *et al.*, 2000).

When the allelic frequencies were estimated, significant differences in that of the –81A/G were detected between alcoholics and controls, as shown in Table 2 ($P = 0.023$). However, the frequencies of the –128 G to T change were not significantly different between the two groups.

Based on the finding that the allelic frequency of –81G was significantly higher in alcoholics than in controls (Table 2), the association between CCK-AR gene –81A/G polymorphism and the clinical features of alcoholics was assessed (Table 3). Comparison of the genotype distributions of the CCK-AR gene –81A/G polymorphism in alcoholics and control subjects revealed that the frequencies of –81A/A were quite similar among the subgroups of alcoholic patients with

Table 1. Distribution of CCK-AR gene –81A/G, –128G/T polymorphisms in alcoholics and control subjects (participants had ALDH2*1/2*1 genotype)

Genotype	Alcoholics ($n = 384$) n (%)	Control subjects ($n = 792$) n (%)
–81A/A, –128G/G	205 (53.3)	470 (59.3)
–81A/G, –128G/G	75 (18.8)	111 (14.0)
–81A/G, –128G/T	76 (19.8)	168 (21.2)
–81G/G, –128G/G	6 (1.6)	9 (1.1)
–81G/G, –128G/T	16 (4.2)	19 (2.4)
–81G/G, –128T/T	9 (2.3)	15 (1.9)

Percentages may not total 100 due to rounding up. The difference between the wild-type genotype and the mutations (the sum of the five different types) was tested by 2 × 2 chi-squared test. $\chi^2 = 3.75$, d.f. = 1, $P = 0.053$.

Table 2. Allele frequencies of CCK-AR gene –81A/G, –128G/T polymorphisms in alcoholics and control subjects (participants had ALDH2*1/2*1 genotype)

Allele	Alcoholics ($n = 768$) n (%)	Control subjects ($n = 1584$) n (%)
–81A	*558 (72.7)	1219 (77.0)
G	210 (27.3)	365 (23.0)
–128G	658 (85.7)	1367 (86.3)
T	110 (14.3)	213 (13.7)

Percentages may not total 100 due to rounding up. $\chi^2 = 5.12$, d.f. = 1, *$P < 0.023$ for the –81A/G polymorphism. Odds ratio = 1.26. There were no differences with respect to –128G/T.

Table 3. Clinical characteristics of alcoholics with CCK-AR gene −81A/G polymorphism (participants had ALDH2*1/2*1 genotype)

Parameter	Genotype of the CCK-AR Gene −81A/G Polymorphism		
	A/A *n* (%)	A/G + G/G *n* (%)	2 × 2 table χ^2 test
Antisocial personality disorder (ASP)			
Negative	204 (54.4)	171 (45.6)	$\chi^2 = 4.99$, d.f. = 1, $P = 0.025$ (continuity adjusted)
Positive	1 (11.1)	8 (88.9)	
Delirium tremens			
Negative	142 (56.8)	108 (43.2)	$\chi^2 = 3.36$, d.f. = 1, $P = 0.067$
Positive	63 (47.0)	71 (53)	
First-degree relatives			
Negative	191 (55.7)	152 (44.3)	$\chi^2 = 6.83$, d.f. = 1, $P = 0.009$
Positive	14 (34.2)	27 (65.9)	
Age of onset of alcohol dependence	41.8 ± 10.7	38.9 ± 10.7	$t = 2.54$, d.f. = 361, $P = 0.012$

Percentages may not total 100 due to rounding up.

negative ASP, with negative delirium tremens, and with negative first-degree relatives, and the control group (55.7, 57.5, and 56.9% for respective subgroups of alcoholics versus 59.3% for control subjects, as shown in Table 1). A comparison among alcoholic subgroups revealed that the frequency of genotype −81A/A was significantly lower in alcoholics with ASP and with first-degree relatives than in those without ASP and without family history (Table 3). The frequency of −81A/A tended to be lower in alcoholics with delirium tremens than in those without delirium tremens, though the difference was not significant ($P = 0.067$). The age at onset of alcohol dependence was significantly earlier in alcoholics with genotypes −81A/G and G/G than in those with wild-type (−81A/A).

DISCUSSION

Our results showed a higher frequency of the G allele of the CCK-AR gene −81A/G polymorphism in alcoholics than in control subjects. Moreover, the allelic frequency of −81G was significantly higher in alcoholic patients with ASP and with family history than in those without ASP and family history. Patients with delirium tremens tended to possess the −81G allele more frequently than did patients without delirium tremens, although the difference was not statistically significant ($P = 0.067$). Furthermore, the age at onset of alcohol dependence was earlier in patients with the −81G allele than in those without it. These findings suggest that the −81G allele of the CCK-AR gene may be associated with intractable alcohol dependence.

The comorbidity rate of antisocial personality disorder was only 2.3% and an average age at onset of alcohol dependence was around 40 years in our samples. These figures are substantially different from those of US alcoholic samples recruited from inpatient treatment settings (Hesselbrock *et al.*, 1986; Raimo *et al.*, 1999). Although reasons are not clear, we have observed a relatively low comorbidity rate of antisocial personality disorder in Japanese alcoholic samples (Yoshino and Kato, 1996; Murayama *et al.*, 1998). In addition, age of onset of our alcoholic samples is comparable to that of other Japanese alcoholic inpatients. (Murayama *et al.*, 1998). These comparisons suggest that our samples did not deviate from general Japanese alcoholic samples.

There have been several previous reports of CCK-AR gene polymorphisms (Inoue *et al.*, 1997; Tachikawa *et al.*, 2000; Okubo *et al.*, 2002). Okubo *et al.* (2002) determined five mutations, −388 $(GT)_8/(GT)_9$, −333G/T, −286A/G, −241G/A, and −85C/G in the promoter region of the CCK-AR gene, and reported a significant association between −85C to G change and alcoholic patients with hallucinations. However, once we had determined the transcriptional start site of the CCK-AR gene (Funakoshi *et al.*, 2000), we discovered that the −85 is not in the promoter region, but is in the 5′ untranslated region. Okubo *et al.* (2002) numbered not from the transcriptional start site but from the initial site of the coding region of exon 1. We examined CCK-AR gene polymorphisms in 50 patients with gallstone and 300 patients with diabetes mellitus before the establishment of the RFLP method (Funakoshi *et al.*, 2000). We found one case with G to A in intron 1, and another case with C to G in exon 3, without any change in amino acid (Thr). The polymorphisms of the promoter region (between −351 and +176) were also examined, and no polymorphisms other than −81A to G and −128G to T were detected. Those designated as −333G/T and −286A/G by Okubo *et al.* (2002) were identical to −128G/T and −81A/G in the present study, respectively. No association of these polymorphisms (−128G/T and −81A/G) with alcohol dependence was observed (Okubo *et al.*, 2002). One possible explanation for the differences between the study by Okubo *et al.* (2002) and our study is that Okubo *et al.* (2002) did not exclude subjects with inactive ALDH2. Inactive ALDH2 (2*1/2*2 and 2*2/2*2) is a strong negative risk factor for alcohol dependence (Higuchi *et al.* 1995). Tachikawa *et al.* (2000) reported an association of the 201A allele (201A/G is identical to −81A/G in the present study) of the CCK-AR gene with schizophrenia. Given the potential differences between alcohol dependence and other psychiatric disorders, our results do not completely contradict their findings.

We recently reported that functional comparison of the A and G variants of the −81 A/G polymorphism by luciferase assay demonstrated a slight decrease in the G variant, but no

significant difference (Takata *et al.*, 2002). However, we used STC-1 (Rindi *et al.*, 1990), established from a transgenic mouse expressing a viral oncogene under the control of the insulin promoter, because no human-derived cell line expressing CCK-AR was available. Further studies employing various experimental conditions are needed before conclusions can be drawn regarding the effect of this polymorphism on expression of the CCK-AR gene.

A recent report mapped the CCK-AR gene to chromosome 4 (4p15.2–15.1), in the vicinity of the dopamine D5 receptor gene (4p16.1–15.1) (Beischlag *et al.*, 1995). The dopamine D5 receptor binds dopamine with a 10-fold greater affinity than that of dopamine receptor 1. The dopamine D5 receptor protein is also localized in the prefrontal cortex. Thus, alterations in the CCK-AR gene may lead to some modification of dopamine release, and alteration of dopaminergic neurotransmission may be involved in alcohol misuse (Crawley, 1991; Marshall *et al.*, 1991; Woodruff *et al.*, 1991; Kalivas, 1993; Ladurelle *et al.*, 1994; Hamilton and Freeman, 1995; Self and Nestler, 1995; Wank, 1995).

In summary, the CCK-AR gene –81A/G polymorphism was found to be associated with alcohol dependence, and the –81G allele of the CCK-AR gene to be possibly associated with intractable alcohol dependence.

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