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Phylogenetic Affiliation of Ancient and Contemporary Humans Inferred from Mitochondrial DNA [and Discussion]

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Phylogenetic affiliation of ancient and contemporary humans inferred from mitochondrial DNA

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SUMMARY

Nucleotide sequence analysis of the major non-coding region of human mitochondrial DNA (mtDNA) from three major races was extended with data from 27 contemporary Mongoloids (20 from southeast Asia, seven from America) and 11 Ancient Japanese bones (five from Jomon Age; 3000–6000 years BP, six from the early modern Ainu; 200–300 years BP). In both cases, the sequence was determined directly from the polymerase chain reaction products. Based on a comparison of the 482 base pair sequences from a total of 128 contemporary humans, the nucleotide diversity is estimated to be 1.46%, which is three times higher than the corresponding value estimated from restriction-enzyme analysis of the whole mtDNA genome. The phylogenetic tree revealed that all lineages are classified into at least five clusters designated as C1–C5. C1 consists exclusively of Africans, and most Asians and Europeans formed C2, C3, C5 and C4, respectively. Phylogenetic analysis also indicated that part of the Asians, including the Japanese, subsequently diverged from the majority of Africans, and that Asians can therefore be separated into two distinct groups. Native Americans, however, appeared only in C3 and C5, suggesting that the size of the founder population was not so large during the peopling of America. Nucleotide sequences derived from ancient bones in a highly polymorphic region were also compared with those of contemporary humans. The nucleotide diversity among the 139 sequences in the region was estimated to be 2.26%. A group of ancient Japanese, including both Jomon peoples and the Ainu, showed a close phylogenetic affiliation with one group of contemporary Japanese and southeast Asians. Moreover, all of the ancient Japanese were clearly placed in a larger cluster, indicating their genealogical difference from another group of contemporary Japanese. This observation supports the immigration theory, which postulates that immigrants from the Asian continent made a considerable contribution to the formation of modern Japanese. Finally, the presence of an Asian-specific deletion was examined by using polymerase chain reaction analysis of native American samples. The deletion was found in one mummy sample. This, together with the present sequence analysis and published information, clearly shows that the Circum-Pacifican populations (Asians, Oceanians and native Americans) can be separated into two distinct mitochondrial lineages.

1. INTRODUCTION

During the past decade, considerable progress has been made in understanding both the molecular and population genetics of human mitochondrial DNA (mtDNA). Human mtDNA is a circular genome of approximately 16.5 kilobase pairs (kb) in length. Its complete nucleotide sequence has revealed an extremely compact organization for this genome (Anderson *et al.* 1981). Over 90% of the genome is a coding region that consists of genes for 13 proteins, a set of ribosomal RNA (rRNA), and 22 transfer RNAs (tRNAs). The remaining genome consists of a major non-coding region and several small non-coding segments. The major non-coding region contains the origin of replication of one strand, the origins of transcription of both strands, and the displacement loop (D-loop) (Anderson *et al.* 1981; Cantatore & Attardi 1980). Maternal inheritance and the predominantly uniclonal nature of mammalian mtDNA within any given individual have been confirmed (Hutchison *et al.* 1974; Potter *et al.* 1975; Giles *et al.* 1980), followed by numerous reports indicating that its nucleotide sequence is evolving much more rapidly than that of single-copy nuclear genes (Brown *et al.* 1979; Ferris *et al.* 1981).

As there are substantial variations in nucleotide sequences among individuals (Brown & Goodman 1979), restriction-enzyme analysis of mtDNAs has become a powerful tool in attempts to elucidate evolutionary relationships among human ethnic groups (Brown 1980; Denaro *et al.* 1981; Blanc *et al.* 1983; Johnson *et al.* 1983; Horai *et al.* 1984; Cann *et al.* 1984; Wallace *et al.* 1985; Horai & Matsunaga 1986; Brega *et al.* 1986; Cann *et al.* 1987; Harihara *et al.* 1988; Stoneking *et al.* 1990). Results from such studies suggest

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that there is a high correlation between mtDNA restriction types and the ethnic origins of individuals.

In previous studies we have shown that the Japanese population can be separated into two distinct groups: one group, with members found at a low frequency (group I), appears to have diverged from another group, with members found at higher frequency (group II) (Horai & Matsunaga 1986; Horai *et al.* 1987; Horai 1991*a*). This grouping can also be applied to other Mongoloid populations as a nine base pair (b.p.) deletion in a small non-coding region of mtDNA (region V; Cann & Wilson 1983), which characterizes the group I Japanese, has also been observed in non-Japanese Mongoloid populations (Stoneking & Wilson 1989; Stoneking *et al.* 1991; Hertzberg *et al.* 1989; Horai *et al.* 1991; Horai 1991*a b*). A phylogenetic analysis of restriction types of mtDNA among three major racial groups has indicated that most Africans and some of the Japanese (equivalent to Japanese group I) appear to have diverged first from the rest of the Japanese and the Europeans (Horai *et al.* 1986, 1987).

Recently, an extensive sequence analysis of the major non-coding region of mtDNA has been undertaken (Horai & Hayasaka 1990). Based on the sequence comparison of over a hundred individuals, several remarkable features of nucleotide substitutions and insertion or deletion events have been revealed. The nucleotide diversity among the sequences is estimated to be 1.45%, three- to sixfold higher than the corresponding value estimated from restriction-enzyme analysis of the whole mtDNA genome. A significantly non-random distribution of nucleotide substitutions and variations in sequence length was also noted. The results confirmed several features of the non-coding region of mtDNA that were previously reported by Greenberg *et al.* (1983) and Aquadro & Greenberg (1983).

Polymerase chain reaction (PCR) analysis has been applied to molecular evolutionary studies not only on contemporary (Wrischnik *et al.* 1987; Vigilant *et al.* 1989) but also on archaeological human samples (Pääbo *et al.* 1988; Hagelberg *et al.* 1989). In such a study we succeeded in amplifying mtDNA extracted from an ancient Japanese bone, the age of which was estimated at about 6000 years BP, and we determined the nucleotide sequence of part of the major non-coding region. Sequence comparison showed that the ancient individual has a close phylogenetic affiliation to southeast Asians (Horai *et al.* 1989).

In this paper we present the design and implementation of an efficient PCR system to detect variations in human mtDNA sequences derived from both contemporary and ancient human samples. The results are discussed with special reference to the evolution of mtDNA sequences at the gene level, as well as at the population level.

2. MATERIALS AND METHODS

(*a*) *Subjects*

(i) *Contemporary humans*

A total of 128 individuals from five ethnic groups (African, Caucasian, Japanese, Asian and native American) were analysed. The mtDNA sequence data for 101 individuals were previously published (Anderson *et al.* 1981; Greenberg *et al.* 1983; Horai & Hayasaka 1990). The newly collected samples of 27 individuals consisted of purified genomic DNA, 20 of which were from southeast Asians (Malay, Indonesian, Thailander, etc.) obtained in Tokyo, and seven were from native Americans (one North American Indian, three Mayas and three Brazilians) provided by A. C. Wilson (University of California, Berkeley).

(ii) *Archaeological samples*

Mummy tissues: 11 specimens of native Americans were examined from two archaeological sites. Six of them were from the southwest United States and five were from the northernmost part of Chile (Arica), provided by C. G. Turner II (Arizona State University). *Bones*: a total of ten specimens of ancient Japanese bones were subjected to DNA extraction, of which four specimens were from the Jomon age (3000–6000 years BP) and six were from the early modern Ainu (200–300 years BP) in Hokkaido.

(*b*) *Polymerase chain reaction* (PCR)

Genomic DNA was prepared from heparinized blood by treatment with SDS and proteinase K and subsequent phenol/chloroform extraction. The method for extraction of DNA from bones has been described elsewhere (Horai *et al.* 1989). DNA extraction from mummified tissues was done by the method described by Pääbo *et al.* (1988).

A fragment of mtDNA was amplified by using the method described by Saiki *et al.* (1988). Three sets of oligonucleotide primers were designed as follows to amplify different regions of mtDNA: primer A
(16011-5′-AAACTATTCTCTGTTCTTTC-3′-16030) and primer B
(41-5′-AAAATACCAAATGCATGGAG-3′-22)
for the major non-coding region, primer C
(16190-5′-CCCCATGCTTACAAGCAAG-3′-16208) and primer D
(16422-5′-ATTGATTTCACGGAGGATGG-3′-16403)
for a highly polymorphic portion of the major non-coding region, and primer E
(8211-5′-TCGTCCTAGAATTAATTCCC-3′-8230) and primer F
(8310-5′-AGTTAGCTTTACAGTGGGCT-3′-8291)
for region V, where the Asian-specific deletion is found. (The notation of Anderson *et al.* (1981) is used for numbering of bases.) These primers were synthesized on an Applied Biosystem model 380B DNA synthesizer. PCR was done for a total of 30 cycles with the use of a thermal cycler (Perkin-Elmer Cetus). The cycle times were as follows: denaturation, 10 s at 94 °C; annealing, 10 s at 45 °C; primer extension, 15 s at 72 °C. The amplified fragments were separated by electrophoresis on 1.5% agarose gels and detected fluorographically after staining with ethidium bromide. In the case of ancient DNAs, one tenth of the first PCR products were used as templates for the second PCR, done in exactly the same manner as the first PCR. Single-stranded

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DNAs were amplified from the agarose extracts of the products from the first or second PCR and direct sequencing (Sanger *et al.* 1977; Gyllensten & Erlich 1988) was done with the Sequenase Kit (U.S. Biochemical).

(*c*) *Analysis of data*

The number of nucleotide substitutions per site between individual sequences was estimated using the six parameter model of nucleotide substitution (Gojobori *et al.* 1982). On the basis of the estimated number of nucleotide substitutions, a phylogenetic tree was constructed using the unweighted pair-group (UPG) method (Sokal & Sneath 1963; Nei 1987).

3. RESULTS AND DISCUSSION

(*a*) *Phylogeny of mtDNA sequences in contemporary humans*

In the present study, the nucleotide sequence analysis of the major non-coding region was extended using data from an additional 27 Mongoloids, mainly from southeast Asia and America. The 482 b.p. sequences from a total of 128 individuals were aligned and compared, and the number of nucleotide substitutions between each pair of sequences was also estimated. The nucleotide diversity among the 128 individuals is estimated at 1.46%, which is three- to sixfold higher than the estimates based on restriction analysis of the entire mitochondrial genome reported in human populations (Brown 1980; Horai *et al.* 1986; Cann *et al.* 1987). Based on the estimated number of nucleotide substitutions between individual sequences, a phylogenetic tree was constructed by the UPG method, as shown in figure 1. On the basis of clustering patterns of the tree, all lineages were classified into five clusters, indicated by brackets with cluster numbers (C1–C5).

The phylogenetic analysis indicates that the diversity among the Africans is much larger than that among the Europeans or the Asians. In fact, the majority of Africans (C1 in figure 1) diverged first from the rest of the individuals in the phylogenetic tree. A group of the Asian population (C2 in figure 1) followed this early divergence. The remainder of the Asians (C3–C5 in figure 1) diverged subsequently, together with the Europeans. To evaluate the features of clustering patterns, the composition of the five clusters and geographic distribution of the 128 human lineages are summarized in table 1. African lineages appeared not only in C1 but also in every other cluster, although there was only one lineage for each cluster. This indicates that Africans possess highly diversified mtDNA sequences. By contrast, the majority of European lineages fell into C4, with the exception of four lineages, two of which appeared in each of C2 and C5. Japanese and Asian lineages, however, were dispersed into every cluster except C1, suggesting that Asians, including Japanese, are much more diverse than are Europeans. For seven native American lineages, three appeared in C3 and four in C5. Although the number of native Americans is small, the absence of these lineages in C2 and C4 may suggest that a somewhat small founder population went across the Bering Strait to the American continent in the past.

We analysed the nucleotide diversities within and between the racial groups on the basis of estimates of the number of nucleotide substitutions between individual sequences. The nucleotide diversity within Africans was much larger than that within Europeans or Asians. It was also larger than that among racial groups. In contrast, the nucleotide differences between Asians-1 (Mongoloid individuals from C2 cluster) and Asians-2 (those from the rest of the clusters), were much larger than those between Europeans and Asians-2. Furthermore, the nucleotide diversity among Asians-1 was much larger than that among Asians-2. The two subpopulations of Asians correspond roughly to groups I and II of the Japanese inferred from restriction-enzyme analysis (Horai & Matsunaga 1986). From restriction-enzyme analysis, nucleotide diversity within group I was found to be larger than that within group II. The present observation, therefore, agrees with earlier findings deduced from restriction-enzyme analysis (Horai *et al.* 1986; Cann *et al.* 1987) and confirms our quantitative study of the nucleotide sequences (Horai & Hayasaka 1990). Thus it is evident that the Asian population can be separated into two subpopulations.

The number of mutated sites in the major non-coding region from three racial groups, Africans, Europeans and Mongoloids (Asians and native Americans) was counted. From the total of 128 individuals, base changes were observed at 131 sites. Of these, 12 sites were common among the three racial groups. Twenty sites were shared between the two racial groups; 14 by the Africans and Mongoloids, two by the Europeans and Africans, and four by the Europeans and Mongoloids. The presence of polymorphic sites shared by two or three racial groups is probably due to an ancient polymorphism, i.e. mtDNAs were already polymorphic at these sites before the divergence of racial groups. By contrast, we also observed many polymorphic sites that were specific for each race; 68 for Mongoloids, 17 for Africans and 13 for Europeans. When these numbers were proportioned to the numbers of individuals tested (98, 10 and 20 respectively), race-specific polymorphic sites were found predominantly in Africans. This indicates that Africans are much more diverse than Europeans or Mongoloids as indicated by the analysis of nucleotide diversity between sequences.

(*b*) *Amplification of mtDNA from ancient Japanese bones*

The development of PCR made it possible to amplify the DNA in targeted regions from a very small amount of template DNA. By applying this technique to archaeological or ancient samples we have successfully amplified the DNA and analysed part of the nucleotide sequence. However, such an analysis was possible only in the case of soft tissues, such as frozen, mummified or stuffed tissue, haired skin and muscle tissue (Johnson *et al.* 1985; Higuchi *et al.* 1984; Pääbo 1985, 1989; Pääbo *et al.* 1988). Such suitable samples have

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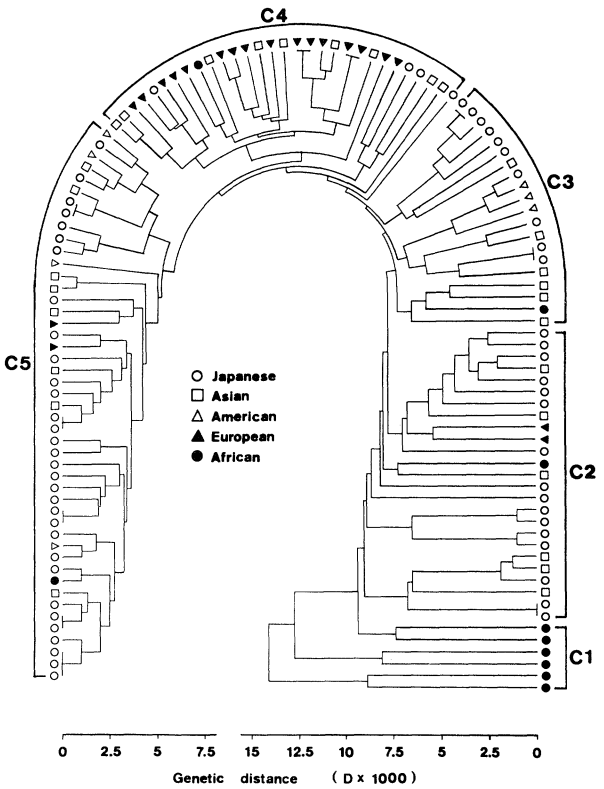

Figure 1. Phylogenetic tree showing the 128 mtDNA lineages from the five ethnic groups, based on sequence data from the major non-coding region (482 b.p.). All lineages are classified into five clusters which are designated C1–C5 according to clustering patterns. Distances (D) are expressed by the number of nucleotide substitutions per site per lineage.

Table 1. *Composition of the five clusters and geographic distribution of 128 human lineages*

	number of lineages from				
cluster	Africa	Europe	Japan	Asia	America
C1	6	0	0	0	0
C2	1	2	16	6	0
C3	1	0	10	6	3
C4	1	16	4	9	0
C5	1	2	32	8	4
Total	10	20	62	29	7

generally been preserved artificially or accidentally. Most of the human remains preserved to date are hard tissues, such as bones. If we can amplify DNA extracted from bones and analyse it, this would probably present some quite important information relevant to the process of human evolution, the divergence of human races, the restoration of details of human populations in the past, and their migration or dispersal (Horai *et al.* 1989; Hagelberg *et al.* 1989).

For this study the nucleotide sequences of the major non-coding region of mtDNA were analysed from 128 contemporary humans of various ethnic origins. These sequences were used to define a region suitable for PCR analysis of archaeological samples, because the distribution of mutations in the major non-coding region is significantly nonrandom (Horai & Hayasaka 1990). To design an appropriate pair of primers for PCR we looked for a highly polymorphic region of less than 250 b.p. This limitation in length is based on a preliminary experiment using archaeological samples; amplifications were successful when targeted regions were less than about 250 b.p. in length (S. Horai, unpublished observation). We therefore chose the set of primers C and D which give rise to a 233 b.p. fragment for amplification by PCR. This amplified region was

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                                                  50                                                  100
 1. ANDERSON  CCCCATGCTTACAAGCAAGTACAGCAATCAACCCTCAACTATCACACATCAACTGCAACTCCAAAGCCACCCCTCACCCACTAGGATACCAACAAACCTA
 2. URAWA-1   ....................................T.....................................................................
 3. TODA-1    ....................................T.....................................................................
 4. TAKA-5    ....................................T.....................................................................
 5. TAKA-13   ....................................T.....................................................................
 6. TAKA-21   ....................................T.....................................................................
 7. KAMO-1    ....................................T.....................................................................
 8. KAMO-2    ....................................T.....................................................................
 9. KAMO-3    ....................................T.....................................................................
10. SUTTU-1   ....................................T.....................................................................
11. SUTTU-2   ....................................T.....................................................................
12. CHITOSE   ....................................T.....................................................................

                                                 150                                                 190
 1. ANDERSON  CCCACCCTTAACAGTACATAGTACATAAAGCCATTTACCGTACATAGCACATTACAGTCAAATCCCTTCTCGTCCCCATGGATGACCCCC
 2. URAWA-1   .....T.....................................................................C..................
 3. TODA-1    ..........................................................................C..................
 4. TAKA-5    ..........................................................................C..................
 5. TAKA-13   ..........................................................................C..................
 6. TAKA-21   ..........................................................................C..................
 7. KAMO-1    ..........................................................................C..................
 8. KAMO-2    ....T.....................................................................C..................
 9. KAMO-3    ....T.....................A...............................................C..................
10. SUTTU-1   ....T.....................................................................C..................
11. SUTTU-2   ..........................................................................C..................
12. CHITOSE   ..........................C.....................................................C..................
```

Figure 2. Nucleotide sequences of the 190 b.p. region (b.p. 16190–16379) from ancient Japanese bones. The sequence reported by Anderson *et al.* (1981) is shown in line 1. Base substitutions observed in respective samples are shown by letters in the lower lines. The origins of each samples are as follows: lines 2 and 3, Jomon age specimens from the Honshu area; lines 4–6, Jomon age specimens from the Hokkaido area; lines 7–12, early modern Ainu of Hokkaido.

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shown to accumulate more than 68% polymorphic positions of the entire 482 b.p. region. Moreover, both 5′ and 3′ flanking portions of 20 nucleotides in the region were almost invariant, so that these two flanking portions were appropriate for oligo-primers of PCR.

In a preliminary experiment, we succeeded in detecting a nucleotide sequence of 190 b.p. directly from the PCR product of a Jomon skull (Urawa-1) (Horai *et al.* 1989). In the present study, sequence analysis of the highly polymorphic region of mtDNA from archaeological specimens was extended with the addition of four bones from the Jomon age, and six from the early modern Ainu in Hokkaido. The determined nucleotide sequences of the 190 b.p. region of these samples are shown in figure 2.

The nucleotide sequences from archaeological specimens were compared with the corresponding sequences from 128 contemporary humans, and the number of nucleotide substitutions between each pair of sequences was also estimated. The nucleotide diversity among the 139 sequences in the 190 b.p. region was estimated to be 2.26%, one and half times higher than that obtained from the entire 482 b.p. region. Based on the estimated number of nucleotide substitutions between sequences, a phylogenetic tree was constructed by the UPG method, as shown in figure 3. Six lineages of ancient individuals, four Jomon and two Ainu, came into a final cluster, which branched off at point 'a' in this tree. This cluster also included 14 contemporary Japanese and four Asians from southeast Asia (Malaysia and Indonesia). This observation suggests that part of the native Japanese (Jomon and Ainu) share a close phylogenetic affiliation with part of the contemporary Japanese as well as southeast Asians. It is also noted that all of Jomon and Ainu lineages were placed in a larger cluster which diverged at point 'b' in the tree, indicating that the Jomon peoples and the Ainu are genealogically different from contemporary Japanese who belong to earlier diverging clusters in the tree.

On the origin of the modern Japanese, two main hypotheses have been presented: the transformation theory and the immigration theory. The former claims that the Neolithic Jomon people evolved without admixture into the modern Japanese, where the latter postulates that immigrants from the Asian continent during the Aeneolithic Yayoi (1700–2400 years BP) and protohistoric Kofun (1400–1700 years BP) periods made a considerable contribution to the formation of modern Japanese. Many of the recent craniometric, non-metric and dental studies investigating the origin of the modern Japanese have yielded results in favor of the immigration theory. The latest cranial non-metric studies have revealed (i) the Jomon peoples and the Ainu are closely related to each other and (ii) the protohistoric Kofun and the Aeneolithic Yayoi peoples are much closer to the modern Japanese of Mongoloid stock than to the Neolithic Jomon (see Dodo & Ishida 1990, and references therein). Our results from the mtDNA sequence analysis support the immigration theory.

It has been shown, both from the restriction-enzyme analysis and from sequence analysis of the major non-coding regions, that the Japanese population can be

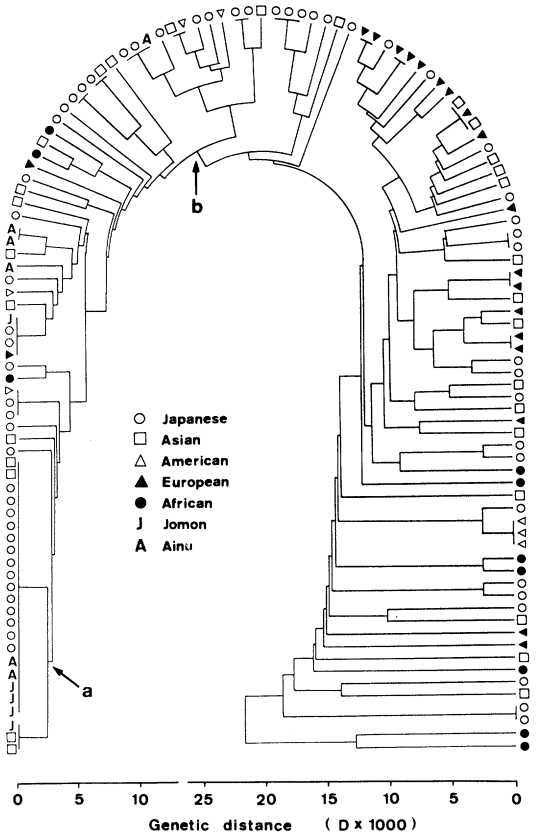

Figure 3. Phylogenetic tree showing the 139 mtDNA lineages from five ethnic groups and ancient Japanese bones of three different ages, based on the sequence data from 190 b.p. in the major non-coding region. Distances (D) are expressed by the number of nucleotide substitutions per site per lineage. Ainu specimens are *ca.* 200–300 years of age. Three specimens of Hokkaido Jomon and two specimens of Honshu Jomon are *ca.* 3000 and 6000 years of age, respectively. Points 'a' and 'b' represent the branching points of ancient Japanese lineages (see text for the explanation).

separated into at least two distinct groups. In this respect, the native Japanese (Jomon people and Ainu) belong to one of the two groups (group II) of modern Japanese. They showed three to eight nucleotide differences in the 190 b.p. region from individuals in group I (data not shown), which suggests that some of the people who migrated from the continent around 2000 years ago (Yayoi age) may be representatives of the other group (group I) of modern Japanese. Although our findings were made by determining the nucleotide sequences from 11 ancient individuals, this archaeological and molecular genetic study adds a new perspective to the evolutionary history of human populations.

(*c*) *Detection of an Asian-specific 9 b.p. deletion in mtDNA*

The deletion of the 9 b.p. fragment in a small non-coding region of mtDNA (region V: Cann & Wilson 1983) is one of the characteristics of individuals who belong to the group I cluster of Japanese. Individuals belonging to group II are likely to have two tandemly repeated copies of the 9 b.p. sequence, as deduced from restriction analysis with *Hae* III.

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Table 2. *Frequency of the Asian-specific deletion in Pacific populations*

population	sample size	deletion frequency (%)	reference
Japan			
Mainland	254	15	Horai (1991*b*)
Okinawa	82	5	Horai *et al.* (1987)
Taiwan			
Chinese	132	17	Horai (1991*a*)
Native	120	28	Horai (1991*b*)
East Asia	34	18	Cann (1982)
Indonesia			
Moluccas	50	16	Stoneking *et al.* (1991)
Nusa Tengarra	96	24	Stoneking *et al.* (1991)
Australia	21	0	Cann (1982)
	30	0	Hertzberg *et al.* (1989)
Highland PNG[a]	64	0	Stoneking & Wilson (1989)
	30	0	Hertzberg *et al.* (1989)
Coastal PNG[a]	55	42	Stoneking & Wilson (1989)
	68	10	Hertzberg *et al.* (1989)
Fiji	28	82	Hertzberg *et al.* (1989)
Tonga	30	77	Hertzberg *et al.* (1989)
Cook Islands	30	87	Hertzberg *et al.* (1989)
Niue	30	100	Hertzberg *et al.* (1989)
Samoa	30	100	Hertzberg *et al.* (1989)
New Zealand (Maori)	30	100	Hertzberg *et al.* (1989)
America			
contemporary	7	0	present study
mummy	11	9	present study

[a] PNG, Papua New Guinea.

Amplification by PCR of the relevant segment, followed by determination of size by electrophoresis, is a rapid and efficient method of screening for the deletion. Recent sequence analysis of the relevant region of mtDNA (region V) from several hominoid species, human, chimpanzee, gorilla and orangutan, revealed that two copies of the 9 b.p. sequence were acquired in the human lineage. The uniqueness of the 9 b.p. tandem repeats to one human lineage suggests that the loss of one of the two copies, as seen in another human lineage, arose once in the ancestor of an Asian lineage (Wrischnik *et al.* 1987; Horai 1991*b*).

By using PCR, targeted regions of mtDNA from seven contemporary native Americans were screened for the deletion. In each individual we observed an amplified fragment of 100 b.p. which corresponded to the presence of two copies of the 9 b.p. sequence, indicating that no Asian-specific deletion was observed in contemporary native Americans. The presence of the Asian-specific deletion was also examined in 11 mummy samples of native Americans from two archaeological sites, one from southwest United States and the other from the northernmost part of Chile (Arica). Although the exact dating of these samples has not yet been determined, information from burial accessories indicate that the U.S. samples were at least prehistoric (i.e. pre-European contact) and those from Chile were between 4000 to 1000 years BP (C. G. Turner II, personal communication). In some mummy samples we observed two bands (91 b.p. and 100 b.p.) at the corresponding position in gel analysis after PCR amplification. However, the lower band of 91 b.p. was an artefact of the PCR procedure, probably the result of degradation of ancient DNA. Consequently, the real deletion was found in one of the six samples from the U.S. and in none of the five samples from Chile. Information on the frequency of the deletion in several Asian and Pacific populations determined from present and previous studies is summarized in table 2. The deletion is absent in Australian aborigines and in highlanders from Papua New Guinea but occurs in every other Asian and Pacific population examined; in particular it is present at a very high frequency in Polynesians. It is, however, worthwhile to note that one archaeological specimen of a native American whose ancestor presumably migrated from the Asian continent also showed the Asian-specific 9 b.p. deletion.

4. CONCLUSION AND PERSPECTIVES

It is clear that the Circum-Pacifican populations (Asians, Oceanians and native Americans) can be separated into two subpopulations according to results from sequence analysis as well as from PCR analysis for detection of the 9 b.p. deletion. This observation confirms the results of our earlier study that clearly showed the existence of two distinct groups of Japanese as determined by restriction-enzyme analysis (Horai & Matsunaga 1986). Most of those belonging to the group I cluster in the Japanese population have a deletion of 9 b.p. in region V (Cann & Wilson 1983). This deletion is observed not only in east Asians (Cann & Wilson 1983), Japanese (Horai & Matsunaga 1986; Horai *et al.* 1987), Indonesians (Stoneking *et al.* 1991), Chinese from Taiwan (Horai 1991*a*), natives of

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Taiwan (Horai 1991*b*) and a native American mummy (present study), but also with a very high frequency in Polynesians Hertzberg *et al.* 1989). The same deletion has never been found in Europeans or Africans with the exception of some Pygmies (Vigilant 1990). Thus, the existence of the two major groups is one of the characteristics found commonly among Mongoloid populations. Recently, an mtDNA sequence derived from a 7000-year-old brain from Florida (considered to be that of an ancestor of American Indians) was partly determined by Pääbo *et al.* (1988). They found a base substitution at position 8251 when compared with the reference sequence (Anderson *et al.* 1981). The base substitution causes the loss of the site specific for *Hae*III and the simultaneous site gain of a site specific for *Ava*II. This variation was also found in five types (five individuals) of Japanese according to our previous study (Horai & Matsunaga 1986). These types are included in group II of our phylogenetic tree and are observed as a small cluster.

Mongoloid descendants are now distributed over a wide area of the Circum-Pacific region, having adapted to a wide variety of environments. An extended analysis of mtDNA polymorphism and its genealogy, using both contemporary and archaeological samples, will provide significant information relevant to our attempts to elucidate the history of the dispersal and adaptation of Mongoloids.

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Discussion

K. A. Joysey (*University Museum of Zoology, Cambridge, U.K.*). We are all aware of the danger of amplification of contaminants when using the PCR technique. It therefore seems fair to enquire whether there are any non-Japanese Asians working within Dr Horai's laboratory?

S. Horai. To avoid contaminations of contemporary human DNA, we are always very careful to perform all the PCR procedures. For example, only a few workers (Japanese) engage in the PCR experiment when amplifying DNA from ancient samples. Furthermore, all disposable plastic wares such as test tubes and tips of pipettes were irradiated by UV light to kill contemporary DNAs, if they exist, before use. Adequate cautions during every step of the PCR analysis give reliable and reproducible results.

G. A. Dover (Department of Genetics, University of Cambridge, U.K.). It could well be that the mechanism generating the 9 b.p. duplication and the subsequent loss of one of the duplicate copies has occurred coincidentally in different human populations. Such mechanisms often occur at a rate of one to two orders of magnitude faster than the point mutation rate, such that the simultaneous appearance of the same 9 b.p. deletion might not signify an ancient common ancestor between migrant populations, without corroborating evidence among the same mtDNA sequences.

S. Horai. Individuals from Asian and Pacific populations who showed the 9 b.p. deletion in region V of mtDNA exclusively appeared in an early diverging Asian cluster of the phylogenetic tree constructed by using sequence data of the major non-coding region. Consequently, as far as Asian and Pacific populations are concerned, the 9 b.p. deletion has arisen once in the ancestor of a Mongoloid lineage.

S. Pääbo (*Institute of Zoology, University of Munich, F.R.G.*). What is Dr Horai's success rate in bone amplification?

S. Horai. Success in this regard is probably dependent on the condition of preservation of bones. In our experience, bones excavated from wet ground give a good DNA amplification.

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S. Pääbo. Does deletion occur in amplification of ancient DNA?

S. Horai. When we amplified region V of mtDNA, in some mummy samples we observed two bands at the corresponding position of the gel. The lower band, which is the deletion form of one of two copies, is the PCR artifact probably due to degradation of ancient DNA.

S. Pääbo. Does deletion also exist in Pygmies?

S. Horai. Linda Vigilant from A. C. Wilson's laboratory observed the 9 b.p. deletion in region V of mtDNA in some African Pygmies. This observation indicates that the 9 b.p. deletion has occurred twice in human evolution.

S. Pääbo. What are the advantages of HCl extraction compared with other techniques?

S. Horai. We also attempted to extract the DNA from bone after decalcification with EDTA. But it took a longer time to complete decalcification than with HCl. Moreover, it is necessary to remove all the EDTA completely for further steps of DNA amplification.

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