Mitochondrial DNA analysis of Jomon dogs from the Kamikuroiwa Rock Shelter site in Shikoku and the Higashimyo site in Kyushu, Japan

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Abstract To investigate the genetic variation of Jomon dogs (Canis familiaris) in Japan, partial sequences of the mitochondrial DNA (mtDNA) control region were determined from dog bone remains, which were excavated from two Jomon-period archaeological sites, the Kamikuroiwa Rock Shelter site in Ehime Prefecture (Shikoku Island) and the Higashimyo site in Saga Prefecture (Kyushu Island), Japan. Of seven individuals from the Kamikuroiwa Rock Shelter site, the mtDNA sequences from two individuals were successfully determined. Comparison of the resultant 215 base pair sequences with previously reported sequences showed that one of these two individuals had a new haplotype, named KRA1, and that the other had the previously reported M1 haplotype. For the Higashimyo site, three of 11 individuals yielded successful sequences. Two of these three individuals shared M1 and the other one had M20, both of which are haplotypes previously reported in modern Japanese dogs, but had not been found in any Jomon dogs. The success rate in the present study was 27.8% (5/18 samples). It is noticed that the three mtDNA haplotypes (M1, M20 and KRA1) were found in Jomon dogs for the first time. In addition, sequence data were obtained from Jomon dogs in Shikoku for the first time. The results suggest that the genetic lineages in the Jomon dog populations were more polymorphic than previously reported, and that at least some maternal lineages shared in the Jomon period descended to modern dogs on the Japanese islands.

Key words: ancient DNA, control region, Japanese islands, Jomon dog, mitochondrial DNA

Introduction

Domestication of the dog (Canis familiaris) occurred during the history of human migration and culture development. Tracing and characterizing the genetic lineages of dogs endemic in local regions has contributed to our further understanding of anthropological history. On the Japanese islands, many dog remains have been found in archaeological sites dating from the Jomon period (16500–2800 years BP). To date, several reports have characterized Jomon dogs based on morphological (e.g. Hasebe, 1952; Saito, 1963; Naora, 1973; Komiya, 1997; Nishimoto, 1983; Shigehara, 1991, 2003; Nishinakagawa et al., 1992) and genetic features (Okumura et al., 1999). Okumura et al. (1999) analyzed many samples of Jomon dogs, which were excavated from archaeological sites of the Jomon period on Honshu and Kyushu Islands of Japan. However, little data exists on Jomon dogs on Kyushu Island, and no genetic information on Jomon dogs of Shikoku Island is available.

Recently Jomon dog bones from two archaeological sites of the early Jomon period, about 7000 years BP, were meticulously re-examined: the Kamikuroiwa Rock Shelter site in Ehime Prefecture (Shikoku Island) and the Higashimyo site in Saga Prefecture (Kyushu Island) (Nishida, 2004; Maruyama et al., 2009; Sato et al., 2015). Any biological information on these Jomon dogs can provide insights that further our understanding of the archaeological and anthropological histories of the Japanese islands.

In the present study, we analyzed mitochondrial DNA (mtDNA) sequences of Jomon dogs from the Kamikuroiwa Rock Shelter site and the Higashimyo sites to ascertain the genetic characteristics and the phylogenetic relationships between those dogs and previously reported ancient and modern dogs on the Japanese islands.

Materials and Methods

Materials
We analyzed ancient DNA from bone remains, which were excavated from two archaeological sites of the Jomon period: the Kamikuroiwa Rock Shelter site, which is located...
in Ehime Prefecture (Esaka and Nishida, 1967; Esaka et al., 1969; Esaka, 1970); and the Higashimyo site in Saga Prefecture (Nishida, 2004; Maruyama et al., 2009). For the Kamikuroiwa Rock Shelter site, bones from seven individuals (four right femurs and three vertebrae) were used, and for the Higashimyo 11 mandibles (three left and eight right) from 11 individuals were analyzed. The individuality of bone samples was identified based on right/left sides, size differences of the same and different portions, and archaeological information about the excavated layers.

DNA extraction and polymerase chain reaction

The DNA extraction from archaeological bone remains was performed according to the method of Masuda et al. (2001, 2006). Bone powders obtained (about 0.2–0.3 g) were used for the DNA extraction. The DNA extracts were concentrated with a VivaSpin6 centrifuge (Sartorius). Part of the mtDNA control region was amplified using the polymerase chain reaction (PCR) and the PCR products were directly sequenced. Two primers, mitL57 and mitH52, reported by Okumura et al. (1999), were used for the PCR. Because no fragments were successfully amplified in several samples, we designed two new primers: DG1 (5’-TCGTCATTAATGGTGTGCCC-3’) and DGR1 (5’-TATTATGACATGCTTATATGC-3’). The fragments (215 base pairs, bp) amplified by a primer set mitL57/mitH52 were sequenced. When this amplification was not successful, semi-nested PCR (second PCR) was performed on the first PCR product with mitL57/DGR1 or DG1/mitH52. Otherwise direct PCR with mitL57/DGR1 or DG1/mitH52 was performed. The fragments (about 150 bp) amplified by primer sets mitL57/DGR1 and DG1/mitH52 were overlapped in a partial region of sequences and combined, resulting in 215 bp sequences useful for sequence analysis.

The PCR was performed in a total volume of 20 μl containing 10 μl of 2 × Multiplex PCR Master Mix (Qiagen), 0.2 μl of each primer (25 pmol/μl), 0.4 μl of bovine serum albumin (20 mg/ml), and 1–2 μl of concentrated DNA extract, adjusted by double-distilled water; cycling conditions were 95°C for 15 min; 40 cycles of 94°C for 30 s, 50°C for 3 min (or 56°C for 1.5 min), and 72°C for 1 min (or 72°C for 1.5 min); and 72°C for 10 min. The above-mentioned semi-nested PCR (second PCR) was performed in a total volume of 20 μl containing 0.2 μl of rTaq polymerase (Takara), 2 μl of 10 × buffer (Takara), 1.6 μl of dNTP (Takara), 0.2 μl of each primer (25 pmol/μl), and 1–2 μl of the first PCR product, adjusted by double-distilled water; cycling conditions were 95°C for 3 min; 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. To check PCR amplification, an aliquot of 10 μl from the PCR product was electrophoresed on a 2% agarose gel, stained by ethidium bromide, and visualized under an ultraviolet illuminator. Then, the PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and applied to a BigDye Terminator v. 1.1 Cycle Sequencing Kit for use in an Applied Biosystems (ABI) 3130 Genetic Analyzer or 3730 DNA Analyzer.

To confirm the authenticity of the ancient DNA sequence data, extracts without any samples obtained by the same method as bone samples were used as negative controls. Disposable gloves were used, and most experiments were performed on an air-filtered benches to prevent any external DNA contamination. Whenever PCRs with bone sample extracts were performed, it was confirmed that no PCR products from negative controls with the same reaction condition were found.

Sequence data analysis

Obtained nucleotide sequences were aligned using MEGA 5.0 software (Tamura et al., 2011). The minimum spanning networks among mtDNA haplotypes were constructed using TCS 1.21 software (Clement et al., 2000). For characterization of mtDNA sequences obtained in the present study, these were compared with those of Japanese dogs previously reported by Okumura et al. (1999) and those registered in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database.

Results and Discussion

In the present study, of seven ancient dogs examined from the Kamikuroiwa Rock Shelter site, the partial sequences (215 bp) of the mtDNA control region from two individuals were successfully determined (28.6% success rate). This is the first time mtDNA sequences have been determined from ancient dog remains from a Jomon-period archaeological site located on Shikoku Island. One individual (KR4901: partial right femur) had a new haplotype and the other (Dog [No. 1]: vertebra) had mtDNA haplotype M1, which was reported by Okumura et al. (1999) (Table 1). The new haplotype had two nucleotide substitutions with haplotype A2, which was shared only by five Jomon dogs of Miyagi Prefecture (northern Honshu Island), reported by Okumura et al. (1999) (Figure 1). This new haplotype identified in the present study was named KRA1. Meanwhile, M1 was reported to be shared by one ancient dog of the Okhotsk period (Susuya Shell Mound, Sakhalin), eight animals of three modern Japanese indigenous breeds (Shiba, Akita, and Kishu) and four animals of non-Japanese breeds (Okumura et al., 1999). The sequence of KRA1 appears in the DDBJ nucleotide sequence database with the accession number AB938311.

For the Higashimyo site, mtDNA sequences were successfully determined from three of 11 individuals examined (27.3% success rate). Two (#6717 and #11843) of the three individuals shared M1 and the other (#15065) had M20, both

| Table 1. Nucleotide sequences of three mtDNA haplotypes (M1, M20 and KRA1) identified in the present study from Jomon dogs from the Kamikuroiwa Rock Shelter site and from the Higashimyo site |
|-------------|---|---|---|---|---|---|---|---|---|---|
| Haplotype   | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| M1          | G | C | C | C | T | A | C | A | A | G |
| M20         | T | T | C | T | G | G | A | A | A | A |
| KRA1        | A | T | C | T | G | T | G | A | A | A |

Numbers show nucleotide site numbers among the 215 bp, which correspond with those of Okumura et al. (1999). Dots indicate identities of nucleotides for M1.
of which were haplotypes previously reported in modern Japanese dogs: M1 was from the Japanese and non-Japanese dog breeds as mentioned above, and M20 was from four animals of two Japanese breeds (Ryukyu and Shiba) and 19 animals of non-Japanese breeds (Okumura et al., 1999).

It is noticed that all three haplotypes (M1, M20 and the new type KRA1: Figure 1, Table 1) found by the present study were identified in Jomon dogs for the first time. These results show that the origins of M1 and M20 currently shared by modern dogs can be traced to dogs of the early Jomon period. This likely supports Okumura et al.’s (1999) suggestion that M1 (and M5, which was not found in the present study) may be a common ancestral type of modern Japanese dogs.

Okumura et al. (1999) reported that all haplotypes identified from the Jomon dogs were phylogenetically classified to cluster 1 (CL1). By contrast, in the present study, we found that one dog from the Higashimyo site in Kyushu had haplotype M20, which is classified to cluster 3 (CL3) (see Figure 1). In addition, the new haplotype KRA1 can be classified to CL1, as shown in Figure 1. These new findings indicate that the maternal lineages in the Jomon dog populations were genetically more polymorphic than those of the Jomon dog population initially reported by Okumura et al. (1999). Okumura et al. (1999) indeed found 14 mtDNA haplotypes only in ancient Japanese dogs, which were excavated from archaeological sites of the Jomon, Yayoi and Okhotsk periods. Okumura et al. (1999) also described that, among the Jomon dogs, the frequency of M2 was higher in southern Japan, whereas that of M5 was higher in northern Japan. However, it is interesting to note that both haplotypes were not found in the Kamikuroiwa site and the Higashimyo site, although the sample numbers were small in the present study. As seen in Figure 1, both M2 and M5 are phylogenetically closely related to M1 found in the Jomon dogs of the present study, because M1 has only one nucleotide substitution with M2 and M5. The genetic information on early Jomon dogs obtained in the present study will contribute not only to further analysis of ancient DNA of Jomon dogs but also to the archaeological study of the Jomon culture on the Japanese islands.

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