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Analysis of mitochondrial DNA HVR1 haplotype of pure-bred domestic dogs in Japan

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ABSTRACT

To develop DNA markers for forensic analysis, we examined the hypervariable region 1 (HVR1) sequences of 447 pure-bred domestic dogs (*Canis lupus familiaris*) that had been bred and raised in Japan. HVR1 is a 660-bp stretch of mitochondrial (mt) DNA. Among the 447 HVR1 sequences examined, we identified 58 haplotypes from 47 single nucleotide polymorphisms (SNPs) and two insertion-deletion (InDel) polymorphisms. The haplotype diversity inferred from inter-breed analysis ($N = 154$, 88 breeds) was 0.929 ± 0.011. Intra-breed analysis showed that the haplotype diversity of Golden Retrievers ($N = 53$), Labrador Retrievers ($N = 67$), Miniature Dachshunds ($N = 61$), Toy Poodles ($N = 62$), and Welsh Corgis ($N = 50$) was 0.624 ± 0.052, 0.722 ± 0.029, 0.922 ± 0.010, 0.877 ± 0.020, and 0.443 ± 0.084, respectively. The results of this genotype analysis were used to construct a dataset consisting of dog mtDNA HVR1 sequences for use in forensic applications in Japan.

1. Introduction

According to the Japan Pet Food Association, approximately 12 million dogs were bred and raised in Japan in 2011 [1]. According to the report, 17.7% of Japanese households had one or more pure-bred dogs, which is equivalent to 1.26 dogs per household. These data imply that the human–dog (or human-companion animal) in Japan bond is strong. The report also states that approximately 6500 dogs were either killed or separated from their owners due to the Great East Japan Earthquake that struck Japan on March 11, 2011 [1]. For those dog owners, forensic dog remains (e.g., hair samples) collected after the tsunami could be used to identify lost or missing animals. In addition, such remains could also be used in criminal investigations to link persons of interest to a crime scene. The utility of mitochondrial DNA (mtDNA) [2–10] and/or short tandem repeat (STR) polymorphisms [11–15] in forensic analysis has been extended in recent years and DNA sequences from dogs can now be used in many of the same ways as DNA sequences from humans. The complete nucleotide sequence of the mitochondrial genome of the domestic dog, *Canis familiaris*, is approximately 16,728 bp, which contains 13 protein-coding genes, 22 tRNAs, and 2 rRNAs [16]. As with human mtDNA, dog mtDNA also contains polymorphic non-coding regions referred to as hypervariable regions (HVRs). One such region, HVR1, was analyzed in this study. In 1997, Savolainen et al. [3] created a population database of the dog mtDNA HVR1 region for forensic investigations and population genetic analyses. Since then, similar databases of the HVR1 and/or HVR2 regions of dogs have been complied by several forensic groups; these databases are considered important resources for forensic genetics.

In Japan, very little research in forensic genetics has focused on DNA polymorphisms in pure-bred dogs [17–19]. Moreover, genetic differences between dogs in Japan and those in other countries are currently unknown. We therefore investigated mtDNA HVR1 diversity among 447 pure-bred dogs in Japan to develop a database of DNA polymorphisms that could be used for forensic analysis.

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2. Materials and methods

2.1. DNA samples

Dog mtDNA samples were selected from our collection of dog DNA samples, which we extracted from peripheral white blood cells using a Gentra Puregene Blood kit (Qiagen, Santa Clara, CA). We prepared dog mtDNA samples from 154 pure-bred dogs belonging to 88 breeds for inter-breed analysis. In addition, we generated a panel of mtDNA samples from 293 pure-bred dogs belonging to five of the most popular breeds in our collection, i.e. Golden Retriever, Labrador Retriever, Miniature Dachshund, Toy Poodle, and Welsh Corgi for the intra-breed analysis (Table 1). The blood samples were obtained from the Department of Veterinary Clinical Pathology at Nippon Veterinary Life Science University (NVLU) in Musashino, Tokyo, they were originally collected at the Veterinary Medical Teaching Hospital at NVLU with the written consent of each owner.

2.2. PCR amplification

The approximately 790-bp HVR1 region was amplified from each dog mtDNA sample using the H15360 (5′-ATTACCTTGGTCTTGTAAACC-3′) and L16106 (5'-AAACTATATGTCCTGAAACC-3′) primer set [3,6]. The 790-bp region corresponded to nucleotides at position 15,458–16,727 in the complete annotated nucleotide sequence of the domestic dog mitochondrial genome [16]. Each 25 μL PCR mixture comprised 1 μl of DNA, 0.5 μL of primer (20 μM each), 12.5 μL of 2x PCR buffer (30 mM Tris/HCl, pH 8.05, 100 mM KCl, 400 μM dNTP, 5 mM $MgCl_2$, 0.05 U/μl, *Taq* Gold), and water. PCR amplification was performed under the following conditions: 5 min at 95 °C for one cycle followed by 36 cycles of 20 s at 95 °C, 30 s at 51 °C, 40 s at 72 °C; each reaction was then held at 4 °C [3]. After amplification, PCR products were separated on 2% agarose gels and stained with ethidium bromide to confirm PCR amplification.

Table 1
List of 447 DNA samples from 93 breeds.

No. sample	No.of Breed	Breeds
Inter-breed analysis		
1 (each)	34	Afghan Hound, Alaskan Malamute, Australian Cattle Dog, Australian Terrier, Basenji, Basset Hound, Belgian Groenendael, Bouvier des Flandres, Brittany Spaniel, Brussels Griffon, Bull Terrier, Bulldog, Chihuahua, Chihuahua (Long coat), Chindo, Chow Chow, Clumber Spaniel, French Mastiff, Japanese Terrier, Kisyu, Kooikerhondje, Leonberger, Plummer Terrier, Pointer, Polish Lowland Sheepdog, Saint Bernard, Samoyed, Schnauzer, Shar Pei, Tibetan Mastiff, Tibetan Terrier, Weimaraner, Welsh Terrier, White Shepherd Dog
2 (each)	43	Airedale Terrier , Bearded Collie, Bichon Frise, Borzoi, Cairn Terrier, Chinese CrestedDog, Dalmatian, Doberman Pinscher, English Cocker Spaniel, English Springer Spaniel, English Setter, Flat Coated Retriever, French Bulldog, Great Pyrenees, German Shepherd Dog, Kai, Keeshond, Irish Setter, Italian Greyhound, Jack Russell Terrier, Japanese Chin, Maltese, Miniature Pinscher, Miniature Schnauzer, Newfoundland, Norfolk Terrie, Papillon, Pekingese, Pomeranian, Pug, Rottweiler, Saluki, Scottish Terrier, Shetland Sheepdog, Shiba, Shiba(Mame), Siberian Husky, Shih Tzu, Welsh Corgi Cardigan, West Highland White Terrier, Whippet, Wire Haired Fox Terrier, Yorkshire Terrie
3 (each)	10	Akita, American Cocker Spaniel, American Pit Bull Terrier, Beagle, Border Collie, Boston Terrier, Cavalier King Charles Spaniel, Dachshund , Japanese Spitz, Poodle
4	1	Bernese Mountain Dog
Intra-breed analysis		
53	1	Golden Retrievers
67	1	Labrador Retrievers
61	1	Miniature Dachshund
62	1	Toy Poodle
50	1	Welsh Corgi

(): number of animals.

2.3. DNA sequencing

The PCR products were purified using a High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Penzberg, Germany) before sequencing with a BigDye™ Terminator Ver.1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The reaction conditions consisted of 40 cycles of 20 s at 95 °C, 10 s at 50 °C, followed by 4 min at 60 °C; each sample was then held at 4 °C. The primers used for sequencing were H15360, L16106, H15422 (5′-CTCTTGCTCCACCATCAGC-3′), H15840 (5′-TACTCCAATCCTACTAATTC-3′), and L16102 (5′-AACTATATGTCCTGAAACCATTG-3′) alternatively [3]. After cycle sequencing, each sample was purified with the BigDye™ X Terminator Purification Kit (Applied Biosystems). Sequencing was performed on an ABI 310 or a 3730 Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems).

2.4. Data analysis

Each of the approximately 790-bp sequences obtained in this study was trimmed to approximately 660-bp (np15453–16112 of the dog mtDNA genome [16]) and analyzed using GENETYX program (Ver. 9, GENETYX Corporation, Tokyo, Japan). The sequence of each HVR1 mtDNA haplotype in this 660-bp region was deposited at NCBI GenBank as part of a database designated "660-bp dog mtDNA HVR1analysis". Random match probability (P), exclusion capacity (PD), and haplotype diversity (h) of the dog mtDNA HVR1 haplotypes were calculated by $\sum x_i^2$, $1-\sum x_i^2$, and $1-\sum x_i^2[n/(n-1)]$, respectively, where x_i is the frequency of the Ith haplotype and n is the number of haplotypes. The Arlequin [20] and Mega 5 [21] software packages were used for statistical analysis, and the Tamura and Nei model of evolution [22] was used for the ANOVA.

3. Results

We identified 47 single nucleotide polymorphisms (SNPs) and 2 insertion-deletion (InDel) polymorphisms in the 447 HVR1 sequences. The 660-bp sequences that were analyzed corresponded to nucleotides 15,453–16,112 of the dog mtDNA sequence [16]. The 447 samples comprised 154 samples for the inter-breed analysis and 293 samples for the intra-breed analysis. Based on our survey of these 447 HVR1 sequences, the SNPs and InDel polymorphisms formed 58 haplotypes. The sequences of these haplotypes were compiled into a dataset, which we designated the 660-bp dog mtDNA HVR1 dataset and deposited in GenBank under Accession Numbers AB622513 through AB622568 and AB700664 and AB700665 (Table 2).

Table 3 lists the frequencies of the 46 different haplotypes that were identified in the intrer-breed analysis ($N = 154$, 88 breeds). Haplotype frequencies ranged from 0.006 to 0.162 in 154 dogs, with the most common haplotype being 031 which was found in 25 dogs belonging to 22 breeds. Haplotype 023 was also widespread, having a frequency of 0.149 and found in 22 pure-breed dogs. The five most common haplotypes (031, 023, 052, 056, and 005) represented 54.5% of all haplotypes identified in the inter-breed analysis.