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PAPER

CRIMINALISTICS

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Forensic Informativity of ~3000 bp of Coding Sequence of Domestic Dog mtDNA*

ABSTRACT: The discriminatory power of the noncoding control region (CR) of domestic dog mitochondrial DNA alone is relatively low. The extent to which the discriminatory power could be increased by analyzing additional highly variable coding regions of the mitochondrial genome (mtGenome) was therefore investigated. Genetic variability across the mtGenome was evaluated by phylogenetic analysis, and the three most variable ~1 kb coding regions identified. We then sampled 100 Swedish dogs to represent breeds in accordance with their frequency in the Swedish population. A previously published dataset of 59 dog mtGenomes collected in the United States was also analyzed. Inclusion of the three coding regions increased the exclusion capacity considerably for the Swedish sample, from 0.920 for the CR alone to 0.964 for all four regions. The number of mtDNA types among all 159 dogs increased from 41 to 72, the four most frequent CR haplotypes being resolved into 22 different haplotypes.

KEYWORDS: forensic science, domestic dog, mitochondrial DNA, coding region, control region, exclusion capacity

Shed hairs are one of the most common types of evidence material found at crime scenes. Humans or animals directly involved in a crime may contribute their own hairs or humans may secondarily transfer animal hair, most often from pets, to the scene. Dogs, one of the most popular pets in the world, are frequently the source of such hairs. Morphological analysis of hair rarely tells more than species type, and it is well known that an individual can possess hairs with varying morphological characteristics. Alternatively, analysis of the DNA collected from hairs has been critical evidence in several forensic cases involving canines, ranging from a dog being excluded from having caused a traffic accident to secondarily transferred dog hair evidence contributing to convictions of murder (1–7).

A single mammalian cell may contain upwards of 1000 copies of the mitochondrial genome (mtGenome) along with one diploid copy of the nuclear genome (8,9). In addition to the increased copy number, the mtGenome is circular, which better protects it from degradation relative to the linear nuclear genome (10). Shed hairs contain minute amounts of DNA that is often degraded making analysis of mitochondrial DNA (mtDNA) generally more successful than analysis of nuclear DNA (11). There are few length polymorphisms in the mtGenome, the normal target for forensic analyses of nuclear DNA *(i.e.*, STRs), and mtDNA is therefore commonly analyzed by DNA sequencing. The ~1300 bp noncoding control region (CR) is the most frequently analyzed portion of the mtGenome as it is the most variable part. The CR contains two hypervariable regions, called HVI and HVII, which are ~670 bp and ~300 bp in length, respectively. In dogs, HVI and HVII are separated by a tandem repeat region, which consists of a 10 bp sequence repeated approximately 30 times. This region is heteroplasmic in terms of both nucleotide composition and repeat copy number and therefore not possible to analyze without cloning (12). The region studied for the largest number of dogs so far is a 582 bp segment (nucleotide positions 15,458–16,039 of the dog mitochondrial genome) of HV1(13,14).

A drawback with the analysis of mtDNA is that the discriminatory power is very restricted as compared to the almost infinite genetic variation of nuclear DNA. Among humans, the exclusion capacity for the combined HVI and HVII regions can be as high as ~0.995 (15). Among dogs, it has been shown to be considerably lower, normally ranging from 0.90 to 0.95 for the HVI region alone and up to 0.961 for studies of the combined HVI and HVII regions excluding the tandem repeat region, and the higher values reported are likely overestimates reflecting nonrandom sampling (13,16–20). The reason for the lower exclusion capacity among dogs than humans is likely a combination

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of a lower substitution rate and a more recent origin of the dog population from its wolf ancestors (~16,000 years ago) (14) than modern humans from their ancestors (~150,000 years ago) (21), implying that fewer haplotypes have accumulated among the dogs.

The relatively low exclusion capacity for the dog CR implies that an inclusive result normally has a limited value as evidence and that exclusions are obtained in only about nine cases of ten. It would therefore be valuable if the exclusion capacity could be increased for analysis of dog mtDNA in forensic investigations. Recent studies have indicated that sequencing the entire mtGenome increases the exclusion capacity to 0.982–0.992, although the studied datasets were not entirely randomly sampled leaving more exact values to be determined (16,22). The mtGenome is ~17 kb, or roughly 17× larger than the combined HVI and HVII regions of the CR. Relative to analysis of the CR, sequencing the entire mtGenome is more costly, more time-consuming, and, most relevantly to forensic investigations, requires a larger amount of starting material, which is often limited and likely degraded when collected from a crime scene.

Here, we have identified the most variable regions of the mtGenome and investigated the extent to which analysis of merely ~3000 bp of the mtGenome increases the exclusion capacity, relative to analysis of 582 bp of the CR. Thus, we sequenced the 582 bp CR segment and the three most variable ~1000 bp regions outside the CR in 100 Swedish dogs. We also combined these sequences with data excised from 59 complete mtGenomes sequenced previously from dogs residing in the United States (US) (22). The Swedish dataset was designed such that it accurately represented the breed diversity of the Swedish dog population, to allow an objective estimation of the exclusion power of the CR and the effectiveness of extending the analysis with 3000 bp of coding sequence. The US dataset was collected in the interest of finding polymorphisms outside the CR that could be used to resolve frequently occurring CR haplotypes (17,22). Our analyses of these datasets demonstrate the utility of assembling regional databases representative of the breed diversity of the population and show that by assessing just ~1/5 of the mtGenome, more powerful exclusion capacities relative to the CR alone can be obtained.

Materials and Methods

Searching for Highly Variable Loci Across the mtGenome

To search for the most variable regions of the mtGenome, in addition to the commonly used 582 bp segment of the CR, we performed a phylogenetic analysis. We assessed the genetic variability across the mtGenome, analyzing a 16,740 character-length alignment of sequences from 112 dogs and 1 coyote representing the entire mtGenome except for the heteroplasmic region, which cannot be sequenced without cloning (12). The sequences were from an earlier study (14) in which the sampled dogs were not randomly collected but instead chosen based on known CR mtDNA types and therefore not ideal for assessing exclusion capacity. To identify not only the polymorphic sites among these sequences, but also the most variable of these sites, a phylogenetic analysis was performed. A phylogenetic tree was reconstructed (data not shown) under an HKY model with a proportion of sites assumed to be invariable (I = 0.6983) and rates for variable sites assumed to follow a discrete gamma distribution (shape parameter = 1.0618). The BioNJ algorithm as implemented in PAUP* was used to search for the best tree (23). Maximum likelihood (ML) and maximum parsimony (MP) were used to infer the most variable sites in the dataset. One of 10 discrete gamma-distributed site rates were assigned to each site of the entire alignment by ML optimization using the same model as in the tree reconstruction, and the most variable sites were identified by high site rates. All high site rates were confirmed by MP analysis (performed with uniformly weighted character positions and transitions), and the number of required substitution steps per site was calculated. Finally, looking outside the 582 bp CR segment, those genomic regions of ~1000 bp that included the highest number of substitutions were identified from the list of variation across the genome.

Building the Swedish Dog Dataset

Sample Collection—For the study of genetic variation in the Swedish dog population, samples were collected from breeds in their approximate frequencies in the Swedish dog population. The frequencies of dog breeds were approximated using the dog registration statistics of the Swedish Kennel Club by counting the number of registered dogs of each breed for the years 1999 to 2003 (table of dog breeds and frequencies available upon request). For each breed, a number of dogs corresponding to the frequency in the Swedish population, rounded off to the nearest integer, were sampled. The list was followed, with a few exceptions, from the most frequent breed to successively less frequent breeds until 100 dogs were sampled. Within each breed, the individuals were randomly sampled during the years 1997–2005 from dog shows, veterinary practices, and ordinary dog owners in Sweden, mostly in the Stockholm area.

DNA Extraction, Amplification, and Sequencing—Blood and hair samples were collected from 100 Swedish dogs. DNA was extracted from blood using the protocol #PT 3628-1 version #PR 22673 of the NucleoSpin Blood Kit (Biosciences Clontech, Saint-Germain-en-Laye, France), and from hairs according to Hopgood et al. (24) with some modifications (6). Generally, 2–5 dog hairs were used for each hair extraction.

Four regions of the mtGenome were analyzed: a 582 bp segment of the CR (nucleotide positions 15,458–16,039 of the dog mitochondrial genome, GenBank Accession Number U96639), and three additional regions identified for high diversity (nucleotide positions 8022–8948, 10,977–11,963 and 14,324–15,374). The PCR amplifications were performed in a nested configuration; an initial reaction with a forward primer and a reverse primer was followed by a second reaction with inner primers (primer sequences available upon request). The initial PCR mixture consisted of 1 μL (for blood samples) or 5 μL (for hair samples) of DNA extract, 0.2 μM of each primer, 2 mM $MgCl_2$, 20 mM TRIS-HCl pH 8.4, 200 mM of each dNTP and 1 unit of Platinum Taq DNA Polymerase (Invitrogen, Stockholm, Sweden) in a total volume of 50 μL. The reactions were performed in a ThermoHybaid MBS 02S (Thermo Electron Corporation, Waltham, MA). The initial PCR program consisted of an initial denaturation step of 2 min at 94°C, 15 cycles of denaturation (94°C, 30 sec), primer annealing (59°C, 30 sec) and extension (72°C, 1.5 min), followed by a final extension at 72°C for 10 min. The inner PCR mixture was identical to the outer PCR mixture except that 1 μL of template was used from the first reaction, and 0.1 μM of each inner primer was used. The inner PCR program was identical to the outer PCR program with the exception that 35 cycles were run. After amplification, the products were confirmed by electrophoresis, using 1% agarose gel.

Both strands of each amplicon were sequenced using sequencing primers (primer sequences available upon request). For the cycle sequencing reaction, 1 μL of the final amplification product was mixed with 17.5 μL of 1× Cycle Sequencing buffer (26 mM TRIS pH 9.0, 6.5 mM $MgCl_2$), 1 μL Big Dye Terminator (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit v2.1 or 3.1, Applied Biosystems, Stockholm, Sweden), and 0.25 μM of primer, in a total reaction volume of 20 μL. The reaction was run in a ThermoHybaid MBS 02S. The cycle sequencing program consisted of 30 cycles of denaturation (96°C, 10 sec), primer annealing (55°C, 15 sec), and extension (60°C, 4 min). The cycle sequencing products were ethanol precipitated and analyzed on an ABI 3700 according to the manufacturer's protocol (Applied Biosystems).

The DNA sequences were edited using Sequencing Analysis (Applied Biosystems), assembled into contigs and further edited in Sequencher 4.1 (Gene Codes). The contigs were aligned in Se-Al (25) and compared in SeqEd (Applied Biosystems). Analysis of the sequences in minimum spanning networks via Arlequin software Ver 2.000 (26) was used to aid in the discovery of artificial recombinants, which can be introduced as a result of clerical errors or sample mix-up when more than one region is amplified for the same individual in separate reactions and later combined after sequencing analysis (27,28).

Building the United States Dog Dataset

Fifty-nine domestic dog mtGenome sequences published previously (17,22) were included as part of this study. The mtGenome sequences were a subset (only those without ambiguous base calls) of those chosen for additional sequencing based on a prior CR study (17). These samples were collected at random from veterinary practices across the US with no direct effort to accurately represent breeds according to their approximate frequencies in the US dog population. Samples were chosen for complete mtGenome sequencing based on forensic and evolutionary analysis of the CR dataset (17,22). In the CR study, large groups of dogs with the same CR type were common. Dogs from these groups were chosen for complete mtGenome sequencing in the hopes of breaking up the large groups. Additionally, dogs of rare breed types were also chosen for complete mtGenome sequencing (22) (Table S1).

Generation of Datasets and Population Genetic Analyses

A total of 16 multiple alignments were created using Sequencher 4.10.1 (Gene Codes) (Table 2). Haplotype frequencies and exclusion capacities were calculated for all alignments. Arlequin 3.5 (29) and DNAcollapser (30) were used to estimate haplotype frequencies. The exclusion capacity was calculated as

$$1 - \sum_{i=1}^{n} f_i^2$$

where f_i is the relative frequency of the i-th mtDNA type and n is the total number of mtDNA types. Additionally, Arlequin 3.5 was used to assess the level of molecular variation among and within the Swedish and United States dog populations using the alignment of the concatenated most highly variable regions of the mtGenome via an AMOVA. Finally, a table of single-nucleotide polymorphisms defining all haplotypes was generated by aligning each haplotype to the reference sequence (31) using Sequencher version 4.10 and confirmed using DNAcollapser. The reference sequence coordinate of the variable positions was recorded in Microsoft Excel.

Results

Identification of Highly Variable mtGenome Regions

Based on a phylogenetic tree reconstructed from the mtGenomes of 112 dogs and one coyote, maximum likelihood (ML) and maximum parsimony (MP)-based methods were used to infer the most variable sites in the dog mtGenome, both agreeing in their classification of the sites that have varied the most during the history of the dog (Table S3). According to the MP analysis, 86% of the variable sites had mutated only once (average number of steps among sites with variation = 1.22, stdev 0.74; where 730/16,740 had varied). The majority of sites with an inferred number of steps greater than one (up to nine steps) occurred in the CR. From this analysis, the three most variable regions of approximately 1000 bp were identified. One of these regions (mtGenome positions 7892–8877) overlaps largely with a region we had previously analyzed in Swedish dogs (unpublished). To exploit existing sequence data, the previously analyzed region was analyzed also in this study, giving a slightly lower genetic variation (65 substitution steps instead of 73) for the analyzed region. Thus, the three highly variable regions used for analysis, in addition to the 582 bp CR segment, were a 927 bp region (mtGenome positions 8022–8948), which includes part of the sequence coding for ATPase subunit 6, *atp6*, and part of *cox3*, a 987 bp region (mtGenome positions 10,977–11,963), which includes part of the sequence of *nd4*, the entire coding sequence for tRNA-His, tRNA-Ser, and tRNA-Leu and a part of *nd5*, and a 1051 bp region (mtGenome positions 14,324–15,374), which includes a majority of *cytB* and a part of the sequence coding for tRNA-Thr.

Identification of Haplotypes – CR Analysis

Domestic dog mtDNA types have earlier been grouped into six principal phylogenetic groups, clades A-F, based on the CR (32), and all CR sequences of this study grouped within four of these clades, A-D (Table 1). Analysis of the 582 bp HVI segment of the CR from the 100 dogs in the Swedish dataset (GenBank Accession Numbers KF002256-KF002355) revealed that the four most common CR types, A11, A17, A18, and B1, were shared by 51% of the dogs. In total, 32 haplotypes were represented, 14 of which were possessed by more than one individual, 20 were not present in the US dataset, and 3 were novel. Also among the 59 dogs of US origin, the four haplotypes A11, A17, A18, and B1 were most frequent, carried by 46% of the dogs. In total, 21 haplotypes were represented among the US dogs, 13 of which were possessed by more than one individual, and 9 were not present in the Swedish dataset. Of the novel types, two grouped with clade A and were named A275 and A276, while one grouped with clade B and was named B57. Naming followed the recommended procedure of Pereira et al. (33). For the combined Swedish and US dataset, a total of 41 types were identified and defined by 40 polymorphic sites across the 582 bp alignment (Table S4).

In a study of the variation in the CR in 867 domestic dogs around the world (13), the eight most frequent CR types, including the top four most common CR types in the Swedish and US datasets, A11, A17, A18, and B1, had a total frequency of 51.7%, and among the 159 dogs from Sweden and the US, 7 of

TABLE 1—*Haplotype frequencies*

Haplotype	Universal Type	mtCR Groups	% Total	Haplotype	mtGenome Groups	% Total
A1*		Ny45A1 Collie m12A1 Chow chow m410A1 Bearded Collie H53A1 Border Collie	2.52	A1 var 1	Ny45A1 Collie	0.63
				A1 var 2	m12A1 Chow chow m410A1 Bearded Collie	1.26
				A1 var 3	H53A1 Border Collie	0.63
A2	X	H67A2 Leonberger EU408275.1FrenchBullDog1P m620A2 Bernese Mountain Dog EU408299.1Schnauzer4P EU408276.1GreatDane2P Y12A2 Great Dane	3.77	A2	H67A2 Leonberger EU408275.1FrenchBullDog1P m620A2 Bernese Mountain Dog EU408299.1Schnauzer4P EU408276.1GreatDane2P Y12A2 Great Dane	3.77
A5*	X	Y59A5 Shetland Sheepdog m31A5 Tibetansk Terrier Y23A5 Shetland Sheepdog	1.89	A5 var 1	Y59A5 Shetland Sheepdog Y23A5 Shetland Sheepdog	1.26
				A5 var 2	m31A5 Tibetansk Terrier	0.63
A11	X	EU408249.1AustralianShepherd7P EU408286.1MiniatureDachshund3P EU408295.1Rottweiler1P EU408296.1Rottweiler2P H22A11 Whippet H3A11 Papillon H44A11 Rottweiler H51A11 Border Terrier H64A11 Golden Retriever H85A11 Fox Terrier H94A11 Norwegian Elkhound L22A11 Pointer L36A11 Swedish Vallhund L43A11 Rhodesian Ridgeback L8A11 Basset Griffon m432A11 Chihuahua m752A11 Miniature Schnauzer Ny78A11 Rottweiler R41A11 Drever R42A11 Drever Y20A11 Yorkshire Terrier	13.21	A11 var 1	Y20A11 Yorkshire Terrier	0.63
				A11 var 2	H3A11 Papillon	0.63
				A11 var 3	L43A11 Rhodesian Ridgeback H64A11 Golden Retriever	1.26
				A11 var 4	H22A11 Whippet	0.63
				A11 var 5	L8A11 Basset Griffon	0.63
				A11 var 6	H85A11 Fox Terrier H51A11 Border Terrier L36A11 Swedish Vallhund m432A11 Chihuahua R41A11 Drever m752A11 Miniature Schnauzer R42A11 Drever EU408249.1AustralianShepherd7P	5.03
				A11 var 7	H94A11 Norwegian Elkhound L22A11 Pointer H44A11 Rottweiler Ny78A11 Rottweiler EU408295.1Rottweiler1P EU408286.1MiniatureDachshund3P EU408296.1Rottweiler2P	4.40
A15*		Y22A15 Giant Schnauzer	0.63	A15	Y22A15 Giant Schnauzer	0.63
A16	X	H10A16 Golden Retriever EU408280.2ItalianGreyhound1P R44A16 Labrador Retriever H54A16 Labrador Retriever EU408257.1BrittanySpaniel1M EU408274.1EnglishMastiff3P	3.77	A16	H10A16 Golden Retriever EU408280.2ItalianGreyhound1P R44A16 Labrador Retriever H54A16 Labrador Retriever EU408257.1BrittanySpaniel1M EU408274.1EnglishMastiff3P	3.77

TABLE 1—*Continued.*

Haplotype	Universal Type	mtCR Groups	% Total	Haplotype	mtGenome Groups	% Total
A17	X	Y82A17 Airedale Terrier Y18A17 Cavalier King Charles Spaniel R47A17 German Shepherd R43A17poscontr Labrador Retriever Ny58A17 Greyhound m747A17 Nova Scotia Duck Tolling Terrier m647A17 German Shepherd m445A17 Staffordshre Bull Terrier m19A17 Pug L2A17 Finnish Lapphund H62KontrollattA17 Norfolk Terrier H57A17 Flat Coated Retriever H19A17 Labrador Retriever EU408304.1unknown1P EU408294.1Pug5P EU408271.1DoguedeBordeaux1P EU408263.1CavalierKingCharlesSpaniel9P EU408253.1Boxer6P EU408250.1BichonFrise3P	11.95	A17 var 1	H57A17 Flat Coated Retriever	0.63
				A17 var 2	Ny58A17 Greyhound Y18A17 Cavalier King Charles Spaniel m19A17 Pug R43A17poscontr Labrador Retriever H19A17 Labrador Retriever m647A17 German Shepherd Y82A17 Airedale Terrier H62KontrollattA17 Norfolk Terrier R47A17 German Shepherd m445A17 Staffordshre Bull Terrier m747A17 Nova Scotia Duck Tolling Terrier EU408263.1CavalierKingCharlesSpaniel9P EU408250.1BichonFrise3P EU408253.1Boxer6P EU408294.1Pug5P EU408304.1unknown1P EU408271.1DoguedeBordeaux1P	10.69
				A17 var 3	L2A17 Finnish Lapphund	0.63
A18	X	EU408246.1AmericanCockerSpaniel1P EU408259.1Cockapoo3M EU408272.1Dachshund15P EU408281.1JackRussell6P EU408298.1Sheltie1M EU408302.1ToyPoodle3P EU408305.1Viszla2P L24A18poscontr Irish Soft Coated Wheaten Terrier m436A18 Poodle m447A18 Samoyed m746A18 Cocker Spaniel m748A18 Welsh Springer Spaniel m750A18 Miniature Schnauzer m751A18 German Spaniel Y36A18 Briard Y52A18 Chinese Crested Y55A18 English Springer Spaniel Y5A18 German Wireharied Pointer Y76A18 Cocker Spaniel	11.95	A18 var 1	Y36A18 Briard	0.63
				A18 var 2	Y55A18 English Springer Spaniel m447A18 Samoyed EU408246.1AmericanCockerSpaniel1P	1.89
				A18 var 3	Y5A18 German Wireharied Pointer m746A18 Cocker Spaniel m748A18 Welsh Springer Spaniel m750A18 Miniature Schnauzer Y52A18 Chinese Crested m751A18 German Spaniel EU408272.1Dachshund15P EU408281.1JackRussell6P EU408259.1Cockapoo3M EU408298.1Sheltie1M	6.29
				A18 var 4	Y76A18 Cocker Spaniel EU408305.1Viszla2P	1.26
				A18 var 5	m436A18 Poodle EU408302.1ToyPoodle3P	1.26
				A18 var 6	L24A18poscontr Irish Soft Coated Wheaten Terrier	0.63
A19	X	EU408270.1Dachshund4P Ny90A19 German Shepherd EU408277.1GermanShepherd12P EU408248.1AustralianShepherd1P m608A19 Lagoto Romagnolo R46A19 German Shepherd	3.77	A19 var 1	R46A19 German Shepherd	0.63
				A19 var 2	m608A19 Lagoto Romagnolo Ny90A19 German Shepherd EU408270.1Dachshund4P EU408248.1AustralianShepherd1P EU408277.1GermanShepherd12P	3.14

TABLE 1—*Continued.*

Haplotype	Universal Type	mtCR Groups	% Total	Haplotype	mtGenome Groups	% Total
A20	X	EU408261.1Chihuahua11M L21A20 Belgian Shepherd H66poscontrA20 Dachshund Ny87A20 Dachshund Ny89A20 Dachshund	3.14	A20 var 1 A20 var 2	L21A20 Belgian Shepherd Ny87A20 Dachshund Ny89A20 Dachshund H66poscontrA20 Dachshund EU408261.1Chihuahua11M	0.63 2.52
A22	X	R37a22 Irish Wolfhound L57A22 St. Bernard EU408289.1NeapolitanMastiff1P EU408290.1NeapolitanMastiff2P	2.52	A22 var 1 A22 var 2	L57A22 St. Bernard R37a22 Irish Wolfhound EU408289.1NeapolitanMastiff1P EU408290.1NeapolitanMastiff2P	1.89 0.63
A23*		L14A23 Russian Wolfhound	0.63	A23	L14A23 Russian Wolfhound	0.63
A26†		EU408287.1Newfoundland1P EU408306.1WestHighlandTerrier4P EU408264.1CairnTerrier4P	1.89	A26	EU408264.1CairnTerrier4P EU408287.1Newfoundland1P EU408306.1WestHighlandTerrier4P	1.89
A27†		EU408283.1Keeshond2P EU408284.1Keeshond3P EU408282.1Keeshond1P	1.89	A27	EU408284.1Keeshond3P EU408283.1Keeshond2P EU408282.1Keeshond1P	1.89
A29*		H90A29 Siberian Husky	0.63	A29	H90A29 Siberian Husky	0.63
A30*		R6A30 Hamiltonstovare	0.63	A30	R6A30 Hamiltonstovare	0.63
A33*		Y16A33 Irish Setter	0.63	A33	Y16A33 Irish Setter	0.63
A65*		m705A65 Shih Tzu	0.63	A65	m705A65 Shih Tzu	0.63
A71†		EU408265.1Corgi2P EU408245.1Akita1P.TXT	1.26	A71 var 1 A71 var 2	EU408245.1Akita1P.TXT EU408265.1Corgi2P	0.63 0.63
A97†		EU408300.1TibetanMastiff1P	0.63	A97	EU408300.1TibetanMastiff1P	0.63
A98†		EU408262.1Chihuahua5P	0.63	A98	EU408262.1Chihuahua5P	0.63
A176*		m621NycaA18 Dalmation	0.63	A176	m621NycaA18 Dalmation	0.63
A275*		2caA80 Small Munsterlander	0.63	A275	2caA80 Small Munsterlander	0.63
A276*		Y6NycaA66 Cavalier King Charles Spaniel	0.63	A276	Y6NycaA66 Cavalier King Charles Spaniel	0.63
B1	X	Y67B1 Dachshund Y34B1 Poodle Y32B1 Golden Retriever R12B1 Finnish Spitz Ny88B1 Dachshund Ny82B1 Doberman m32B1 Tibetan Spaniel H45B1 Golden Retriever H38B1 Golden Retriever EU408301.1TibetanSpaniel1P EU408292.1Poodle7M	11.95	B1 var 1	Y32B1 Golden Retriever R12B1 Finnish Spitz m32B1 Tibetan Spaniel Ny82B1 Doberman H45B1 Golden Retriever H38B1 Golden Retriever EU408269.1DobermanPinscher5P EU408285.1Labradoodle1P EU408301.1TibetanSpaniel1P EU408252.1Bolognese1P EU408292.1Poodle7M	6.92

TABLE 1—*Continued.*

Haplotype	Universal Type	mtCR Groups	% Total	Haplotype	mtGenome Groups	% Total
		EU408285.1Labradoodle1P EU408278.1GreatPyrenees1P EU408269.1DobermanPinscher5P EU408260.1CardiganCorgi2P EU408256.1BassetHound4P EU408254.1BassetHound2P EU408252.1Bolognese1P EU408247.1AustralianTerrier1P		B1 var 2	Y67B1 Dachshund Ny88B1 Dachshund	1.26
				B1 var 3	Y34B1 Poodle	0.63
				B1 var 4	EU408256.1BassetHound4P EU408254.1BassetHound2P	1.26
				B1 var 5	EU408260.1CardiganCorgi2P EU408278.1GreatPyrenees1P	1.26
				B1 var 6	EU408247.1AustralianTerrier1P	0.63
B3*		m435B3 Miniature Poodle	0.63	B3	m435B3 Miniature Poodle	0.63
B6	X	EU408307.1WalkerHound1P m455B6 Parson Jack Russell Terrier EU408297.1Schipperke1P	1.89	B6	m455B6 Parson Jack Russell Terrier EU408307.1WalkerHound1P EU408297.1Schipperke1P	1.89
B7*		Ny83B7 Danish–Swedish Farmdog	0.63	B7	Ny83B7 Danish–Swedish Farmdog	0.63
B8*		H30B8 Flatcoated Retriever	0.63	B8	H30B8 Flatcoated Retriever	0.63
B10†		EU408268.1CockerSpaniel8P	0.63	B10	EU408268.1CockerSpaniel8P	0.63
B11*		m749B11 American Cocker Spaniel	0.63	B11	m749B11 American Cocker Spaniel	0.63
B14†		EU408303.1unknown1M	0.63	B14	EU408303.1unknown1M	0.63
B18*		m434B18poscontr Bichon Havanese	0.63	B18	m434B18poscontr Bichon Havanese	0.63
B27*		m609NycaB3 Coton de Tuléar	0.63	B27	m609NycaB3 Coton de Tuléar	0.63
B28†		EU408258.1Cockapoo1M	0.63	B28	EU408258.1Cockapoo1M	0.63
B57*		4caB8 English Springer Spaniel	0.63	B57	4caB8 English Springer Spaniel	0.63
C1*	X	H98C1 German Shepherd H95C1 German Shepherd	1.26	C1	H95C1 German Shepherd H98C1 German Shepherd	1.26
C2*		H48C2 West Highland White Terrier	0.63	C2	H48C2 West Highland White Terrier	0.63
C3	X	L32C3 Finnish Hound EU408291.1Pomeranian2M EU408267.1CockerSpaniel3P EU408279.1Havanese3P H91C3poscontr Swedish Elkhound Y35C3 Jack Russell Terrier	3.77	C3 var 1	H91C3poscontr Swedish Elkhound Y35C3 Jack Russell Terrier EU408291.1Pomeranian2M	1.89
				C3 var 2	L32C3 Finnish Hound	0.63
				C3 var 3	EU408279.1Havanese3P	0.63
				C3 var 4	EU408267.1CockerSpaniel3P	0.63
C8†		EU408293.1PitBullTerrier1M	0.63	C8	EU408293.1PitBullTerrier1M	0.63
D1		EU408288.1NorwegianElkhound1P L33D1 Sweddish Lapphund H37KontrollattD1 Swedish Elkhound R4KontrollattD1 Norwegian Elkhound H65KontrollattD1 Swedish Elkhound	3.14	D1 var 1	L33D1 Sweddish Lapphund	0.63
				D1 var 2	H65KontrollattD1 Swedish Elkhound R4KontrollattD1 Norwegian Elkhound	1.26
				D1 var 3	H37KontrollattD1 Swedish Elkhound	0.63
				D1 var 4	EU408288.1NorwegianElkhound1P	0.63

*Haplotype comprised of Swedish dogs only.
†Haplotype comprised of US dogs only.

these types were represented at a frequency of 63.4% (Table 1). It has also been shown that dogs in Europe, SW Asia as well as East Asia share 15 CR types, including A11, A17, A18, and B1, which are accordingly referred to as Universal Types (UTs) (14,34). These types are universally frequent, carried by around 75% of individuals in most dog populations. Of these UTs, all but two (A3, C5) were found in the Swedish and US dataset at a combined frequency of 74.8% with each being present in at least two individuals (Table 1).

Identification of Haplotypes – Analysis of Highly Variable mtGenome Regions

The 100 dogs from Sweden and 59 dogs from the US were analyzed as a concatenated sequence of the three most variable regions of the mtGenome plus the HVI region of the CR. When considering all four mtDNA regions, the number of haplotypes in the Swedish dataset rose to 55, an almost 2-fold increase relative to the HVI region of the CR alone. The number of haplotypes in the US dataset was 32 and in the combined dataset 72. The most common type in the combined dataset, as well as in each country when considered separately, was A17 var 2, which was possessed by 11 dogs from Sweden (11%) and 6 dogs from the United States (10.7%) (Table 1). There were 40 types in the Swedish dataset not present in the US dataset, while 17 types found in the US dataset were not present in the Swedish dataset (Table 1). An alignment of the resulting mtDNA types is available upon request. A total of 155 polymorphic sites were found across the 3548 bp alignment.

Importantly, analysis of the three additional regions separated the most frequent CR haplotypes into a large number of subtypes. The four most common CR types, A11, A17, A18, and B1, with a combined frequency of 49.1% in the total dataset, were separated into 22 haplotypes, and all but 4 of the 13 UTs were resolved into subtypes. For example, the most common CR type, A11, which had a frequency of 13.2% in the combined Swedish and US dataset, was separated into seven different types, the most frequent of which had a frequency of just 5.0%, demonstrating the increased discriminatory power that can be obtained through targeted mtGenome sequencing and analysis (Table 1).

Resolution of Breed-specific Groups

There were 23 CR haplotypes that were carried by more than one dog of the same breed. In six cases, these dogs obtained different haplotypes through analysis of the three additional mtGenome regions (Table 1). For example, type A19 is frequently found in German Shepherds (14 of 27 German Shepherds sampled in Europe in a previous study) but rarely in other European breeds (13). The combined US and Swedish dataset contained three German Shepherds of CR type A19. Following analysis of the additional mtGenome regions, one of the three German Shepherds could now be distinguished from the others as A19 was divided into two subtypes. For Scandinavian forensic casework, an important result was that type D1, which has been found in ~70% of the common Scandinavian breeds Jämthund, Norwegian Elkhound, and Lapphund (13,35), was divided into four subtypes among the five dogs having CR type D1. Conversely, the largest assemblage of dogs of the same breed in the combined dataset consisted of four Rottweiler dogs carrying CR type A11. Despite the additional mtGenome sequencing, no subtypes were created for this breed as they all possessed mtGenome type A11 var 7. A similar result was obtained for three Keeshonds all possessing CR type A27 (Table 1).

Exclusion Capacities

The exclusion capacity for the 582 bp CR segment among the Swedish samples was 0.920. As the samples were collected according to the breed frequency in Sweden, this is probably a relatively accurate estimate of the exclusion capacity of the mtCR in the Swedish dog population.

The three additional mtGenome regions each had moderate exclusion capacities, ranging between 0.814 and 0.845, but gave a considerable increase in the total exclusion capacity (Table 2). In the Swedish dataset, adding the three additional mtGenome regions increased the discriminatory power from 0.920 to 0.964. For the US dataset, the improvement was more moderate, from 0.921 for the HVI region to 0.939 for the combined HVI and HVII regions, and to 0.953 including the three additional mtGenome regions. Finally, for the combined Swedish and US dataset, discriminatory power improved from 0.928 to 0.965.

Analysis of Molecular Variance

Despite the two datasets being comprised of dogs residing on opposites sides of the world, there was little genetic variation to group dogs by country of sample origin. In the combined Swedish and US HVI CR dataset, 74.8% of the dogs carried one of 12 haplotypes that were represented among dogs from both Sweden and the US, and these haplotypes were carried by between 3 and 21 individuals (Table 1). This trend persisted when all four mtDNA regions were analyzed. In the combined dataset, 56.6% of the dogs grouped into one of 15 haplotypes that contained dogs from both countries, the most frequent of which was carried by 17

TABLE 2—*Exclusion capacities and number of haplotypes across the four investigated regions, in three populations.*

	Alignments					
Dataset	15,458–16,039 (HVI)	15,458–16,727 (HVI and HVII)	8022–8948	10,977–11,963	14,324–15,374	All Regions Combined
Swedish						
Ex. cap	0.92	n/a	0.845	0.842	0.837	0.964
No. of haplotypes	32	n a	18	22	16	55
US						
Ex. cap	0.921	0.939	0.82	0.816	0.814	0.953
No. of haplotypes	21	25	15	13	15	32
Swedish and US						
Ex. cap	0.928	n/a	0.839	0.838	0.831	0.965
No. of haplotypes	41	n/a	23	26	23	72

All coordinates listed correspond to those of the domestic dog mtDNA reference sequence (31).

TABLE 3—*Results of by country AMOVA.*

	Source of Variation	Degrees of Freedom	Percentage of Variation
Swedish vs. United States	Among countries	1	−0.15
	Within countries	157	100.15
	Total	158	100
		FST = −0.00154, p = 0.39980	

The significance, reported as a p-value, was derived from 1023 permutations.

dogs. Accordingly, an AMOVA revealed no significant difference in the haplotype distributions of the two investigated countries (Table 3).

Handling Error and Heteroplasmy

A concern in population analyses of several separate genomic regions is that, because of a number of possible handling errors (28,29), the different regions may become mixed between individuals, resulting in final sequences that are a mosaic of sequences from more than one individual. To aid the discovery of such artificial recombinants, minimum spanning networks were created separately for each of the four sequenced regions (data not shown). In the Swedish dataset, two discrepancies were discovered when the networks were compared, one due to a clerical error and the other due to sample mix-up, both of which were in sequences from an earlier study (32).

Point mutation heteroplasmy in dog mtDNA has previously been described in blood and hairs from 1 of 105 individuals in a study of 595 bp of the mtDNA control region (36). In this study of 100 newly sequenced Swedish dogs, in a total of 3,547 bp, we saw indications (at the detection level) of heteroplasmy in five individuals (one position per individual). For the calculations in this study, the majority peak was recorded and the possible heteroplasmy was not further investigated.

Discussion

Mitochondrial DNA has been employed widely in the field of canine forensics toward determining the donor of dog hair found at a crime scene and/or concluding that hairs collected from different locations were contributed by the same individual (1–7). In these investigations, the noncoding CR of the mtGenome has so far been analyzed, giving exclusion capacities normally between 0.90 and 0.95 (13,16–20). Outside of the CR, there exists approximately 15,500 bp of mitochondrial coding region sequence, which in combination with the CR gives exclusion capacities above 98% (22). While giving a clear improvement compared with evaluations of the CR alone, collecting the DNA sequence of the entire mtGenome can be costly and time-consuming. Often only one or a few shed hairs are found at a crime scene, limiting the amount of starting material available. The need to amplify multiple overlapping segments toward collecting the DNA sequence of the entire mtGenome requires a larger amount of starting material relative to collecting just the CR, which can be amplified in a single reaction. Additionally, the DNA in shed hairs is mostly degraded to stretches of maximally a few hundred bp (37) making PCR amplification of the entire ~17 kb mtGenome less than optimal for shed hairs. Here, we demonstrate that by sequencing just 20% of the mtGenome, considerably improved exclusion capacities relative to the CR alone can be obtained.

Dataset Composition

When creating a dataset for forensic analysis, random sampling is critical for accurately representing the haplotype distribution and estimating the exclusion capacity of the population being assessed. However, random sampling from the normal dog population has normally not been performed in previous studies of dog mtDNA. In the present study, three datasets with different sampling strategies were considered. The first was comprised of mtGenomes from dogs of disparate geographic regions including Europe, the Middle East and the Far East, sampled in a previous study (14) for estimating the timing and location of wolf domestication. We used this dataset to identify the most highly variable 1 kb regions within the mtGenome, but as the samples were not randomly collected but instead chosen based on known CR haplotypes to obtain representation of a large number of different haplotypes, it is not suitable for assessing the exclusion capacity. To assess the exclusion capacity, we therefore collected a second dataset consisting of 100 Swedish dogs. These samples were collected to accurately reflect breed frequencies in Sweden, to obtain an objective assessment of diversity and exclusion capacity in the Swedish dog population. Finally, the US dataset, which had been collected and analyzed previously (22), represents a collection strategy where most samples were chosen for complete mtGenome sequencing because they possessed a CR type with a high prevalence in the US based upon a previously published dataset (17,22).

Powerful Variation Found Outside of the CR

The CR is the most commonly exploited region of the canine mtGenome for forensic casework due to its high amount of diversity within a relatively short DNA segment. However, as expected, a large proportion of the dogs sampled from Sweden and the US resolved into one of just a few common CR haplotypes. These results are typical for domestic dog mtCR analyses, making it less informative relative to human CR analysis. In hopes of obtaining more powerful data, researchers have begun exploring the utility of the coding region of the mtGenome toward forensic analysis, most often looking at the entire coding region. Here, we have improved upon the CR exclusion capacity by sequencing just a fraction of the mtGenome, giving a decrease in the probability of a random match from 1 in 14 to 1 in 30. Most importantly, analysis based on the CR alone gives exclusions in nine cases of ten, but inclusions have a limited value in almost 50% of the cases, when they involve one of the four most frequent types, which have frequencies of around 10% or more (Table 1). With the addition of the three coding regions, these four mtDNA types are divided into 22 subtypes, giving a considerably increased value of inclusions.

Resolution of Breed-specific CR Haplotypes

With the analysis of larger regions of the mitochondrial genome, the probability increases in finding recent mutations, acquired after the formation of different morphological types and breeds of dogs. This kind of polymorphism may be important if a breed is common in the dog population and has a dominating CR haplotype that became frequent through genetic drift at the forming of the breed. This is exemplified in the current study by the resolution of frequently occurring CR haplotypes within the German Shepherd and Scandinavian spitz breeds through analysis of the additional mtGenome regions (Table 1). The recent

mutations may also create breed-specific mtDNA types, an example of which was found for the Rottweiler and Keeshond, which may be used to indicate the likely breed of dog from which a hair originates.

A Global Population

While previous forensic studies have looked at population structure in the domestic dog based on CR and mtGenome sequences within the United States (17–19,22) as well as in Sweden, the UK, Germany, Japan, and China (13), this was the first to compare dogs from the Old World to the New World in a forensic context. An insignificant amount of variation was detected among the countries. This supports the notion that dogs in the US constitute a random sampling of the larger world-wide dog population, thought to have been first domesticated ~16,000 years ago and subsequently introduced to North America, largely from Europe in post-Columbian time (14,32,38).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Dataset Information.

Table S2. Primer Information.

Table S3. Variable sites in the dog mtGenome.

Table S4. Haplotypes and Defining Polymorphic Sites.