Dogs carrying the B1 control region haplotype are also identical at second mutational hotspot

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ABSTRACT

Canine mitochondrial DNA (mtDNA) can be used in criminal cases as evidence to convict or free a suspect. The most common way this is done is through haplotype analysis. Databases of haplotype diversities for populations both inside and outside of the contiguous United States have already been compiled. One example of a database outside of the contiguous United States can be seen in Hawaii, where the haplotype distribution is vastly different from that of the contiguous U.S with overrepresentation of the control region haplotype B1. Finding variable regions in mtDNA outside of the control region (CR) is important because dog hair is often found at crime scenes and mtDNA is the only type of DNA that can be used. One variable region, ranging from bases 10,251-10,354 of the mtDNA, outside the Hyper Variable 1 (HV1) region was studied to look for variability of samples with the B1 haplotype from Hawaii. Of 24 samples, the target region was successfully amplified and sequenced from 14. Only 1 single nucleotide polymorphism (SNP), common to all 14 samples, was found at position 10,257. This lack of diversity could be due to many factors such as evolutionary constraints on coding regions of DNA or founder effect.

INTRODUCTION

Forensic Utility of Mitochondrial vs. Nuclear DNA

There are two types of DNA, mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). Both types of DNA are found in most cells; however hair cells are an exception. nDNA differs from mtDNA in that it is found in the hair follicle and not the hair shaft (Graham 2007). This difference between the two types of DNA is important considering the two ways hair can fall out, one of which is being shed and the other is being pulled out. The main difference between the two is if a hair is shed then it will likely not have the hair follicle attached (Graham 2007). DNA can offer great utility in forensics. Since hair is so common, hair is often found at crime scenes and DNA is extracted from these hairs for use as forensic evidence. However, hairs found at a crime scene usually have been shed and do not contain the hair follicle (Himmelberger et al. 2008). This means that the only type of DNA available is mtDNA. This proves beneficial because mtDNA, with more than 1,000 copies of the genome per cell (Bogenhagen and Clayton 1974), is more common than nuclear DNA, which has only about 2 copies of the genome per cell (Parsons and Coble 2001). Thus, mtDNA is more likely to be present and better preserved than nDNA and hence is far better for analysis (Verscheure et al. 2013). However, due to strict maternal inheritance and lack of recombination, mtDNA is not as variable when compared to nDNA.

Use of animal DNA in forensics

DNA extracted from hair is a useful forensic tool. The hair doesn't even have to be human hair; it can come from any type of animal, most commonly a cat or a dog. According to the Humane Society, in 2012 83.3 million dogs were owned as pets in the United States

(http://www.humanesociety.org/issues/pet_overpopulation/facts/pet_ownership_statistics.html?referrer=https://www.google.com/?referrer=http://www.humanesociety.org/issues/pet_overpopulation/facts/pet_ownership_statistics.html). Anyone who has ever owned a dog can testify that dog hair gets everywhere. It is very difficult for an individual to walk into a home with a dog and not get hair on them. Since it is so abundant, dog hair can be discovered at crime scenes and can be used as evidence in criminal investigations. There are two ways that dog hair can end up at a crime scene. The first is primary transfer, where the dog is actually present at the scene of the crime. The second is secondary transfer, which is when hair is brought to and dropped at the crime scene by a person involved in the crime. For example, dog hairs can be transferred from a victim's clothes to the trunk of a perpetrator's car during transportation of a body (Verscheure et al. 2013).

One example of canine DNA being used in forensics is the case of the State of Washington v. Kenneth Leuluaialii and George Tuilefano (Halverson and Basten 2005). These two men were accused in the shooting a couple in December of 1996. The couple's dog was also shot. Jackets and shoes were recovered from the suspects, on which blood and hairs were found. The hairs phenotypically resembled the victims' dog's hair and the blood was found to be both human and canine (Halverson and Basten 2005). DNA was extracted and after being sequenced was matched to the victim's dog, consequently incriminating the suspects and leading to their conviction (Halverson and Basten 2005).

Hyper Variable 1 Region

The most utilized region of the mtDNA in forensics is the hyper variable 1 (HV1) region. The HV1 region is a 672 base pair (bp) region of the mitochondrial control region (mtCR), or D-loop, that is a non-coding portion of the mtDNA (Parsons and Coble 2001).

While small compared to the entire 16,800 bp of the entire mitochondrial genome, the HV1 region is the most used region of the mtDNA in forensic analysis due to its high variability because of its high mutation rate (Himmelberger et al. 2008; Webb and Allard 2009a; Webb and Allard 2009b; Savolainen et al. 1997; Angleby and Savolainen 2005; Kim et al. 1998). In forensics, it is best to have a highly identifiable sample to look at, therefore regions that have a high mutation rate have the highest chance of not being identical between individuals and can lead to greater forensic information.

Mitochondrial Haplotypes and Mitochondrial Haplotype Diversity

The HV1 region is also the region of the mtDNA used to determine a dog's haplotype. A mitochondrial haplotype is a specific sequence of mtDNA. Once the mtDNA has been sequenced, one can determine the haplotype of the evidence hair by comparing it to known haplotypes from previously published forensic and evolutionary studies of the domestic dog. There are six major haplogroups that have been previously defined, named A-F (Savolainen et al. 2002). Each haplogroup is thought to be associated with a different domestication event of the domestic dog (Savolainen et al. 2002). The origin of haplogroup A is a domestication event that occurred in East Asia and haplogroup B occurred in Europe or Southwest Asia (Savolainen et al. 2002). These haplogroups can have haplotypes, such as A11 and B1, which have all the single nucleotide polymorphisms (SNPs) of the original haplogroup but also contain unique SNPs and are therefore denoted as a separate haplotype within the haplogroup. As the name suggests, SNPs are single variations in the nucleotide sequence that occur at a specific point in the sequence. For example, if the reference sequence has a C at base number 113 and the sample sequence has a G at that same position, this would be considered a SNP. Currently, 278 distinct haplotypes have been described for

haplogroup A, 59 have been described for haplogroup B, and 18 have been described for haplotype C (Spadaro et al. 2015).

Dog Mitochondrial Haplotype Diversity

Dog mitochondrial haplotype diversity has been studied extensively in the contiguous United States where mitochondrial haplotype A has been found to be the most common in previous studies by a large margin (Webb and Allard 2009a; Webb and Allard 2009b; Savolainen et al. 1997; Angleby and Savolainen 2005). These studies have found that more than 60% of dogs fall into the A haplotype while less than 25% fell into the next most common haplotype, B (Webb and Allard 2009b; Savolainen et al. 1997; Angleby and Savolainen 2005). Therefore, the A haplotype is the most common in the contiguous United States by greater than 35%.

Hawaii Haplotype Diversity

While the haplotype distribution of the contiguous United States has been studied, there is considerable variation between populations and not all populations have the same haplotype distribution as the contiguous Untied States. Haplotype distribution has also been studied in populations outside of the contiguous United States. A shockingly reduced genetic diversity has been found in the domestic dog population of Hawaii (Spadaro et al. 2015). For this study, 61 dogs were sampled from two veterinary hospitals on the Hawaiian Islands. One practice was in Honolulu on the island of Oahu and the second was in Kailua-Kona on the island of Hawaii. Of these 61 samples, 20 different haplotypes were found with 15 of these haplotypes also being represented in the contiguous United States population (Spadaro et al. 2015). It was also found that over 39% of these samples shared the same haplotype, B1, which shows extreme regional haplotype frequency variation (Spadaro et al. 2015). This is an

unusually high frequency for haplotype B, which normally has less than a 25% prevalence rate in the contiguous United States (Webb and Allard 2009b; Savolainen et al. 1997; Angleby and Savolainen 2005). The results of this population survey found that haplotype B is only slightly less common, 45.9%, than the A haplotype, 50.8% (Spadaro et al. 2015). This lose of genetic diversity could have grave implication for the use of canine DNA in criminal investigations, where a less common haplotype is preferred. For example, if a dog hair in Hawaii was found to carry the B1 haplotype, it greatly decreased the value of the evidence. This loss of evidentiary value is due to the match being not as significant because if a dog has a common haplotype, there is a higher chance that the dog will be matched with another dog simply by chance.

Importance of Databases

The evidentiary value of DNA found at a crime scene almost entirely depends on the distribution of the population sample (Verscheure et al. 2013). Therefore, the frequencies of these haplotypes in the area where the crime occurred must be known for the mtDNA evidence to be useful (Himmelberger et al. 2008; Budowle et al. 2005). It is more convincing if a sample hair is found to have a rare haplotype and is matched to a dog that has a same haplotype than if the sample is more common and could be matched to many different dogs (Himmelberger et al. 2008). To know whether a certain haplotype is rare or not, studies need to be performed on the dogs in the area to determine the haplotype distribution. These studies lead to the creation of databases where the distribution of haplotypes can be easily ascertained and used for forensic purposes. This makes identification of common and rare haplotypes possible. Regional variation of haplotype distribution must be considered when creating these databases (Spadaro et al. 2015). Due to many factors, such as migration or

founder effect, haplotype distribution can vary from region to region. Since the forensic evidence of dog mtDNA is directly correlated to how rare the haplotype is; it is imperative to have accurate databases that depict the haplotype distribution of a region before dog mtDNA is used as forensic evidence.

Important Region to Investigate

Currently the most common way canine mtDNA is used for forensic evidence is the haplotype is determined from the HV1 region of the mtCR. However, this is only one small, non-coding section of the mtDNA and most of the mtDNA codes for different proteins, rRNAs and tRNAs (Kim et al. 1998). Using only one region can create difficulty if the dog in question carries a common haplotype of the region such as a dog carrying the B1 haplotype in Hawaii. Due to the lack of diversity found in the HV1 region of the mtDNA in the dogs sampled from Hawaii, it would be beneficial to look for other areas of the mtDNA that could also be used to distinguish between Hawaiian dogs carrying the B1 haplotype. If regions outside of the HV1 region prove to be variable when the HV1 haplotype is the same, forensic analysis of canine DNA would be greatly improved and could be used in populations where the haplotype diversity is skewed such as the Hawaiian population. A region of the mtDNA that is not the HV1 region but is also highly variable has identified (Table 1) (Webb and Allard 2009a).

Table 1: Important Region Outside the HV1 Region to Survey for SNPs

Region of mtDNA	Size of Region	SNP frequency	Coding region?	What is coded for
HV1	672	1 in 2.7 bases	No	N/A
10,251-10,354	103	1 in 11.5 bases	Yes	*ND4* gene

The region surveyed of non-control region mtDNA from Webb and Allard (2009) along with the HV1 region of the control region are shown. Single Nucleotide Polymorphism (SNP) frequency and what the region codes for are also given.

Webb and Allard looked at the entire mtDNA genome of 79 dogs to look for variable regions outside of the HV1 region and found one region spanning bases 10,251-10,354 that had a SNP frequency of 1 in 11.5 bases (Table 1) (2009a). To calculate variation, the number of SNPs throughout the genome was counted. While this non-control region is not as variable as the HV1 region, which has a SNP frequency of 1 in 2.7 bases (Table 1), it is the most variable region outside of the HV1 region and could provide useful information in populations where the HV1 region is not variable. It was found that while the HV1 region has more SNPs proportionally, the mtGenome has more haplotypes (Webb and Allard 2009a). This means that other regions besides the HV1 region are variable and SNPs can be found there. While Webb and Allard (2009a) found SNPs in dogs with the same haplotype, the dogs sampled in their study were all from the contiguous United States. This region has not yet been studied in a small, isolated population such as Hawaii.

Here we studied the 10,251-10,354 region of the mtDNA in a sample of domestic dogs from Hawaii that all carried the mitochondrial haplotype B1. This region could prove to be variable when the HV1 region does not show variability, in this case all samples having the same mitochondrial haplotype, B1. The results of this study could especially help Hawaiian criminal investigations but other small, isolated populations would also benefit greatly. If this region of the mtDNA proves to be variable when the mitochondrial haplotype is the same in this type of population, the value of the hair as forensic evidence would exponentially increase. This could lead to more widespread use of dog mtDNA in criminal investigations, as dog hair is not as utilized as it could be in forensic investigation.

MATERIALS AND METHODS

Sample Collection

DNA extracts from 24 domestic dogs from Hawaii carrying the B1 haplotype were stored at -20°C (Spadaro et al. 2015). Fifteen of the samples came from the practice in Honolulu while 9 samples came from Oahu (Spadaro et al. 2015). In the fall of 2015, extracts were then removed from the freezer and thawed and have since been stored at 4°C.

Assessment of DNA degradation

1% agarose gel electrophoresis of five randomly chosen samples was used to make sure the DNA was not degraded during its time at -20°C. DNA Marker (Bio Basic INC, Markham, Ontario), a ladder that had bands of known size, was used for comparison to samples to determine the samples' sizes. Once samples were loaded, the gel was run at about 70 volts for 45 minutes. The gel was then imaged. The samples were compared to the ladder to determine if the DNA extracts were above the 2000 bp band of the ladder.

Amplification of Target Loci

The 10,251- 10,354 region of 11 samples was amplified using a protocol based off of that used by Webb and Allard (2009b). The primers that were used were 11021R and 9190F, previously published by Webb and Allard (2009a) (Table 2). Based on these primers, the expected PCR product size was 1,851 bp. A primer map is also provided for the primer pair used to amplify the target region (Figure 1). The reaction conditions were as follows: 75 ng total DNA, 1× Buffer (ThermoFisher Scientific, Grand Island, NY), 5 mM $MgCl_2$ (ThermoFisher Scientific, Grand Island, NY), 0.4 mM dNTP mix (ThermoFisher Scientific, Grand Island, NY), 0.1 μM of each primer, and 2.5 units of Taq polymerase (ThermoFisher Scientific, Grand Island, NY) in a total volume of 25 μL (Webb and Allard 2009b). The thermal cycler conditions began with an initial denaturing step of 96°C for 10 min, 39 cycles of amplification which included denaturing at 94°C for 15 sec, annealing at

58°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 7 min (Webb and Allard 2009b). Another 3 samples were amplified using the same reagent concentrations and similar thermal cycling conditions but with an annealing temperature of 52.8°C. Each set of PCRs was run with a positive control using 100 ng of sample HI1-3 with the conditions found above and previously published primers for the entire mtCR from Webb and Allard (2009b) (Table 2). A negative control was also run for each set of PCRs. Gel- electrophoresis was performed to assess if DNA amplification occurred for each PCR run. This was performed in accordance with the procedure above, however the ladder used was a Low Mass ladder (Life Technologies, Grand Island, NY) and 4 µL of sample was loaded instead of 5 µL. The remaining 10 samples were attempted however amplification was never successful.

Table 2. Primers that were used to amplify target regions of mtDNA

Primer Name	Primer Sequence (5' → 3')	mtDNA Region Amplified	Expected Product Size (bp)
11021R	CTGTTTGACGGAGACAGATAG	10,251-10,354	1,851
9190F	GAGACATCTTTTACAATCTCCG		
R51	TATGTTTATGGAGTCGTGCGA	mtCR	1,214
F15406	TTTGCTCCACCATCAGCACC		

Primer names are the base within the domestic dog mitochondrial genome where the primer anneals at the 5' end. F and R signify whether the primer is forward (F) or reverse (R).

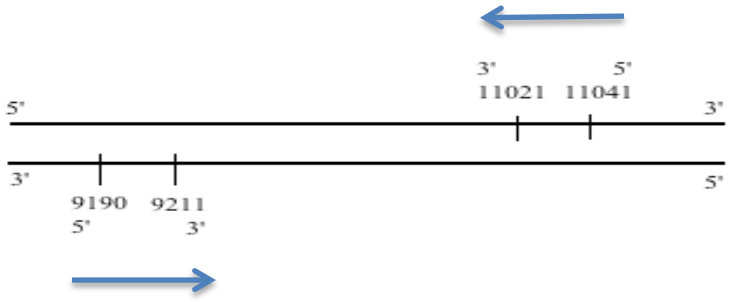

Figure 1: A primer maps is shown for the primer pair used to amplify the 10,251-10,354 region of the mtDNA. The 5' and 3' of each primer is shown as well as arrows indicating which way the primer is running.

DNA Sequencing

Bidirectional sequencing of the first 11 samples was performed using previously published primers 9190F and 11021R (Webb and Allard 2009a). To prepare samples for sequencing, 20 μL of each PCR product was mixed with 8 μL of ExoSAP-IT (Affymetrix, Santa Clara, CA). The samples were then put in the thermal cycler with the following parameters: 37 C for 15 min. then 80 C for 15 minutes to activate the ExoSAP-IT. 14 μL of each sample was then transferred to its own sequencing tube. The final three samples were only sequenced in one direction, using the primer 11021R. For these samples, 10 μL of each sample was mixed with 4 μL of ExoSAP-IT and subjected to the same conditions as the previous 11 samples. The total 14 μL was then transferred to a sequencing tube. All sequencing reactions were performed by Eurofins MWG Operon (Hunstsville, AL).

Statistical Analysis

The resultant sequences were then aligned to the canine mitochondrial genome reference sequence (Genbank Accession #: NC_002008.4) using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI) to determine SNPs. Only the 103 bp target sequence between bases 10,251-10,354 was surveyed for SNPs. The exclusion capacity of the dataset was also calculated using the equation

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

where *fi* is the relative frequency of the ith haplotype and n is the total number of haplotypes.

RESULTS

Assessment of DNA degradation

Gel-electrophoresis revealed concentrated bands for all five randomly chosen samples. Bands for all samples were above the first band of the ladder. Since these samples were DNA extracts that included the entire genome, which is about 2.5 billion bp (https://www.genome.gov/12511476), this is what was to be expected from DNA that was intact. Based on the appearance and placement of the bands, it was concluded that the DNA was not degraded and appropriate to use in PCR.

Amplification of Target Loci

All successfully amplified samples revealed bands around the expected length of the PCR product, which was 1851 bp. From this, it was concluded that amplification of the target sequence was successful. All PCR products were determined to be at a concentration of 50 μM, meaning that the samples were ready to send out for sequencing.

DNA Sequencing

After editing and aligning all 14 sequences, it was determined that all sequencing reactions were high quality and a clean sequence was obtained for the target region of all samples. There sequences were determined to be high quality by the lack of double peaks and ambiguous bases and the presence of clear, single peaks from which a particular base could be identified.

Statistical Analysis

After a pairwise alignment to the reference genome, only one SNP was found in the target region for all 14 samples sent out for sequencing. This SNP occurred at position 10, 257 and included a change from G to A. The exclusion capacity was calculated to be 0, which was to be expected since all samples had identical sequences.

DISCUSSION

The purpose of this study was to find SNPs outside of the HV1 region in the mtDNA of canine mtDNA samples. Samples were chosen by their haplotype and location of origin as only samples that carried the B1 haplotype from Hawaii were used to try and resolve the problem of skewed haplotype diversity in Hawaii. Fourteen out of 24 samples were amplified for the mtDNA region spanning bases 10,251-10,354 and all samples were sequenced successfully. These sequences were then aligned to the reference canine mtDNA sequence and SNPs were recorded. However, only one SNP was found and it was common to all 14 samples (Table 3). This SNP has been previously reported in Webb and Allard (2009a). It is not surprising that Webb and Allard (2009a) would also report this SNP as their study was done on dogs from the contiguous United States, which is geographically very close to Hawaii and where the dogs that were first introduced to Hawaii came from. However, Webb and Allard also found 8 other SNPs in this region (2009a). This raises the question of why these other SNPs were not seen in the samples from Hawaii.

The exclusion capacity of 0 calculated for this population shows that this particular region is not discriminating for these particular samples. This lack of discriminatory power is also in contrast to the findings of Webb and Allard, who calculated an exclusion capacity 0.982 for the entire mtGenome for their samples (2009a). However, variability could be found in other regions of the coding mtDNA. The lack of diversity could also be indicative of something happening in the Hawaiian population, such as genetic drift, that is lowering variability in that population.

The lack of diversity found in the samples has some interesting implications. It may not be surprising that this region did not show variability as it is a coding region and therefore has selection against mutations in this region (Björnerfeldt et al. 2006). The gene

coded for by the region of interest is the *ND4* gene. In humans, mutations in this gene are associated with age-related macular degeneration (Restrepo et al. 2014) and cystic fibrosis (Valdivieso et al. 2007). Since there are severely debilitating disease, there would be a lot of evolutionary constraints against mutation in this gene.

While finding SNPs in this region may not be impossible, the SNPs that were found in this region by Webb and Allard were found to be unique or possessed by only a few individuals (2009a). While the dogs in this study did not show variability in this region, it is still imperative to find mutational "hotspots" in the mtDNA that can be used for small, isolated populations. Small regions of mtDNA are important to find because forensic samples are often degraded and sequencing of the entire mtDNA is usually impossible or impractical even if it has been previously shown that full mtDNA sequencing would allow for the most discrimination (Angleby et al. 2014). It is also not financially prudent to sequence the entire mitochondrial genome as it is very large and would be vastly more expensive to sequence then a 1000 bp part of the mtDNA (Angleby et al. 2014). Two additional small, variable regions outside of the mtCR have been identified (Webb and Allard 2009a) (Table 4).

Table 4. Variable Regions outside of the mtCR that should be surveyed for variability

Region of mtDNA	SNP frequency	Coding region?	What is coded for
11,800-12,006	1 in 12.9 bases	Yes	*ND5* gene
8,661-9,028	1 in 16 bases	Yes	*Cox3* gene

The two additional variable regions found by Webb and Allard (2009a) are shown along with the SNP frequency of the region and what the region codes for.

There may be other explanations for the lack of diversity besides evolutionary constraints on coding regions of DNA. Small, isolated populations tend to be less genetically diverse. This could be due to multiple causes, including founder effect and bottleneck effect. The fact that Hawaii is a small isolated population means that founder effect is much more likely to have occurred. The difference in haplotype diversity from the contiguous United

States supports the notion that founder effect has occurred in Hawaii since the population is so skewed for the B haplotype, in particular B1 (Spadaro et al. 2015). It is likely that the population of dogs that originally colonized the Hawaiian Islands had many individuals that carried the B1 haplotype. This overrepresentation of the B1 haplotype has become even more exaggerated over the generations and has led to the significantly skewed population of today. Founder effect often happens on islands and therefore finding a way to distinguish between samples of the same haplotype, especially in regards to populations where founder effect has occurred or is likely to occur, is imperative. DNA in these populations needlessly loses much of its value as evidence and if new regions are found it would greatly increase a sample's evidentiary value.

Founder effect has been previously shown to exist in Hawaii. Coral reef fish populations in Hawaii have been found to exhibit the lack of genetic diversity indicative of founder effect (Planes and Lecaillon 1998). This study looked at two different species of fish that were introduced to Hawaii between 1956 and 1958. They compared the genetic diversity of these fish to the genetic diversity of fish from populations from where the fish were native. They found a distinct lack of heterozygosity in the Hawaiian populations when compared to native populations. They concluded that the lack of genetic variation was due to the small number of fish introduced to the Hawaiian Islands (Planes and Lecaillon 1998). Small population size and an unrepresentative sample population are the two things required for founder effect to occur.

Finding regions outside of the HV1 would also help in cases where the DNA is significantly degraded. These other variable regions would provide options in the event that the HV1 region is degraded. With multiple regions, there are multiple chances to amplify the

DNA from the sample. Likewise, even if the HV1 region is not degraded in the sample, using more that one region for forensic analysis would be very beneficial, as it would lead to more concrete evidence. It has already been determined that sequencing the entire mtDNA would be most useful for forensic analysis, but it is normally impossible (Verscheure et al. 2014). Therefore, analysts must find small regions that are both not susceptible to degradation and variable enough to be able to distinguish between dogs. For most analysts, this region is the HV1. However, in the event that the population is highly skewed, such as the Hawaii population, this region does not provide much discriminatory power and it has rather low evidentiary value (Verscheure et al. 2014).

The original goal of the study was to look at three previously found variable regions outside of the HV1 region found by Webb and Allard (2009a). However, due to a variety of constraints the study was reduced to only one region. Since this region was not found to be variable, the other two variable regions should be surveyed for variability (Table 4). The potential gains of finding variable regions outside of the HV1 region are vast. For instance, it would allow much more animal DNA to be used as evidence and the increased use of animal DNA could lead to quicker, more accurate trials. It would also help ease the burden placed on forensic analyst working with degraded DNA, giving more regions to sequence in the event that one is degraded.

The use of canine DNA is forensics could be vastly improved with the use of variable regions of the mtDNA outside of the HV1 region in instances where the HV1 haplotype is the same. These regions could prove useful in populations with skewed haplotype diversities such as the B1 haplotype in Hawaii (Spadaro et al. 2015) and the A11 haplotype in Germany, which has been found to account for about 29% of the population (Angleby and Savolainen

2005). Three such regions have previously been identified and one has been surveyed in this study. Due to the lack of diversity found in this region, looking for variability in the remaining mtDNA, particularly the two regions not surveyed in this study but surveyed in Webb and Allard (2009a), is imperative.

While only possible if blood or tissue is found, there are other options, such as microsatellites, that could be surveyed instead. Microsatellites are a series of dinucleotide repeats in the nuclear genome with unique flanking sequence that can be easily amplified (Weber and May 1989). Microsatellites are an older method for forensic analysis but if the mitochondrial genome proves to not show variability they are still a viable option. Microsatellites have been shown to be very polymorphic and abundant in genome (Müller et al .1999). The idea of microsatellites is that a few are chosen to create a "DNA fingerprint" pattern than is in theory unique to an individual (Müller et al. 1999).

In summary, canine mtDNA use in forensics is vastly underused in forensics today. This could be due to the lack of established databases for particular regions. Another problem could be that a certain population has an overrepresentation of one haplotype, lessening the evidentiary value of the DNA. Since the HV1 region is so frequently used in forensics, it is imperative to find regions outside of the HV1 region that prove to be variable and are able to discriminate between individuals. If these types of regions are found, it would allow for more pervasive use of canine mtDNA in forensics. If there are no such regions of the mtDNA, other routes of investigation, such as microsatellites, should be looked at.

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