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Developmental validation of Mini-DogFiler for degraded canine DNA☆

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ABSTRACT

Dogs (*Canis lupus familiaris*) are kept as pets in 39% of American households and are, therefore, a significant source of potentially probative biological evidence. As with any biological evidence, degradation can occur as a consequence of environmental exposure causing fracturing of the DNA and a resulting loss of intact template. Degraded human DNA analysis has benefited from the application of primer sets that amplify shorter nuclear sequences for core STR loci (miniSTRs), resulting in improved DNA profiles. This same approach was applied to our core canine STR loci. The 16-locus "DogFiler" panel was redesigned into three panels of miniSTRs for analysis of degraded canine DNA, with all primer pairs producing amplicons below 205 base pairs in length. These new miniSTR marker panels – known as Mini-DogFiler – were validated according to SWGDAM guidelines, and concordance with the original 16-locus multiplex was demonstrated through genotyping 1244 samples. The combination of these miniSTRs and a half-volume reaction increased the amplification success of degraded and low copy number canine biological samples resulting in a near three-fold increase in reportable alleles. This assemblage of miniSTRs along with the DogFiler panel and associated allelic ladder are the first non-human DNA profiling system to parallel the human forensic paradigm.

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1. Introduction

Due to man's co-habitation with domesticated animals, use of animal evidence has become a valuable resource in linking perpetrators to the crimes of murder, burglary, and sexual assault; in cases of animal cruelty and dog fighting; and in animal attacks on humans or other animals (e.g. pets, livestock, or protected wildlife species). Short tandem repeat (STR) markers have become the standard tool for individual DNA profiling not only in human forensic casework [1–3] but also for cases involving animal evidence [4–8]. The implementation of non-human STR profiling has given investigators the ability to identify an individual animal and compare it to crime-scene evidence with a high degree of confidence.

With any biological evidence, the condition of that evidence is critical to successful DNA profiling. Sample degradation can occur from exposure to various conditions including low pH, heat, ultraviolet (UV) light and soil microbes [9–12]. Obtaining a full nuclear STR profile can be challenging for degraded DNA due to extensive DNA fragmentation caused by such environmental exposure. The successful amplification of shorter DNA fragments from degraded samples was first observed in STR analysis of victims in the 1995 Waco Branch Davidian fire [13–15] and then again in the attack on the World Trade Center Towers on September 11, 2001 [16,17]. While those markers were not designated as "mini STR" markers, it was noted that, when working with degraded samples, the amplification of shorter PCR products resulted in reduced allelic drop-out and increased profiling success. Because the CODIS [9] loci are used extensively in human forensic DNA testing in the U.S., these markers were the ideal candidates for conversion to miniSTRs for degraded human samples. Due to extensive databases of standardized alleles, special care was taken during development of the miniSTRs to ensure database compatibility. Database compatibility between standard and miniSTR panels for canine forensic analyses is equally essential in order to utilize the large established database maintained by the University of California, Davis, Veterinary Genetics Laboratory (VGL) Forensic Unit. The DogFiler panel was developed as a single-reaction multiplex consisting of 15 novel

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unlinked tetra-repeat STR markers [18] and the sex-linked SRY gene [19]. The panel has been validated according to SWGDAM revised guidelines for developmental validation [20] and found to be robust, sensitive and discriminating. An associated allelic ladder has been assembled with all alleles sequenced and named according to the International Society for Forensic Genetics (ISFG) recommendations [21] and the "one-change rule" [22]. The panel has been used on forensic casework that has been adjudicated and, therefore, accepted by the U.S. court system (e.g., State of California vs, Huynh, CD #222832). This panel was also instrumental in establishing the nation's first dog-fighting database [23,24], thereby making it the ideal candidate for development of a complementary suite of miniSTRs.

Canine DNA evidence in forensic casework can be degraded due to recovered evidence being compromised (e.g. hairs from decomposing bodies), because dogs themselves spend a significant amount of time out-of-doors (environmental insult), and because the sample type may be inherently challenging. Fecal samples are often encountered in investigations related to canids, and such samples are notorious for degradation and inhibition. Mini-DogFiler was developed to meet the challenges of degraded DNA analysis in dogs and to provide continuity with the established VGL Forensics canine database. Our objective of enhancing genotyping success through a significant reduction in amplicon sizes while keeping the number of multiplexes to a minimum was achieved through primer redesign and comprehensive validation studies, thereby increasing amplification success and providing for increased stringency in forensic analysis of domestic dogs. Here we present the results of our developmental validation of Mini-DogFiler based upon the revised SWGDAM guidelines [20]. Characterization (SWGDAM 2.1) and population (SWGDAM 2.7) studies were fully addressed in the original DogFiler manuscript and are not included in this validation.

2. Materials and methods

2.1. Primer design

Fourteen of the sixteen oligonucleotide primer pairs from the original panel were redesigned using Primer3 [25] with the goal of reducing the amplicon size to less than 200 bp. Due to the already small amplicon size of STR VGL1063 and the SRY sex identification marker, primers were not redesigned for those loci.

2.2. Samples

The reference database consists of opportunistic blood samples ($n = 1027$) that were collected by venipuncture into EDTA anti-coagulant tubes from IDEXX Laboratories, Inc., in OH, TX, CA, FL, OR, IL, NV, AZ, MD and GA. Sample information, including breed and sex, was owner-identified. Spleen samples from the Cornell Canine Reference Family ($n = 217$) (NIH Grant EY006855) [26] were included in the accuracy study for concordance. Whole blood from a three-year-old yellow male Labrador Retriever was collected by venipuncture into EDTA anti-coagulant tubes for the degradation study. Bones used for the degradation study were exhumed from the remains of a two-year-old male Dalmatian dog that had been hit by a car and buried for 16 yrs.

For reference database samples, the buffy coat was washed in buffer until the pellet was white. DNA extraction was performed by incubation in NaOH and subsequent neutralization with Tris–HCl (French National Institute for Agricultural Research, personal communication, 1995; comparable to the protocol reported by Graffy [27], but without the final concentration and washing steps). For spleen samples, a 2 mm × 2 mm section was excised and digested overnight in Proteinase K followed by organic extraction [28] and concentration with Microcon® centrifugal filters (Millipore, Billerica, MA). Blood samples spotted on material were digested in Proteinase K overnight followed by organic extraction [28] and concentration with a Microcon centrifugal filter. Bones were sonicated in Tergazyme and then 10% bleach followed by sterile water. The bone was dried and then powdered in a blender in the presence of liquid nitrogen. 5 mL of crushed bone was decalcified in EDTA/Sarkosyl [29], digested overnight in Proteinase K and bone lysis buffer followed by organic extraction [28], and the extract concentrated with Amicon® Ultra-4 centrifugal filter units (Millipore). Hairs from closed casework were extracted by digestion in Proteinase K followed by organic extraction [28] and concentration with a Microcon centrifugal filter.

All sample extracts and their dilutions were quantified using a real-time canine-specific quantitative PCR (qPCR) assay [30] on an AB 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) incorporating an Internal positive Control (IPC) to identify inhibition [31].

2.3. PCR amplification

The 25 μL reactions contained 0.06–0.65 μM primers (Applied Biosystems and Operon) balanced for peak height, 200 μM dNTPs (ISC Bio Express, Kaysville, UT), 1× Titanium Taq™ PCR Buffer (Clontech Laboratories, Inc., Mountain View, CA), 0.4× Titanium Taq™ polymerase (Clontech Laboratories, Inc.),up to 3 μL of DNA extract, and sterile DNA grade water (Fisher Scientific, Pittsburgh, PA) to volume. The 12.5 μL half-volume reactions [32] included 0.06–0.65 μM primers, 200 μM dNTPs (ISC Bio Express), 1.5× Titanium Taq™ PCR Buffer, 0.8× Titanium Taq™ polymerase, and 5 μL of DNA extract.

PCR was performed on Applied Biosystems 2720 Thermal Cyclers. The thermal cycling parameters consisted of an initial 1 min denaturation at 95 °C followed by 31 cycles of: 30 s denaturation at 95 °C, annealing for 30 s at 62 °C, extension for 1 min at 72 °C. A final extension was performed for 30 min at 72 °C with a 5 °C hold until the plate was removed from the thermal cycler. Due to the redesign of the primers, a range of annealing temperatures from 60 to 64 °C was evaluated (SWGDAM 2.10.1).

2.4. Capillary electrophoresis and data analysis

Samples were prepared for fragment separation by diluting 1 μL of PCR product into 10 μL of ddH_2O, diluting 1 μL of that dilution into 10 μL of HiDi formamide (Applied Biosystems), and adding 0.0625 μL GeneScan™ 500 LIZ® size standard (Applied Biosystems). Samples were denatured for three min at 95 °C. Capillary electrophoresis was carried out on an AB 3730 DNA Analyzer (Applied Biosystems) using the GeneMapper36_POP7 run module. Electropherograms were analyzed using both STR and [33] and GeneMapper® (Applied Biosystems) software.

3. Developmental validation

3.1. Species specificity, SWGDAM 2.2

DNA samples from dog (*Canis lupus familiaris*), cat (*Felis catus*), Mule deer (*Odocoileus hemionus*), cow (*Bos taurus*), horse (*Equus ferus caballus*), elk (*Cervus elaphus*), black bear (*Ursus americanus*), pig (*Sus scrofa*), sheep (*Ovis aries*), llama (*Lama glama*), red fox (*Vulpes vulpes*), California sea lion (*Zalophus californianus*), mouse (*Mus musculus*), coyote (*Canis latrans*), Yellow-backed duiker (*Cephalophus silvicultor*), human (*Homo sapiens*), *Escherichia coli*, *Staphylococcus aureus*, and *Pasteurella aerogenes* were tested for amplification using the Mini-DogFiler panels. Extract concentrations ranged between 1 and 5 ng/μL and all mammalian species were male animals.

3.2. Sensitivity, SWGDAM 2.3

To evaluate the sensitivity of the Mini-DogFiler panels, two known DNA samples from a Husky–Shepherd mix and a Bichon Frise were quantified and serially diluted to yield samples concentrations ranging from 500 pg/μL to 31 pg/μL. One μL of each dilution was amplified using the 25 μL and the 12.5 μL reaction volumes to determine the lower detection limit for each reaction.

3.3. Stability, SWGDAM 2.4

Whole blood, 100 μL, was spotted onto laundered white cotton jersey material. Twenty-four spots were allowed to air dry overnight at room temperature in a fume hood. The cloth was divided into four strips with each strip containing six spots. As a control, 200 μL of whole blood was subjected to organic extraction and the extracts were stored at −20 °C. The sample strips were placed in the following locations: attached to the east side of a house, inside a dark storage shed, inside the trunk of a vehicle, and buried under five cm of soil. Blood spots were cut from the material at two-month intervals.

A degradation series was prepared by digesting an extract of Husky–Shepherd DNA with DNase I (Fermentas Life Sciences) for varying lengths of time. The reaction contained 80 μL of 195 ng/μL DNA, 11 μL of 10× DNase I reaction buffer and 19 μL of DNA grade water (Fisher Scientific), for a reaction volume of 110 μL. From the reaction mix, 10 μL was removed, to serve as the 0 time point, and 2 μL DNase I (1 U/μL) was added to the remaining mix. A degradation series was produced by incubating the reaction at room temperature (24 °C) and removing 10 μL volumes at 1–5, 7.5, 10, 12.5, 15, and 20 min time points. The DNase activity was stopped by mixing the DNA with 2 μL of 25 mM EDTA and heating at 65 °C for 15 min for each of the time points including the 0 min sample [34]. Samples were visualized on a 2% agarose gel and then diluted 1:3 prior to PCR amplification.

All samples from each degradation study were amplified with both the DogFiler [18] and the Mini-DogFiler panels.

3.4. Reproducibility, SWGDAM 2.5

Eighteen DNA samples from the reference database were provided to three analysts to independently genotype using two different Applied Biosystems 3730 Genetic Analyzer instruments on different days. The data from each analyst were compared to determine run-to-run consistency.

3.5. Casework samples, SWGDAM 2.6

Extracts from two cases were re-examined using the Mini-DogFiler multiplexes. The first case involved an alleged wolf hybrid that had escaped and attacked neighborhood dogs. The sample consisted of hairs snagged on a doghouse. The second case involved a child who was attacked by a dog while riding his bike. That sample consisted of hairs collected from the child's jacket, of which seven white hairs were co-extracted.

3.6. Mixture study, SWGDAM 2.8

In animal forensic casework, mixed samples are encountered in cases such as dog-on-dog attacks or in dog fighting cases. A mixture study was performed to determine the level at which a minor contributor could be deduced. Mixtures of two individuals were created at 1:1, 1:2, 1:3, 1:4, 1:10 and 1:20 ratios with the total amount of input DNA for each reaction maintained at 1 ng.

3.7. Precision and accuracy, SWGDAM 2.9

The precision of the sizing was demonstrated by evaluating twelve injections of amplified product from the DNA ladder on the AB 3730 DNA Analyzer. The mean and standard deviation was calculated for the common alleles at each locus. Accuracy was verified through concordance with samples from the reference database and the Cornell Canine Reference Family, both of which had been previously profiled with the DogFiler panel.

3.8. PCR-based procedures, SWGDAM 2.10.2

To evaluate the effects of DNA concentration on heterozygous peak imbalance (defined, for the purpose of this study, as the peak height of the longer amplicon exceeding the peak height of the shorter amplicon), two samples were amplified in duplicate using a DNA template concentration of 125 pg, 63 pg, 31 pg, 16 pg, 8 pg, and 4 pg in a 12.5 μl reaction.

4. Results

4.1. Characterization, SWGDAM 2.1

Table 1 details the primer sequences, fragment size ranges for the miniSTRs, and the overall size reduction for each locus compared to the original DogFiler fragment size. All primer pairs except K9 SRY and VGL2136 contained one primer with a seven-base tail (GTTTCTT) [35] to promote adenylation. The number of multiplexes was minimized to reduce the number of amplification reactions required and conserve sample and reagents while still keeping the product for all loci below 200 bp. Four of the sixteen loci produced amplicons exceeding 200 bp: VGL2918, VGL1828, VGL1165, and VGL1541, with the maximum fragment sizes for these loci being 201, 202, 203 and 204 bp, respectively. However, all of these loci were within the 200 bp target when the 7-base primer tail was accounted for. VGL2918 has two reverse primers to promote annealing at a point mutation in the primer-binding region as described by Wictum et al. [18]. Designing primers around the SNP was impractical due to its proximity to the repeat region.

The primers were assembled into three panels containing seven, five, and four loci. While most primers multiplexed with little problem, primers for VGL0910 caused formation of numerous small artifact peaks when paired with VGL1828 from panel A or VGL3438 from panel B. Artifact peaks created by residual FAM dye were initially noted throughout the panel A electropherograms, but this was resolved by changing the vendor for the dye-labeled primers.

4.2. Species specificity, SWGDAM 2.2

All of the samples of bacteria – *E. coli*, *S. aureus*, and *P. aerogenes* – failed to produce any detectable product. Non-specific peaks were noted in mule deer (*O. hemionus*), in horse (*E. caballus*), in elk (*C. elaphus*), in mouse (*M. musculus*) and in black bear (*U. americanus*) at VGL1165; in black bear at VGL3112; and in sea lion (*Zalophus californianus*) at VGL1063. Black bear exhibited polymorphism at locus VGL1063, and both the bear and seal samples amplified product for the SRY gene. No product was detected in the remaining species tested.

As expected, coyote (*C. latrans*) and wolf (*C. lupus*) amplified at all loci, and red fox (*V. vulpes*) amplified at twelve of the fifteen loci. In testing 24 coyotes, microvariants and out-of-range alleles were observed in VGL1063, VGL2409, VGL1165, VGL1828, VGL3438, VGL3235 and VGL0760. In 24 wolves, microvariants and out-of-range alleles were observed in VGL1063, VGL2409, VGL1828, and

Table 1
Primer details for Mini-DogFiler.

Locus	Panel		Dye label	MiniSTR primer sequence (5′ to 3′)	Fragment range (bp)	Size change (bp)
K9 SRY	A	F	NED	GAACGCATTCTTGGTGTGGT	80	0
		R		TGATCTCTGAGTTTTGCATTTGG		
VGL1063	A	F	PET	AGCCACAGAGCCTGAGAGTG	86–138	0
		R	GTTTCTT	CAATCACCACCTTCCCTCCT		
VGL2136	A	F	VIC	TTAAAGGTAACAAGGATGTACTGATGG	77–121	−13
		R		GCATGGAGAAAAAGCAGGTG		
VGL1541	A	F	NED	CCTGATGGAAGAGCTTACTGAGTTT	147–203	−37
		R	GTTTCTT	TGAAGGTCTAGAGAGCAACTCCTG		
VGL1165	A	F	6FAM	ATCTTCCTCTGGCACCACCT	120–202	−70
		R	GTTTCTT	TGAAGATACATAGAAAATAAGGCCAGA		
VGL1828	A	F	VIC	TCCCTTCATTTCCTTTCCTCTC	135–199	−84
		R	GTTTCTT	GGAGCCTGCTTCTCCTTCTC		
VGL2409	A	F	NED	GTGCTTCAACCTCTGTTTCTGAAT	100–148	−8
		R	GTTTCTT	GATAGACCTCCATAACTGACCATAGG		
VGL3008	B	F	6FAM	AGAACACGGTTATTTGCTAGGC	112–180	+2
		R	GTTTCTT	CCAACAGCAGCAGAGGAAGT		
VGL3438	B	F	VIC	TGCTACACTATATGTTGGCAAATTGA	100–152	−36
		R	GTTTCTT	GGACTGCCCTCACAAGCAT		
VGL2009	B	F	PET	GCTCTTAAATTTTCTGGGTTTGG	105–145	−39
		R	GTTTCTT	GAATTTTCGGTGTTGTATATCCAGT		
VGL3112	B	F	PET	CCAATAGAGCATTAAGTAGAGCTGAAA	160–192	−25
		R	GTTTCTT	TTCAGAATTAATCTCCTCACTTTTAATAATATC		
VGL1606	B	F	NED	TCTGATTCTAAAGGGTAGTAAAAAGGA	112–176	−160
		R	GTTTCTT	TAAGCTAAAAAGATACTATTTGCATTGG		
VGL2918	C	F	PET+GTTTCTT	TGCTCCTCCCTCTGCCTATG	130–202	−58
		R-1		GGAAACATGTGTTTTCCCTTCA		
		R-2		GGAAASATGTGTTTTCCCTTCA		
VGL0760	C	F	VIC	GCATTCTGCTTACTCACATGACG	122–186	−154
		R	GTTTCTT	TCCTGTCCCATCAAACACAGA		
VGL3235	C	F	6FAM	TTTCTCCCAATCATTTTCAGGA	95–149	−175
		R	GTTTCTT	TTCTCTCAGCAAAAAGCTCAGG		
VGL0910	C	F	NED	CCCTCTGCTTCTCTCTCTCTGTG	121–181	−165
		R	GTTTCTT	CCAAAGCGTGTTCCTCCTCT		

VGL1606. These same variants were found during the validation of the DogFiler panel. A red fox sample known to amplify target DNA with the original panel primers for VGL1828, VGL1606, and VGL0760 did not yield product when amplified with the mini versions of those primers. This indicates significant sequence variation between red fox and domestic dogs adjacent to the repeat regions of those loci.

4.3. Sensitivity, SWGDAM 2.3

For the standard reaction volume of 25 μL, full profiles were obtained down to 63 pg with dropout occurring at 31 pg. A number of alleles at 31 pg exhibited peaks below the laboratory's minimum threshold and were not scored. In the 12.5 μL half-volume reaction where the amount of DNA was effectively doubled, both samples produced full profiles at all DNA concentrations. At 31 pg in the 12.5 μL reaction, the average RFU values across all loci were 1161 and 939 for the two samples tested – well above the laboratory's analytic threshold (Fig. 1).

4.4. Stability, SWGDAM 2.4

During the year-long study of blood spotted onto a cotton substrate and placed in various environments, amplification success increased significantly when those samples were amplified with the Mini-DogFiler panels at the 12.5 μL reaction volume as compared to the DogFiler panel (Fig. 2). The greatest degradation of DNA – other than the soil sample set that was immersed in water due to excessive rain during the first two months of the study and was fully disintegrated – was noted in exterior samples that were exposed to UV light and moisture, where full degradation occurred at the two-month time point for DogFiler and the six-month time point for Mini-DogFiler. QPCR data (not shown) did not exhibit a depression of the IPC (internal positive control), revealing that the amplification failure in those samples was due solely to environmental degradation.

Large weight-bearing bones (e.g. femur) have been shown to yield more DNA than less dense bones (e.g. rib) [36]. QPCR indicated that both bone samples were fully inhibited through depression of the IPC. A 1:10 dilution of each extract was tested with the DogFiler panel and Mini-DogFiler panels to attempt to dilute out the inhibitors that were present. Unexpectedly, the rib extract provided results at ten of the sixteen loci while the femur extract produced results at just three loci for the standard panel. When amplified with the Mini-DogFiler panels at the 12.5 μL reaction volume, the femur yielded results at fourteen of the sixteen loci while the rib produced a full profile.

In the controlled enzymatic degradation study, the DogFiler panel failed to amplify seven of the sixteen loci at the 3-min

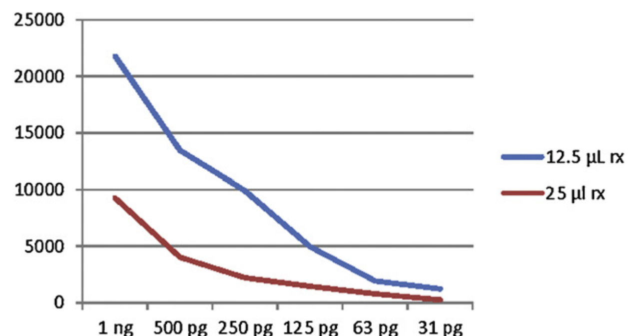

Fig. 1. Average relative fluorescence units (RFU) as a function of template concentration in full and half-volume reactions.

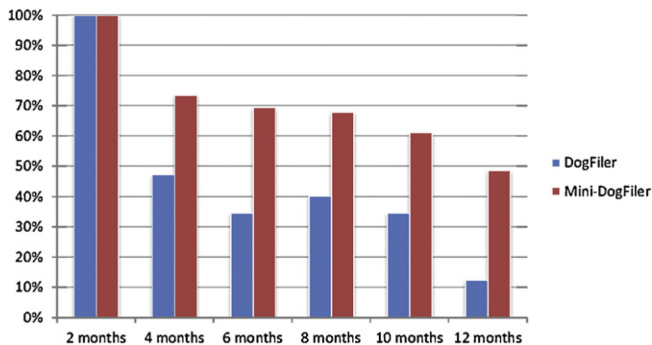

Fig. 2. Percentage of total alleles obtained from all substrates over the 12-month degradation study of blood spotted into cotton for the DogFiler and Mini-DogFiler panels.

time point. Six of the failed loci were the largest amplicons in the panel (VGL2918, VGL1828, VGL0760, VGL3235, VGL1606 and VGL0910). At each subsequent time point, additional loci continued to drop out. At the 10-min. time point, all loci failed with the exception of VGL1063 which continued to amplify until the 15-min time point. When amplified with the Mini-DogFiler panels, all alleles were present and well balanced at the 3-min. time point, and more than half of the loci were still present at the 10-min time point. Total failure occurred for both panels at the 20-min. time point (Fig. 3).

4.5. Reproducibility, SWGDAM 2.5

Each analyst obtained full profiles from the eighteen dog samples. Allele calls were identical between analysts, across days, and between the two different AB 3730 DNA Analyzer instruments for all samples.

4.6. Casework samples, SWGDAM 2.6

The hair samples submitted for the wolf hybrid case were shed hairs without visible root follicles. One of the three hairs selected for testing contained 37 pg/μL and the remaining two hair extracts each contained 8 pg/μL. The extract having 37 pg/μL was tested with the DogFiler panel and yielded alleles for nine of the sixteen loci. The seven largest loci (VGL1165, VGL2918, VGL1828, VGL0760, VGL3235, VGL1606 and VGL0910) ranging from 182 bp to 349 bp did not amplify. When the extract was analyzed with Mini-DogFiler in the 12.5 μL reaction volume, a full DNA profile was obtained (Fig. 4).

In the case of a child being attacked by a dog, the hairs recovered from the jacket worn during the attack again appeared to be shed hairs with no visible root follicles. One hair from the jacket was selected for testing. The DNA extract quantified at 25 pg/μL canine DNA. The extract was first tested with the DogFiler panel. Four loci (VGL3008, VGL3438, VGL1606, and VGL2136) did not amplify, and there was suspected allelic dropout at two more loci (VGL2409 and VGL3112). When analyzed with the Mini-DogFiler panels in the 12.5 μL reaction volume, results were obtained at all loci except VGL2136. The two loci that had exhibited allelic dropout in the original analysis were heterozygous when amplified with the Mini-DogFiler primers in the half-volume reaction.

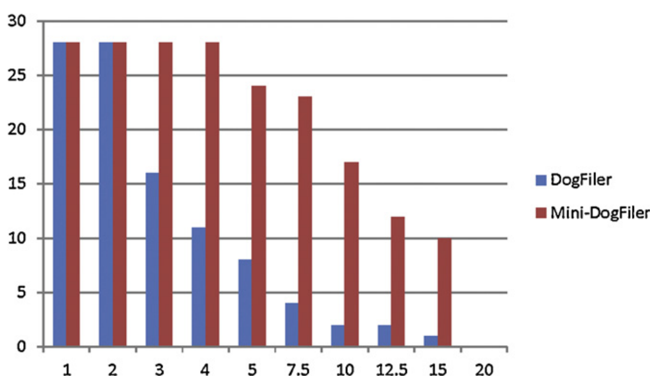

Fig. 3. Percentage of total alleles obtained over time in the DNase degradation study for the DogFiler and Mini-DogFiler panels.

4.7. Mixture study, SWGDAM 2.8

For the 1:1, 1:2, 1:3 and 1:4 mixtures, complete profiles for both animals were discernible. At the 1:10 mixture, some of the minor contributor alleles were obscured by the stutter product of the major contributor preventing characterization of the two genotypes at all loci. For the 1:20 ratio, one of the alleles in the Mini-DogFiler panel C, locus VGL0910, failed to amplify. However, the remaining alleles were above the laboratory's RFU analytic threshold.

4.8. Precision and accuracy, SWGDAM 2.9

The standard deviation for multiple injections of the same sample ranged from 0.03 in loci VGL0709 and VGL0910 to 0.19 in locus VGL1828. All alleles fell within three standard deviations of the mean demonstrating the precision of the profiling system and providing for run-to-run reliability.

The 1244 reference samples analyzed with the miniSTR panels produced full genotypes. A discrepancy was identified at locus VGL1606 where microvariant alleles became whole repeats with the mini primers due to the revised reverse primer lying adjacent to the repeat region. Sequencing of those rare alleles revealed the following indels in the downstream flanking sequence: an insertion (D25Tins) for alleles 14.1, 15.1, 17.1, and 18.1; and a deletion (D99Gdel) for alleles 12.3, 13.3, and 14.3. Some of the microvariants appear to be fixed as to breed (private alleles) and so may provide additional information in forensic investigations or phylogenetic studies. No null alleles were identified.

4.9. PCR-based procedures, SWGDAM 2.10

When evaluated over a range of annealing temperatures from 60 °C to 64 °C, no locus dropout or artifacts were observed. At 60 °C, a decrease in the peak heights of VGL2136, VGL1165, and VGL0910 was observed; at 63 °C a decrease in the peak heights of VGL1828, VGL3438, VGL1606 and VGL3235 was observed; and at 64 °C a further decrease in peak heights at VGL1828 and VGL3235 was observed. For both 61 °C and 62 °C, peak heights were balanced within each of the panels with little difference between the two temperatures, so an annealing temperature of 62 °C was chosen to maintain continuity with the original DogFiler panel.

However, heterozygous peak imbalance was observed across all loci at low copy number (LCN) concentrations (4–125 pg), increasing as input template DNA decreased. Allelic drop-out began to occur at 31 pg template and also increased as template DNA decreased, with 66% of alleles dropping out at 4 pg template. Of greater concern was the incidence of allelic drop-in with decreasing template and 31 cycles of PCR. One drop-in allele was observed at 31 pg DNA (0.008); four occurrences at 16 pg DNA (0.033); two occurrences at 8 pg DNA (0.017); and one occurrence at 4 pg DNA with each appearing in a different locus.

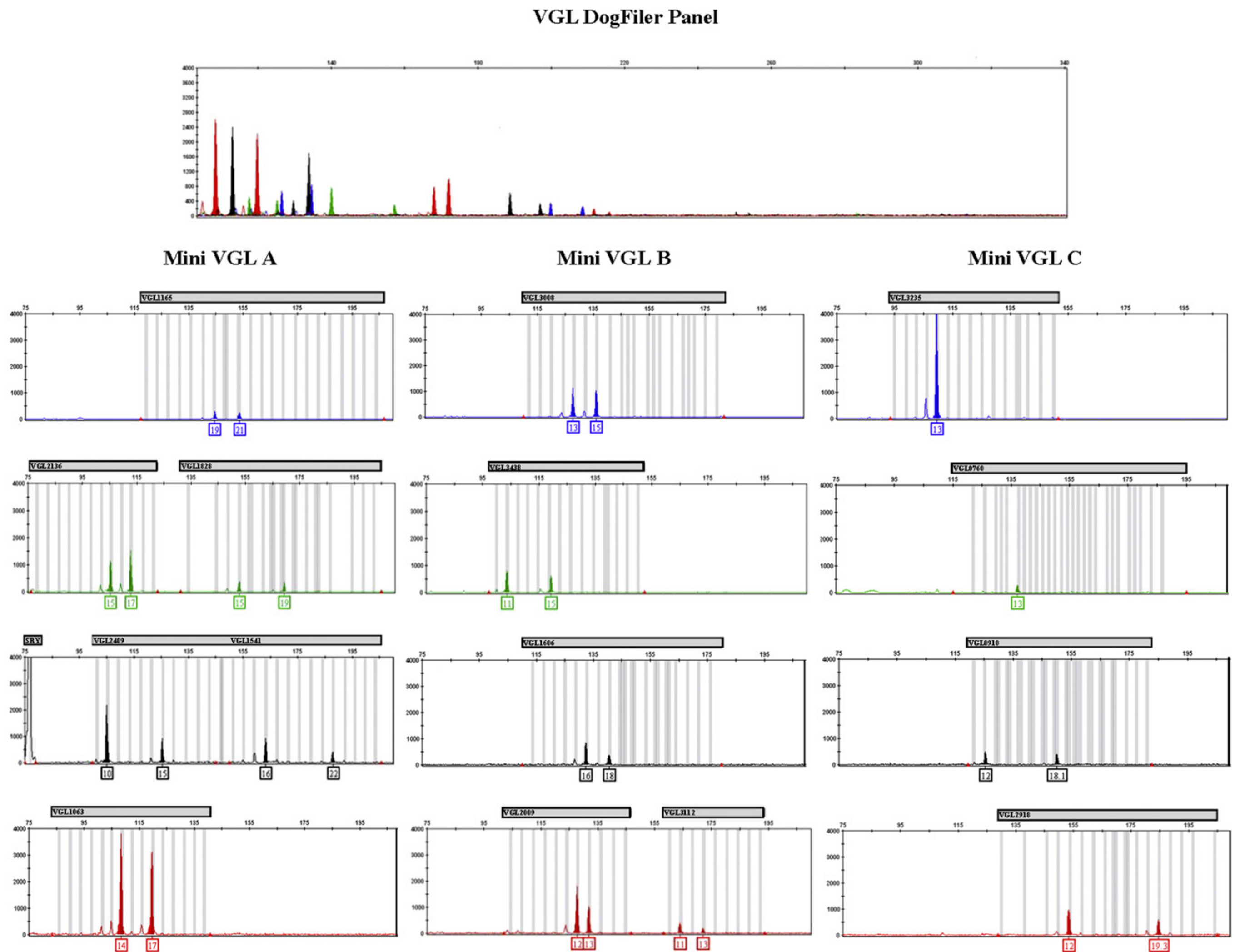

Fig. 4. Casework example exhibiting degradation in the 16-locus DogFiler profile while a full profile was obtained when the sample was amplified with the three miniSTR panels.

5. Discussion

Our primary objective of improving the analysis of degraded canine DNA was achieved through a reduction in size of the amplicons, multiplexing markers into three panels, and integrating a half-volume PCR that effectively increases template DNA for degraded and LCN samples commonly seen in forensic casework. This approach has resulted in a 66% increase in reportable alleles for compromised samples.

The sensitivity of this assay was shown to be comparable to DogFiler with full profiles obtained down to 62 pg in 25 μL reactions and down to 31 pg in 12.5 μL reactions. However, the stochastic effects of low template DNA at 31 PCR cycles resulted in significant peak imbalance with both allelic drop-out and allelic drop-in appearing at 31 pg DNA. The use of miniSTRs may promote drop-in alleles, and increased allelic drop-in has been reported for low molecular weight markers in humans [37]. When sample volume allows, re-amplification of LCN samples to confirm allele calls and reporting a consensus profile is recommended [37]. In no instance were drop-in alleles replicated in this study, and the likelihood of replicate drop-in alleles occurring is very low and would not necessarily result in an exclusion [37].

However, the strength of Mini-DogFiler lies in its ability to amplify degraded samples. Just as was found in human DNA profiling, tissues exposed to heat, cold, and moisture were successfully genotyped with miniSTR primer sets when the use of a large multiplex failed. Validation studies incorporating environmentally degraded samples have demonstrated a doubling of alleles obtained under this system at 6 months and more than three-fold yield at 12 months. A controlled enzymatic degradation study demonstrated not only improved genotyping but also improved peak balance using Mini-DogFiler.

Accuracy was demonstrated through concordance between the original and the miniSTR panels on 1244 samples representing 95 breeds and a variety of mixed-breed dogs. However, microvariant alleles at or below 18.1 in locus VGL1606 of the DogFiler panel (frequency = 0.0067 in our database) should be binned with whole repeats for comparison with results obtained using the miniSTR primers due to indels within the flanking regions encompassed by the original DogFiler primers. Alleles 12.3, 13.3, and 14.3 became 13, 14 and 15 when run with the miniSTRs, while 14.1, 15.1, 17.1, and 18.1 became 14, 15, 17, and 18 when run with the miniSTRs. Microvariant alleles at or above 18.3 repeat units were not affected, but we recommend full consideration be given to any microvariant alleles at this locus. Concordance was further established by amplification of the ladder alleles for the precision portion of the validation. All alleles exhibited correct sizing with no microvariants that would reflect

additional indels, and no significant variation in peak height was observed that would indicate incomplete primer annealing due to primer binding site mutations.

The casework examples exhibited more allelic dropout in the first case (12 alleles) than the second case (7 alleles), although the first case had actually quantified at a slightly higher concentration. This indicates greater degradation as a consequence of the amount of time the first sample was exposed to the external environment. Through application of the miniSTRs and increasing the DNA template by reducing the reaction volume, full profiles were achieved for samples that were both degraded and LCN. Quantification data may not indicate the presence of degradation due to the short (72 bp) amplicon produced by the assay, so samples evidencing degradation by drop-out of the longer alleles when analyzed with the DogFiler panel could be reanalyzed with just the mini primer set incorporating those missing loci. Future research could be directed towards incorporating VGL1828 and VGL1606 into mini panel C and thus combining the six largest amplicons from the DogFiler panel into a single reduced-amplicon multiplex for use as a supplemental panel for degraded samples.

This panel has also shown promise for use in studies of wild canids. While the species specificity study showed that the allele ranges may differ for wild canids when compared to domestic dogs, the loci, nevertheless, are highly polymorphic and have proven useful for that purpose (Supplemental Table S1). Because fecal samples are a noninvasive source of DNA routinely collected for genetic profiling of wildlife, the Mini-DogFiler panels would be useful for countering the degradation often found in feces.

6. Conclusions

The DogFiler panel of markers and its complementary Mini-DogFiler panels are analogous to the AmpFℓSTR® Identifiler® and AmpFℓSTR® MiniFiler™ kits used in human forensic casework. Implementation of Mini-DogFiler has significantly increased the amplification success of canine samples that have been subjected to environmental insult just as the AmpFℓSTR® MiniFiler has done for degraded human samples. The admission of animal biological evidence into courtrooms is increasing, and it must come under the same scrutiny and meet the same standards as those applied to human evidence. Accordingly, this validation study provides confirmation that these miniSTR panels are precise, specific, sensitive, reproducible, and robust, and has defined the limitations of this assay. While many canine loci have been proposed for forensic casework, this study represents the first mini primer sets to be constructed from a panel of canine markers that has been genotyped on a large population database by an ASCLD-LAB accredited laboratory, validated according to SWGDAM guidelines, and accepted in court.

Conflict of interest statement

The authors do not have any known conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2012.09.002.

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