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## PCR MULTIPLEXED MICROSATELLITE PANELS TO EXPEDITE CANINE GENETIC DISEASE LINKAGE ANALYSIS

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### ABSTRACT

Modern dog breeds possess large numbers of genetic diseases for which there are currently few candidate genes or diagnostic tests. Linkage of a microsatellite marker to a disease phenotype is often the only available tool to aid in the development of screening tests for disease carriers. Detection of linkage to a specific disease phenotype requires screening of large numbers of markers across known affected and unaffected animals. To establish high throughput genome scanning this study placed 100 canine microsatellite markers, arranged by fragment size and fluorescent dye label, into 12 PCR multiplexed panels. The highest degree of multiplexing was 11 markers per panel while the lowest was five markers per panel; each panel was run in one gel lane on automated DNA sequencers. Selection of the markers was based upon chromosomal or linkage group locations, degree of polymorphism, PCR multiplex compatibility and ease of interpretation. The marker set has an average spacing of 22.25 centiMorgan (cM). Marker polymorphism was evaluated across 28 American Kennel Club (AKC) recognized breeds. The utility of buccal swab vs. blood samples was also validated in this study as all template DNA was derived from swabs obtained and submitted by participating dog breeders and owners. The PCR multiplexed microsatellite panels and sampling method described in this report will provide investigators

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with a cost effective and expedient means of pursuing linkage studies of specific canine genetic diseases.

*Key Words:* Canine DNA; Polymorphisms; Genome screen; Linkage; Microsatellites

## INTRODUCTION

Comparative gene mapping continues to provide clues to candidate genes from analogous human diseases,<sup>[1-3]</sup> but any and all means of controlling canine genetic disease must be put to use. Disease phenotypes related to either single gene defects or polygenic disorders with a dominant gene effect lend themselves to linkage studies with type II markers.<sup>[4-7]</sup> Integration of the canine linkage and radiation hybrid (RH) maps provided nearly 500 mapped microsatellite markers for potential use in disease linkage studies.<sup>[8-10]</sup> Although these markers provide reasonable coverage of the canine genome they are not all equally effective in genome screening applications. Inadequacies are encountered when public domain markers are rigorously evaluated. These include published PCR primer sequences that fail to amplify detectable product under standard PCR parameters or a lack of polymorphism when markers are examined across a variety of breeds. The present study addressed these concerns and extensively characterized numerous markers for polymorphism on 28 American Kennel Club (AKC) recognized breeds. These breeds are a random cross section of the 150 AKC breeds, currently divided by the AKC into seven distinct groups. Four breeds from each group were selected to provide an accurate assessment of marker polymorphism across all AKC breeds. In addition, the AKC Canine Health Foundation has specifically targeted many of these breeds for genetic disease research.

The use of fluorescence-labeled microsatellites sized on automated DNA sequencers is standard for gene mapping, disease linkage analysis, forensic investigations, and paternity testing.<sup>[11-15]</sup> The degree of automation allowed by this methodology, combined with high-speed DNA fragment analysis and data acquisition software, provides an efficient means for testing large sample numbers. This high throughput process can be further enhanced by multiplex PCR, which allows simultaneous amplification of multiple markers that are identified during gel electrophoresis by unique fluorescent labels. Genetic analyses requiring large number of markers are expedited by this technique, with the added advantage of dramatically decreasing PCR reagent costs. A majority of published markers have not been evaluated for reliable amplification in a multiplex PCR or for PCR products that can be easily interpreted and sized by DNA fragment analysis software. Unreliable markers increase test costs while those that are hard to interpret, relative to allele sizing, increase analysis time, and may yield erroneous linkage information. All markers screened for this study were assessed for reliability of amplification and ease of interpretation; only those that performed well in both areas were selected for inclusion in the project.



## MATERIALS AND METHODS

### Animal Selection

Breeds were selected to provide four representative breeds from each of the seven AKC recognized groups. The breeds screened were: Airedale Terrier, Akita, American Eskimo Dog, Australian Shepherd, Borzoi, Belgian Tervuren, Bernese Mountain Dog, Border Collie, Boxer, Brittany, Bulldog, Bull Terrier, Chow Chow, Doberman Pinscher, Golden Retriever, Greyhound, Jack Russell Terrier, Keeshond, Labrador Retriever, Miniature Bull Terrier, Norwegian Elkhound, Papillon, Pembroke Welsh Corgi, Pomeranian, Pug, Rhodesian Ridgeback, Weimaraner, and Yorkshire Terrier. An average of 41 unrelated animals was tested for each breed. Individual animals were defined as unrelated if they shared no common ancestors within the first generation.

### Marker Selection and Panel Design

Markers were selected from the 1999 canine genetic linkage map<sup>[9]</sup> based on their map location and their reported polymorphism and allele size ranges. Additional unmapped markers were also evaluated based on known high polymorphism values. In total, 117 markers were selected for evaluation.

Forward primers were synthesized and dye labeled with Fam, Hex or Tamra [Applied Biosystems, Inc. (ABI), Foster City, CA]. Reverse primers were synthesized by Operon (Alameda, CA). All primer pairs were initially tested at final concentrations of 0.2  $\mu$ M and 0.4  $\mu$ M and at an annealing temperature of 64°C. Primer pairs that did not amplify at 64°C were tested under identical conditions but at successively lower annealing temperatures until an optimum temperature was determined. Primers were arranged into mini-panels of three to six markers, based on their annealing temperature and dye label and then tested against a minimum of 40 samples from each of 16 breeds to provide an accurate estimate of their allele number and size range. Once parameters were determined for each marker the final multiplex panels were assembled. Markers were grouped by annealing temperature and then organized by dye color and size range as to not allow any overlap of markers labeled with the same dye. As necessary, final primer concentration adjustments were made to provide optimal product yield and the lowest degree of non-specific artifact peaks. Completed panels were tested against the remaining 12 breeds.

### Sample Preparation and PCR Conditions

All samples used in this study were derived from buccal cell swabs taken with nylon bristle cytology brushes (Medical Packaging Corp., Camarillo, CA) collected by owners and submitted directly to the laboratory. DNA was extracted



by heating a single swab for 10 min at 95°C in 400  $\mu$ L 50 mM NaOH and then neutralized with 140  $\mu$ L 1 M *Tris*-HCl, pH 8.0. A 2  $\mu$ L aliquot of this extraction was then used in each PCR reaction. All markers were tested with a PCR reagent mix of 1X PCR buffer (ABI), 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M of each dNTP (Hoffmann-La Roche Inc, Nutley, NJ), 0.7 unit AmpliTaq (ABI), and 2% DMSO. Thermal cycler parameters differed depending on the annealing temperature used; all PCR work was done on MJ Research PTC-100 thermal cyclers (Waltham, MA). Due to the volume of information, all markers, primer sequences, appropriate primer concentrations, and complete PCR protocols for the 12 panels are provided at the Veterinary Genetics Laboratory website: [http://www.vgl.ucdavis.edu/research/canine/paper\\_data/index.jsp](http://www.vgl.ucdavis.edu/research/canine/paper_data/index.jsp).

### Gel Electrophoresis Conditions and DNA Fragment Analysis

One  $\mu$ L aliquots of PCR product were mixed with 2  $\mu$ L Fluorescent Ladder (CXR) 60–400 bases (Promega 400) or Internal Lane Standard 600 (Promega 600, Promega, Madison, WI) fluorescent size standard, denatured on MJ Research PTC-100 thermal cyclers for 3 min at 95°C, then held at 5°C or placed on ice for at least 1 min before gel loading. Two  $\mu$ L aliquots were then loaded onto a 6% denaturing polyacrylamide gel and run on an ABI 377 Automated Sequencer using ABI 10''  $\times$  7 $\frac{1}{8}$ '' short plates (12 cm). Gels were run at 1.10 kV (constant) voltage, 60.0 mA current, 200 W power, 51°C, and 40.0 mW (constant) laser power for up to 2 hours when using Promega 400, up to 3 hours using Promega 600. DNA fragment analysis was performed with in-house designed STRand software<sup>[16]</sup> that replaces ABI Genotyper and Genescan software and is available from the VGL website at: <http://www.vgl.ucdavis.edu/STRand>. This data was then transferred to an in-house database compatible with the STRand software.

### Statistical Analysis

Marker polymorphism was determined by the relative number and frequency of alleles for a specific locus within each breed.<sup>[17,18]</sup> Allele frequencies and observed heterozygosity ( $H_O$ ) were determined by direct counting; expected heterozygosity ( $H_E$ ) was calculated from the allele frequencies. Polymorphism information content (PIC)<sup>[19]</sup> and probability of exclusion (PE)<sup>[20]</sup> were determined for all markers in each of the 28 breeds.

### Linkage Analysis

Accuracy of pedigrees, quality of data, and linkage analysis were performed with the various program options of CRIMAP-prepare, twopoint, build, and chrompic.<sup>[21,22]</sup> A lod score of 3 was used as a minimum threshold for linkage.



## RESULTS

## Marker Multiplex PCR Performance

Of the 117 markers originally selected for evaluation, ten would not amplify under standard PCR parameters, three did not amplify under PCR multiplex conditions, three were difficult to score (Table 1), and one proved uninformative after extensive testing on multiple breeds. The remaining 100 markers were multiplexed into 12 panels, ranging from five to 11 markers

**Table 1.** Listing of Markers by Chromosomal Location. Also Included Are the Number of Alleles, Average Observed Heterozygosity and Ease of Scoring Across All 28 Breeds

Locus	Chromosome	#Alleles	OH	Ease of Scoring
C01.424	CFA01	8	0.472	4
FH2313	CFA01	11	0.694	4
FH2326	CFA01	9	0.636	4
AHT111	CFA02	11	0.649	4
AHT132	CFA02	8	0.489	5
FH2274	CFA02	8	0.598	4
C03.877	CFA03	9	0.493	4
FH2137	CFA03	10	0.650	4
FH2145	CFA03	9	0.469	4
PEZ12	CFA03	10	0.608	4
AHT103	CFA04	7	0.563	3
C04.140	CFA04	9	0.473	3
PEZ13	CFA04	11	0.660	4
C05.771	CFA05	9	0.524	5
CPH14	CFA05	10	0.593	5
FH2140	CFA05	8	0.592	4
C06.636	CFA06	10	0.450	5
CPH03	CFA06	8	0.611	5
FH2164	CFA06	9	0.652	4
C07.620	CFA07	8	0.469	5
FH2201	CFA07	11	0.641	3
PEZ22	CFA07	9	0.645	4
VIASD10	CFA07	8	0.550	4
C08.410	CFA08	10	0.582	5
C08.618	CFA08	8	0.521	5
FH2138	CFA08	14	0.672	3
PEZ11	CFA08	10	0.653	4
C09.173	CFA09	8	0.511	5
C09.250	CFA09	8	0.576	4
C10.404	CFA10	7	0.515	3
FH2293	CFA10	11	0.719	4

(continued)

*Table 1.* Continued

Locus	Chromosome	#Alleles	OH	Ease of Scoring
AHT136	CFA11	2	0.152	4
AHT137	CFA11	8	0.641	4
C11.750	CFA11	2	0.544	3
FH2004	CFA11	9	0.568	4
FH2054	CFA12	10	0.653	5
FH2200	CFA12	10	0.723	4
FH2202	CFA12	10	0.747	4
PEZ05	CFA12	8	0.500	4
AHT121	CFA13	11	0.616	5
C13.365	CFA13	7	0.568	5
C13.391	CFA13	9	0.491	5
C13.758	CFA13	2	0.467	5
C14.866	CFA14	9	0.596	3
PEZ10	CFA14	10	0.720	4
AHT139	CFA15	7	0.471	5
C15.608	CFA15	8	0.469	5
RVC1	CFA15	8	0.574	4
C16.147	CFA16	7	0.339	4
FH2175	CFA16	11	0.607	4
C17.402	CFA17	8	0.551	4
PEZ02	CFA17	10	0.658	4
PEZ08	CFA17	10	0.578	4
AHT130	CFA18	7	0.611	5
AHTk292	CFA18	9	0.511	5
FH2356	CFA18	10	0.473	5
Wilms-TF	CFA18	10	0.607	4
CPH08	CFA19	10	0.549	4
PEZ03	CFA19	10	0.668	5
C20.253	CFA20	7	0.297	5
C20.446	CFA20	8	0.529	3
CPH16	CFA20	8	0.570	5
FH2148	CFA20	10	0.518	4
FH2161	CFA21	10	0.657	5
FH2233	CFA21	13	0.712	4
INRA21	CFA21	7	0.471	5
C22.279	CFA22	10	0.631	4
C22.763	CFA22	9	0.554	4
AHTk253	CFA23	8	0.564	4
C23.123	CFA23	8	0.502	4
FH2001	CFA23	8	0.560	5
FH2283	CFA23	9	0.629	4
FH2079	CFA24	7	0.209	4
C25.213	CFA25	8	0.542	4
FH2324	CFA25	12	0.620	4



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Table 1. Continued

Locus	Chromosome	#Alleles	OH	Ease of Scoring
AHTk211	CFA26	9	0.478	5
FH2130	CFA26	8	0.572	3
C27.671	CFA27	7	0.458	5
FH2289	CFA27	8	0.690	4
LEI002	CFA27	11	0.548	5
PEZ18	CFA27	8	0.680	3
C28.176	CFA28	9	0.507	5
C29.002	CFA29	8	0.456	4
FH2328	CFA29	9	0.583	5
INRA02	CFA29	8	0.564	3
FH2305	CFA30	12	0.656	4
C31.646	CFA31	9	0.533	4
FH2199	CFA31	13	0.775	3
CPH02	CFA32	7	0.498	5
FH2165	CFA33	14	0.677	4
FH2361	CFA33	11	0.672	4
AHT133	CFA37	10	0.498	5
LEI004	CFA37	7	0.538	4
CXX130	Not mapped	8	0.358	4
CXX161	Not mapped	7	0.438	5
CXX263	Not mapped	9	0.559	3
FH2247	Not mapped	12	0.769	4
LEI003	Not mapped	8	0.456	4
LEI005	Not mapped	7	0.459	5
LEI007	Not mapped	8	0.521	3

each. Nine of the panels were each amplified in one PCR reaction and run in one gel lane. The remaining three panels each required two separate amplifications, which were then co-loaded into one gel lane. Variation of annealing temperature and primer concentration proved to be the most effective means to achieve simultaneous amplification of multiple primer pairs. Approximately 90% of the markers amplified at an annealing temperature of 60°C or above; 11 markers required temperatures below 60°C. High annealing stringency made it possible to multiplex up to 11 loci in a single reaction without generating excessive non-specific artifact peaks. More than 86% of the original markers were successfully amplified and multiplexed despite fairly stringent PCR conditions. Approximately one third of the markers were represented by each fluorescent dye: 36 for Fam, 34 for Hex, and 30 for Tamra. Of the markers that did not amplify, two were labeled with Fam, three with Hex and five with Tamra. Failure to amplify was not thought to be a function of the dye label.



The original panels were optimized using fluorescent dye labels, Fam, Hex, and Tamra. In January 2001, Ned and Vic became commercially available. Tamra and Hex were replaced respectively with labels Ned and Vic. The new labels provide for less dye shedding during gel runs, particularly noted with Tamra, and consistently allow for a lower primer concentration to be used. Subsequently, the final panels presented on the web site were optimized using the new labels. Result data remain unchanged.

### Marker Analysis and Allele Sizing

Markers were evaluated for PCR products that could be easily interpreted and sized by DNA fragment analysis software. Ease of allele sizing, with a score of 5 representing the optimum, was based on several factors including lack of non-specific artifact peaks, consistency of sizing predicted by the repeat type, lack of allelic dropout, and lack of differential peak heights. A majority of the markers, 84%, scored 4 or higher with 34 receiving a score of 5 and 51 receiving a score of 4 (Table 1). A marginal score of 3 was given to 14 markers; two received a score of 2 and may be replaced if alternative markers become available near their map locations. Slightly more dinucleotide repeats than tetranucleotide repeats, 57 vs. 42, and only one trinucleotide fulfilled selection criteria. Some reports<sup>[23]</sup> indicate that dinucleotide repeats are difficult to interpret due to stutter peaks; this has proven not to be an issue when using fluorescent dye detection. Although canine dinucleotide repeats tend to be less polymorphic than tetranucleotides,<sup>[24]</sup> when all criteria for inclusion in this study were considered, the two repeat types often ranked equally. Some tetranucleotide loci received low scores because they possessed alleles differing in size by 1, 2, and 3 bases, in addition to the usual multiples of 4 base differences, which made accurate interpretation of allele sizes difficult. In all, the majority of the markers successfully multiplexed were readily scored, providing for high throughput gel analysis. Figure 1 is an electropherogram image of the K9-1 panel during assembly of that panel.

### Marker Statistics and Resolution

It was not feasible to present the large volume of statistical data, 114,737 genotypes, generated by this study in a journal format. Therefore, the complete statistical information for each marker on all 28 breeds can be found at the Veterinary Genetics Laboratory web site [http://www.vgl.ucdavis.edu/research/canine/paper\\_data/index.jsp](http://www.vgl.ucdavis.edu/research/canine/paper_data/index.jsp). Table 1, presented in the text, lists the average number of alleles and average heterozygosity across breeds for each marker to give a preliminary indication of the possible informative value of the markers. However, providing an overall average PIC value for these markers across 28 breeds can be misleading due to the high degree of genetic heterogeneity between modern dog breeds and the resultant wide variation in marker polymorphism.<sup>[17,18]</sup> For example,





the overall average PIC for FH2238 was 0.572 yet the range across the breeds was from 0.085–0.806. Therefore, PIC, PE, and heterozygosity values for each marker are presented individually by breed on the web site. Listed in Table 1 and on the web site is the chromosomal distribution of markers according to the latest integrated canine map;<sup>[25]</sup> the present panels cover all but four chromosomes. In-house linkage analysis shows an average distance between markers of 22.25 cM with the smallest gap being 0.8 cM and the largest being 56.4 cM. The panels also contain seven markers, which currently remain unmapped.

### Sampling Methods

A major obstacle to the completion of this project was the large number of samples required for accurate statistical data. The non-invasive buccal swab sample collection technique resulted in a significantly higher rate of owner participation than usually seen with requests for blood sample donations. Template DNA derived from buccal cell swabs rather than blood samples proved not to be an issue. Greater than 99% of samples submitted to the laboratory yielded more than adequate amounts of template DNA, generally enough for 200 plus PCR reactions per swab. In addition, consistent amplification of PCR product was obtained from the relatively crude buccal swab preparations. The swabs were shipped by regular post with no restrictions on temperature or transit time.

### DISCUSSION

The extensive statistical data presented in this study, 100 markers across 28 AKC breeds, have provided confirmation of the expected disparity in marker polymorphism between phenotypically diverse domestic dog breeds. A considerable number of the breeds tested were quite homogenous, many unrelated individuals possessed similar DNA profiles, and most markers had unusually low numbers of observed alleles. Lack of canine genetic diversity may eventually necessitate selective out-crossing among breeds. Expanded profiling of all recognized breeds and feral populations using the 100 marker panels will help determine the extent of existing genetic variation. The substantial variations in allele numbers and frequencies among the 28 targeted breeds dictate the need to determine baseline polymorphism values on any breed prior to beginning linkage analysis studies.

The 100 markers presented here represent a significant first step towards a minimum 300-marker set, placed in PCR multiplexes, and which will provide a 10 cM low-resolution genome screen assuming a canine genome size of 2650 cM.<sup>[9]</sup> Screening hundreds of samples, from affected and unaffected animals, for a specific disease investigation will be significantly more cost effective and expedient through the use of PCR multiplexed panels of well-characterized markers. The statistical data presented here, combined with the PCR multiplex



parameters, will allow many investigators to quickly initiate linkage analysis for their specific disease study.

Detection of marker linkages will aid in development of indirect diagnostic tests to screen for carriers of genetic disorders<sup>[26]</sup> or in the determination of the actual chromosomal location of the disease gene(s).<sup>[4,5,27,28]</sup> The availability of diagnostic tests for inherited diseases will help breeders make informed breeding decisions with an ultimate goal of decreasing the incidence of canine genetic diseases.

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