TRANSMISSION GENETICS OF PANCREATIC ACINAR ATROPHY IN THE GERMAN SHEPHERD DOG AND DEVELOPMENT OF MICROSATELLITE DNA-BASED TOOLS FOR CANINE FORENSICS AND LINKAGE ANALYSIS

A Dissertation

by

LEIGH ANNE CLARK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Veterinary Microbiology

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Approved as to style and content by:

Keith E. Murphy (Co-Chair of Committee)	Jörg M. Steiner (Co-Chair of Committee)
Kelly M. Credille (Member)	David A. Williams (Member)
Ann B. Kier (Head of Department)	

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ABSTRACT

Transmission Genetics of Pancreatic Acinar Atrophy in the German Shepherd Dog and Development of Microsatellite DNA-based Tools for Canine Forensics and Linkage Analysis. (May 2004)

Leigh Anne Clark, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Keith E. Murphy
Dr. Jörg M. Steiner

The domestic dog, *Canis lupus familiaris*, has emerged as a model system for the study of human hereditary diseases. Of the approximately 450 hereditary diseases described in the dog, half have clinical presentations that are quite similar to specific human diseases. Understanding the genetic bases of canine hereditary diseases will not only complement comparative genetics studies but also facilitate selective breeding practices to reduce incidences in the dog. Whole genome screens have great potential to identify the marker(s) that segregate with canine hereditary diseases for which no reasonable candidate genes exist. The Minimal Screening Set-1 (MSS-1) was the first set of microsatellite markers described for linkage analysis in the dog and was, until recently, the best tool for genome screens. The MSS-2 is the most recently described screening set and offers increased density and more polymorphic markers. The first objective of this work was to develop tools to streamline genomic analyses in the study of canine hereditary diseases. This was achieved through the development of 1)

multiplexing strategies for the MSS-1, 2) a multiplex of microsatellite markers for use in canine forensics and parentage assays and 3) chromosome-specific multiplex panels for the MSS-2. Multiplexing is the simultaneous amplification and analysis of markers and significantly reduces the expense and time required to collect genotype information.

Pancreatic acinar atrophy (PAA) is a disease characterized by the degeneration of acinar cells of the exocrine pancreas and is the most important cause of exocrine pancreatic insufficiency (EPI) in the German Shepherd Dog (GSD). Although the prognosis for dogs having EPI is typically good with treatment, many dogs are euthanized because the owners are unable to afford the expensive enzyme supplements. The second objective of this work was to determine the mode of transmission of EPI in the GSD and conduct a whole genome screen for linkage. Two extended families of GSDs having PAA were assembled and used to determine the pattern of transmission. The results of this indicate that PAA is an autosomal recessive disease. The multiplexed MSS-1 was used to conduct an initial whole genome screen, although no markers were suggestive of linkage.

For Preston and the dogs

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My extensive network of family and friends is too lengthy to mention here. Nonetheless, I extend my sincere thanks to each of you.

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CHAPTER I

INTRODUCTION

The dog as a model

In recent years the domestic dog, *Canis lupus familiaris*, has emerged as a model for the study of human hereditary diseases, gene expression, and development. The dog, believed to be the first domesticated animal, is in the genus *Canis*, along with the coyote, jackals, and various wolves. All species in this genus are phylogenetically closely related and are capable of interbreeding (Wayne and Ostrander 1999). Although it may seem that the vast phenotypic diversity of the dog must be the result of multiple founding species, current theory holds that the dog diverged exclusively from the wolf, *Canis lupus*. Morphologically, dogs are most similar to Chinese wolves (Wayne and Ostrander 1999) and recent studies point to an East Asian origin (Savolainen et al. 2002). These studies also suggest that a single domestication event occurred roughly 15,000 years ago, a date that is consistent with archaeological records that indicate a date of 9,000 to 14,000 years ago (Savolainen et al. 2002).

Centuries of selective breeding practices by humans have created more than 300 breeds of dog, each with its own distinctive morphological and behavioral characteristics. It is believed that only a small number of genes is responsible for the unique characteristics of each breed. In essence, each breed is an isolated, highly inbred,

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This dissertation follows the style and format of Mammalian Genome.

young population (most breeds have been developed in the past 250 years) (Ostrander and Giniger 1999). Thus, breeds exhibit substantial interbreed genetic homogeneity and remarkable intrabreed phenotypic homogeneity. Factors that have accentuated these developments are founder effects, population bottlenecks, and popular sire effects.

As a consequence of certain breeding practices, more than 450 hereditary diseases of the dog have been described to date (OMIA 2003). More than half of these diseases have clinical presentations that mimic a specific human hereditary disease, and mutations in the same genes are often responsible (Ostrander and Giniger 1997; OMIA 2003). Unlike rodent models in which mutations are generally induced, the dog affords researchers the opportunity to study naturally occurring models of human diseases. Importantly, large litter sizes and a short gestational period make the dog more amenable to genetic study than the human. Furthermore, our canine companions share our environment and also have a level of medical surveillance second only to the human (Ostrander et al. 2000).

Evolutionarily and physiologically, the dog is more closely related to the human than is the mouse. Analyses using a 1.5X coverage of the canine genome sequence revealed that nearly two times as much human sequence could be aligned with the dog than with 8X sequence from the mouse (Kirkness et al. 2003). This sequence, assembled from a Standard Poodle by Celara, represents the first large scale sequencing effort for the dog. The potential of the canine sequence to contribute to understanding genetics of hereditary disease and comparative genetics was recognized by the National Human Genome Research Institute (NHGRI) (http://www/genome.gov/11008069). That is, the

dog was selected over other species for genome sequencing. After several breeds were analyzed for high levels of genetic homogeneity, the Boxer was chosen to be the breed sequenced. In 2003, assembly of a 6.5X coverage sequence of the dog commenced. Data are publicly available through the daily deposition of trace sequences into Ensembl (ftp://ftp.ensembl.org/pub/traces/canis_familiaris/) and NCBI Trace Archive (http://www.ncbi.nlm.nih.gov/Traces/trace).

History of the canine map

Today, construction of the ultimate map of the canine genome is nearly complete, but less than a decade ago researchers were struggling with standardizing the canine karyotype. This task was complicated by the dog's 38 small, acrocentric autosomes that could not be readily distinguished by standard cytogenetic techniques. It was not until 1999, with the development of chromosome-specific fluorescence in situ hybridization (FISH) paint probes, that the canine karyotype was standardized (Breen et al. 1999a; Breen et al. 1999b). This development was an important step towards the construction of a high-resolution map of the canine genome, an essential tool for genetic studies in the dog.

In 1997, Mellersh and colleagues published the first linkage map of the dog, which included 139 microsatellite markers and 30 linkage groups. Another milestone in development of the canine map was the construction of a radiation hybrid (RH) panel, using canine-rodent hybrid cell lines (Vignaux et al. 1999). The first RH map consisted of 218 gene markers and 182 microsatellite markers (Vignaux et al. 1999). More

recently, there was construction of an integrated linkage-radiation hybrid map of the canine genome (Breen et al. 2001).

The integrated map allowed for identification of microsatellite markers suitable for whole genome linkage scans. Successful linkage studies require a set of markers that is distributed evenly across all chromosomes. The Minimal Screening Set–1 (MSS-1) is the first screening set providing full coverage of the canine genome (Richman et al. 2001). It is comprised of 172 microsatellite markers, 64 di-, 3 tri-, and 104 tetra-nucleotide markers, with an average polymorphic information content (PIC) value of 0.74 (Richman et al. 2001). Average spacing for the MSS-1 is 10cM and no gaps greater than 20 cM exist (Richman et al. 2001).

In 2003, an updated RH panel, RHDF5000-2, was used to map 3,270 markers with an average intermarker distance corresponding to ~1 Mb (Guyon et al. 2003). This facilitated the characterization of a more comprehensive screening set, the Minimal Screening Set -2 (MSS-2) (Guyon et al. 2003). The MSS-2, comprised of 327 microsatellite markers, offers an average spacing of 9 Mb and has no gaps larger than 17.1 Mb (Guyon et al. 2003). The MSS-2 includes 151 di-, 3 tri-, and 171 tetra-nucleotide repeats with an average heterozygosity value of 0.73 (Guyon et al. 2003). Only 64 markers from the MSS-1 are part of the MSS-2 (Guyon et al. 2003).

Linkage analysis in the dog

Though the unique population structure of the dog lends itself to the study of human hereditary diseases, it is important to note that there are many diseases unique to

the dog. Because of the many roles of the dog in society, from guardian to companion, elimination of hereditary diseases is desirable. Approximately two-thirds of hereditary diseases in the dog are transmitted in an autosomal recessive fashion (Ostrander and Kruglyak 2000), which makes it difficult for breeders to eliminate deleterious alleles from the breeding stock. In addition, many hereditary diseases have late onset and are therefore difficult to prevent because dogs reach reproductive maturity before symptoms arise. Thus, identification of markers linked with diseases allows for the development of PCR-based tests that can identify carrier and affected animals before symptoms occur.

There exist two basic strategies to identify genes harboring mutations that are causative for diseases. The candidate gene approach is a more direct method and focuses on specific genes that are suspected to be involved in the diseases. For some diseases, many candidate genes have been identified, however, for others, the limited knowledge of the pathobiology of the underlying disease process prevents the selection of suitable candidate genes. In these situations, recombination mapping strategies, such as classical linkage analysis and linkage disequilibrium (LD), may be employed. After linkage has been established, identification and subsequent positional cloning of candidate genes is possible.

Classical linkage studies use known relationships to trace inheritance through a family. Although it is necessary to construct multigenerational pedigrees for these studies, collaborations with breeders can obviate the need to maintain expensive colonies. An alternative to classical linkage studies is LD, which uses small numbers of unrelated affected and unaffected dogs. Purebred dogs are well suited for LD, which

requires a young population with certain structures, including genetic isolation, a small number of founders, expansion by growth, and rare disease alleles (Hyun et al. 2003).

Multiplexing

The recent characterization of highly polymorphic microsatellite markers is an important advance in the study of canine genomics. Microsatellites are useful as markers for linkage analysis, as well as for evolutionary studies and forensics investigations (Leopoldino et al. 2002). Variability of microsatellites within and among breeds allows for their use in identification of individuals and determination of parentage (Sutton et al. 1998; Zajc and Sampson 1999; Muller et al. 1999; Clark et al. 2004). High sequence conservation within the family *Canidae* allows for comparative studies of microsatellites across canine species (Fredholm and Winterø 1995). The generation of microsatellite profiles for individuals is termed genotyping.

A limiting factor in the use of microsatellites is the availability of DNA from subjects of interest. Thus, it is desirable to minimize the number of PCRs necessary to collect genotypes because the amount of DNA is often limited and because materials, reagents, and resolution of PCR products are costly. One mechanism to address this issue is the multiplexing of markers. Multiplexing is the simultaneous amplification and resolution of PCR products. Multiplex PCR has been developed for linkage studies in the human (Beekman et al. 2001), and for forensics investigations in several mammals, including the dog (Koskinen and Bredbacka 1999, Altet et al. 2001, Clark et al. 2004). In

short, multiplexed sets of markers reduce both the expense and time necessary for collection of genotype data.

Pancreatic acinar atrophy

Pancreatic acinar atrophy (PAA) is a disease characterized by the degeneration of acinar cells of the exocrine pancreas that leads to exocrine pancreatic insufficiency (EPI) and occurs primarily in the German Shepherd Dog (GSD) (Westermarck et al. 1993). Pancreatic acinar cells synthesize digestive enzymes such as lipase and amylase, and also inactive precursors, zymogens, of proteolytic and phospholipolytic enzymes necessary for proper digestion (Williams 1989). The aforementioned enzymes and zymogens are secreted into the small intestine and are instrumental in the breakdown of fats, carbohydrates, and proteins (Williams 1989). The pancreas also functions as an endocrine gland, wherein the islets of Langerhans secrete the hormones insulin and glucagon, which regulate glucose homeostasis in the body. This function is not disrupted as a result of PAA (Archibald and Whiteford 1953).

Ninety-six percent of affected dogs present with symptoms of EPI by five years of age, although many dogs show signs as early as 6 months of age (Raiha and Westermarck 1989; Westermarck et al. 1993). More than 90% of the secretory capacity of the pancreas has to be lost before clinical symptoms ensue (Dimagno et al. 1973). Clinical signs include a ravenous appetite, weight loss, and voluminous soft stools (Westermarck et al. 1989). Steatorrhea, borborygmus, coprophagia, and polydipsia are also associated with EPI (Raiha and Westermarck 1989).

Histological studies

PAA is unique to the dog (Westermarck and Wiberg 2003). Histologic evaluation of pancreatic biopsy specimens from dogs having PAA reveals atrophy, scattering, and disorganization of pancreatic acinar cells (Rogers et al. 1983; Westermarck et al. 1993). Abnormal acinar cells can be identified as early as 6 weeks of age (Westermarck et al. 1993). Degenerating acinar cells exhibit dilation of the rough endoplasmic reticulum (RER) and extensive fusion of zymogen granules (Westermarck et al. 1993). These changes progressively affect larger portions of acinar tissue, and ultimately result in a severely diminished exocrine pancreatic function (Westermarck et al. 1993).

One group recently examined the histopathology of pancreatic tissue during the subclinical phase, that is, before the complete destruction of the exocrine pancreas (Wiberg et al. 1999). They observed a marked infiltration of the exocrine pancreas with lymphocytes (Wiberg et al. 1999). During the clinical stage, only a mild inflammatory reaction is observed. Thus, these investigators proposed that PAA progresses through two stages: 1) lymphocytic pancreatitis, during which there is active destruction of acinar tissue, and 2) end-stage EPI, during which atypical parenchyma, ductal structures, and adipose tissue replace acinar tissue (Wiberg et al. 1999).

Diagnosis and treatment

Previously, the fecal soybean stimulation test (SST), fecal proteolytic activity (FPA), and N-benzoyl-L-tyrosyl-P-aminobenzoic acid absorption test (BT-PABA) have been used for the diagnosis of EPI (Westermarck 1982; Westermarck et al. 1993).

However, these tests are either cumbersome to perform and/or are unreliable (Williams and Batt 1988). Today the measurement of serum canine trypsin-like immunoreactivity (cTLI) using a radioimmunoassay, which determines the amount of trypsinogen released into the bloodstream from the pancreas, serves as a gold standard (Williams and Batt 1988). The reference range for this assay is 5.0 μg/L to 35.0 μg/L, and values below 2.5 μg/L are diagnostic for EPI (Williams et al. 1988). This assay has been reported to be 100% sensitive and specific for EPI and thus is a clinically effective diagnostic tool (Williams and Batt 1988).

The prognosis for dogs having EPI is typically good with treatment (Wiberg et al. 1998). Expensive enzyme supplements must be administered with each meal for the duration of the animal's life (Williams 1989). However, one fifth of dogs having EPI are euthanized because owners are unable to afford the enzyme supplements (Hall et al. 1991).

Heritability studies

Many studies have been conducted in an effort to understand the mode of inheritance of PAA. Weber and Freudiger (1977) studied a multigenerational pedigree of GSDs and hypothesized that chronic exocrine pancreatic insufficiency is an autosomal recessive trait. Westermarck (1980) investigated the inheritance of PAA in a Finnish family of GSDs and also reported that it may have an autosomal recessive mode of inheritance. The above investigations, however, are problematic because statistical analyses necessary to conclusively determine a mode of inheritance were not conducted.

Specific objectives

There were two major objectives of this work. The first was to develop tools for genomic analysis in the study of hereditary diseases of the domestic dog. This objective was achieved through the development of 1) multiplexing strategies for the MSS-1, 2) a multiplex of microsatellite markers for use in canine forensics (e.g., parentage identification of individual dogs, etc.), and 3) chromosome-specific multiplex panels for the MSS-2. The second objective of this work was to use these tools to study the genetics of EPI in the GSD. To accomplish this objective, it was necessary to 1) determine the mode of inheritance of PAA and 2) conduct a whole genome screen of pedigrees of GSDs segregating PAA. These two objectives have been met but linkage of markers with PAA has not, to date, been met.

CHAPTER II

INHERITANCE OF PANCREATIC ACINAR ATROPHY IN GERMAN SHEPHERD DOGS*

Overview

Our objective was to assess the heritability of PAA in the GSD in the US. Two multigenerational pedigrees of GSDs with family members with PAA were identified. The clinical history of each GSD enrolled in the study was recorded and serum samples for cTLI analysis were collected in 102 dogs. Dogs with a cTLI concentration ≤ 2.0 μg/L were diagnosed with EPI and were assumed to have PAA. Pedigree I consists of 59 dogs and pedigree II of 76 dogs. Serum cTLI concentrations were measured in 48 dogs from Pedigree I and 54 dogs from pedigree II. A total of 19 dogs (14.1%) were diagnosed with EPI, 9 in pedigree I (15.3%) and 10 in pedigree II (13.6%). Of the 19 dogs with EPI, 8 were male and 11 were female. Evaluation of data by complex segregation analysis were strongly suggestive of an autosomal recessive mode of inheritance for this disease in GSDs in the US. Currently, linkage analysis is being performed in order to identify a genetic marker that co-segregates with PAA. Ultimately, this work is expected to lead to the development of a PCR-based assay for a genetic marker that co-segregates

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* Reprinted with permission from Moeller EM, Steiner JM, Clark LA, Murphy KE, Famula TR, Williams DA, Stankovics M, Vose A (2002) Inheritance of pancreatic acinar atrophy in German Shepherd Dogs. Am J Vet Res 63, 1429-1434

with PAA in the GSD. Such a test may help to decrease the incidence of this disease through directed breeding programs.

Introduction

PAA is a degenerative disease of the exocrine pancreas, mainly seen in GSD and Rough-coated Collies, that leads to EPI (Weber and Freudiger 1977; Rogers et al. 1983; Raiha and Westermarck 1989; Westermarck et al. 1989; Westermarck et al. 1993). Affected dogs typically have clinical signs of EPI by five years of age, but some dogs may have signs as early as 13 months of age (Raiha and Westermarck 1989; Westermarck et al. 1989; Westermarck et al. 1993). Clinical signs include polyphagia, weight loss, voluminous stools, and steatorrhea (Archibald and Whiteford 1953; Anderson and Low 1965; Anderson and Low 1965; Rogers et al. 1983; Westermarck et al. 1989). Feces are light in color, loose in texture, and can be quite malodorous (Archibald and Whiteford 1953; Anderson and Low 1965; Anderson and Low 1965; Rogers et al. 1983; Westermarck et al. 1989).

Findings on histologic evaluation of pancreatic biopsy specimens from dogs with PAA include atrophy, scattering, and disorganization of pancreatic acinar cells (Rogers et al. 1983; Westermarck et al. 1993). Electron microscopy of pancreatic tissue reveals degenerative changes of acinar cells as early as 6 weeks of age (Westermarck et al. 1993). Abnormalities include dilation of the rough endoplasmic reticulum (RER) and extensive fusion of zymogen granules (Westermarck et al. 1993). As the disease progresses, the tissue loss becomes more extensive and leads to a rapid loss of exocrine

pancreatic function (Westermarck et al. 1993). Islets of Langerhans are usually unaffected by the degenerative process (Archibald and Whiteford 1953).

A number of tests have been developed to aid in the diagnosis of EPI. The fecal soybean stimulation test, fecal proteolytic activity, and N-benzoyl-L-tyrosyl-P-aminobenzoic acid absorption have all been used for the diagnosis of EPI (Westermarck 1982; Westermarck et al. 1993). Unfortunately, all of these tests are either cumbersome to perform, unreliable, or both and have been replaced by the measurement of serum cTLI by use of a radioimmunoassay (Williams and Batt 1988). The reference range for this assay is 5.0 to 35.0 μg/L, with a value of < 2.5 μg/L being diagnostic of EPI (Williams and Batt 1988). Serum cTLI concentration has been reported to be 100% sensitive and specific for EPI and, thus, is clinically highly useful for the diagnosis of EPI (Williams and Batt 1988). In fact, the high sensitivity and specificity of serum cTLI concentration for a diagnosis of EPI make this disease an ideal candidate for evaluation as a hereditary disease. By using this assay, the disease status of any family member can be assessed easily. Recently, an assay for measurement of fecal elastase has been introduced (Spillmann 1998). However, this assay is associated with some false positive results, making it inferior to the measurement of serum cTLI concentration.

In 1977, Weber and Freudiger analyzed a pedigree composed of 19 GSDs with EPI and 33 unaffected GSDs. All 19 affected dogs were found to have a common ancestor born in 1918. Eighteen of the dogs were inbred more than once with a descendant of this dog. On the basis of the degree of inbreeding within this pedigree,

Weber and Freudiger hypothesized that chronic EPI was an autosomal recessive trait (Weber and Freudiger 1977).

In 1980, Westermarck et al investigated the inheritance of PAA in GSDs in Finland (Westermarck 1980). Measurement of fecal proteolytic activity was performed by use of radial enzyme diffusion to determine the disease status of each dog (Westermarck 1980). This study included 59 GSDs from 2 different kindreds that had the same male progenitor. The first kindred had at least 1 affected dog in each of 4 litters. This evidence further supported an autosomal recessive inheritance of PAA in GSDs. However, Westermarck pointed out that on the basis of his data, the mode of inheritance could also be dominant with incomplete penetrance (Westermarck 1980).

Recent work indicates that PAA might be an autoimmune-mediated disease. More specifically, it is theorized that PAA progresses through the following 2 stages: 1) lymphocytic pancreatitis, when there is active destruction of acinar tissue and 2) end-stage EPI, during which atypical parenchyma, ductal structures, and adipose tissue replace acinar tissue (Wiberg et al. 1999). Thus, PAA in GSDs may represent an autoimmune disorder that is caused by a gene inherited in an autosomal recessive fashion. Because previous studies have been conducted outside the United States, the purpose of the study presented here was to determine the inheritance of PAA in GSDs in the US.

Materials and methods

A questionnaire was sent to veterinarians who had GSD patients with low serum cTLI concentrations as determined by previous analysis of serum samples at the Gastrointestinal Laboratory at Texas A&M University. Veterinarians were asked for permission to contact the owners of the dogs. Owners were then asked to provide information about the breeders they obtained their dogs from. Finally, breeders were contacted for family information and for participation in our study. Several families of GSDs having family members with EPI were identified, and 2 pedigrees were selected because dogs belonging to several generations were available. Many dogs related to these dogs previously determined to have EPI were identified, and as many dogs as possible were tested for EPI. No discrimination was made between dogs that had clinical signs of EPI and those that did not. Dogs previously determined to have EPI were retested when possible.

A single serum sample was collected from each dog, stored in a 10 ml red-top red-top evacuated tube, and sent to the Gastrointestinal Laboratory for measurement of serum cTLI concentration by radioimmunoassay. The serum cTLI concentration was used to determine the disease status for each dog. Dogs with a serum cTLI concentration of ≤ 2.0 μg/L were considered to have EPI, and it was assumed that EPI was caused by PAA. For dogs that were retested (i.e., because they had no clinical signs of EPI but did have a low serum cTLI concentration previously), the most recent serum cTLI concentration was reported. Additional blood samples for future extraction of DNA were also collected and sent to the Gastrointestinal Laboratory.

Statistical analysis methods

Logistic regression models developed for complex segregation analysis were used to assess the possible segregation of a single locus with a large effect on PAA in our pedigrees (Bonney 1986). For a review of complex segregation analysis, see Lynch and Walsh (Lynch and Walsh 1998). This technique, which contrasts possible modes of inheritance, is purely statistical, using pedigree information, disease status, and sex to identify a pattern of transmission. The data are fit to various models of transmission, and a likelihood ratio is calculated for each of these models. The likelihood ratio is a measurement of how well the data fit the model, and a *P* value is calculated to determine a significant difference between likelihood ratios. The data were fit to the different models by use of Statistical Analysis for Genetic Epidemiology (S.A.G.E.) software (Release 3.1). All dogs belonging to either of the pedigrees were used in the complex segregation analysis. Dogs with a serum cTLI concentration ≤ 2.0 μg/L were considered affected and all other dogs were considered unaffected. A value of $P \leq 0.05$ was considered significant.

Results

A total of 135 togs were evaluated in our study. Serum cTLI concentration was measured in 102 of the 135 (75.6%) dogs. Nineteen of these 135 dogs (14.1% or 18.6% of the 102 dogs tested) had EPI, 8 of which were male and 11 female. The first family of GSDs consisted of 59 dogs, 48 of which had serum cTLI concentrations measured (Figure 1). Nine of those 59 (15.3%; 18.8% of the 48 dogs tested) dogs, including 4

males and 5 females, had serum cTLI concentrations ≤ 2.0 µg/L. Two dogs had serum cTLI concentrations of ≤ 2.0 µg/L but were asymptomatic for EPI. The second family of GSDs consisted of 76 dogs, 54 of which had serum cTLI concentrations measured (Figure 2). Ten of the 76 (13.2%; 18.5% of the 54 dogs that were tested) dogs, including 4 males and 6 females, had serum cTLI concentrations of ≤ 2.0 µg/L. Thirty-three of the dogs were not tested for various reasons, including death at birth, death before clinical signs warranted testing, and lack of cooperation by the owner for sample collection.

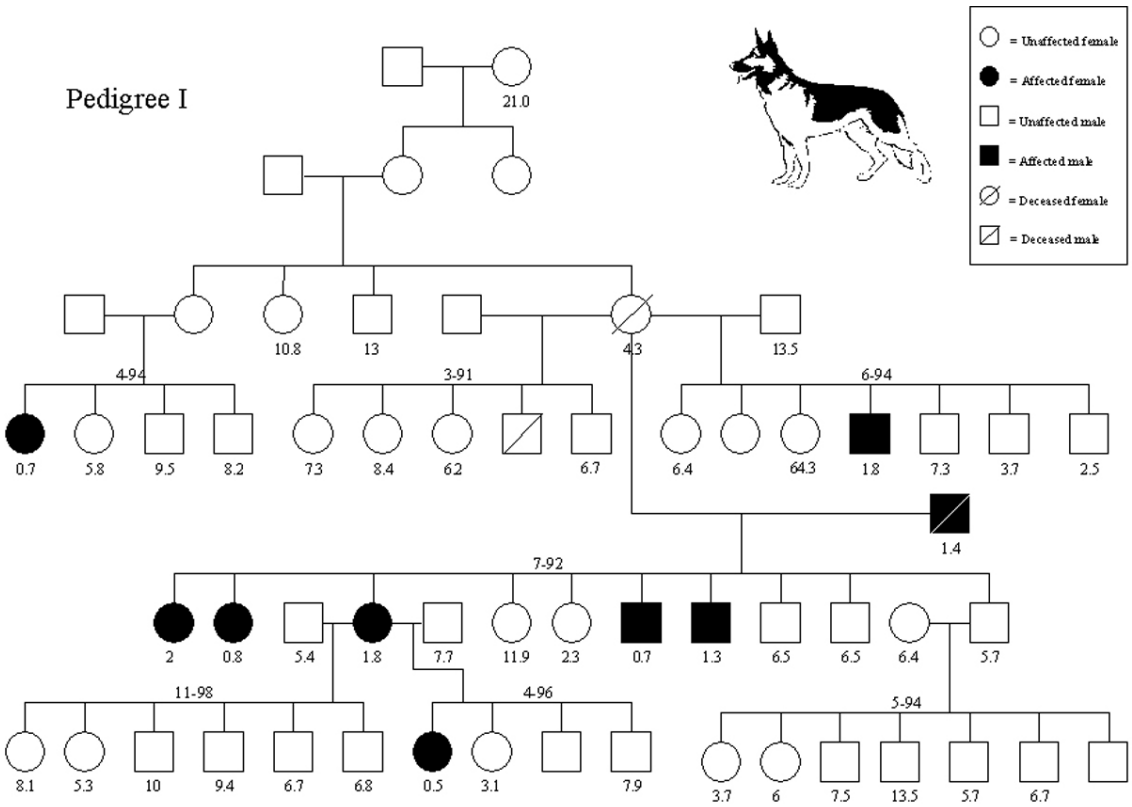

Figure 1. Pedigree I.

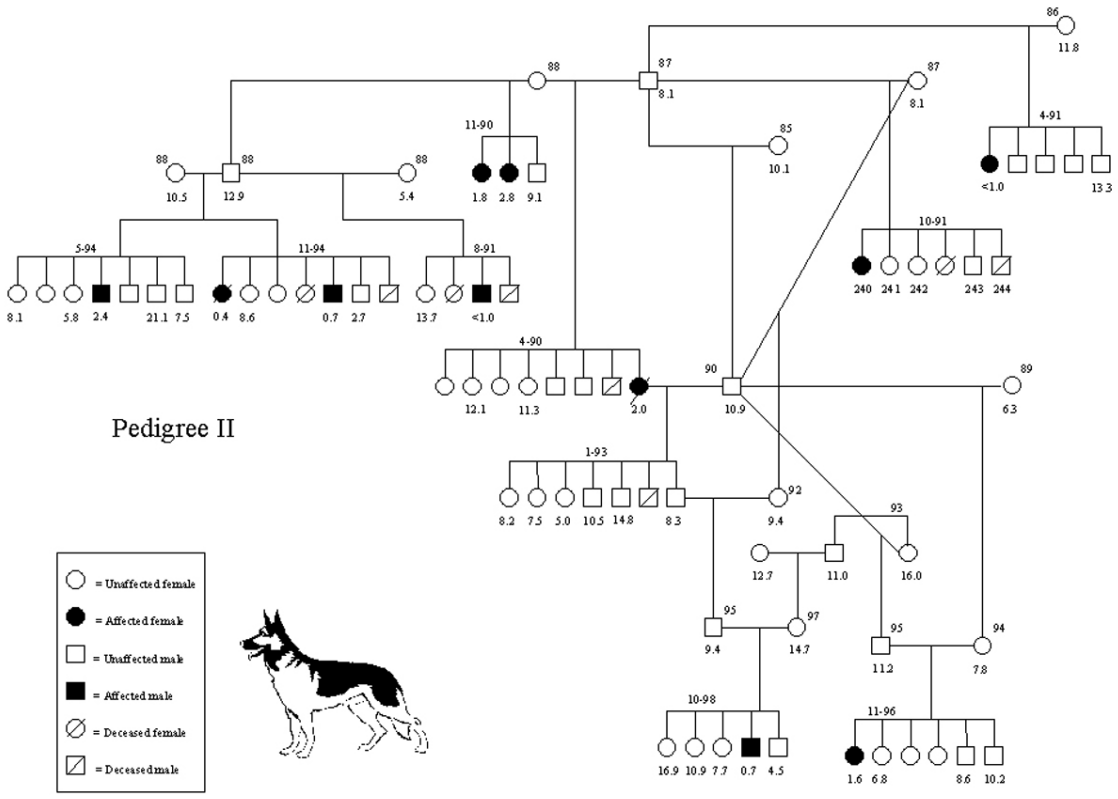

Figure 2. Pedigree II.

Several litters from parents, of which at least 1 parent was affected, had unaffected individuals. Conversely, there were several litters with affected individuals from unaffected parents. Pedigree I represents data from 7 complete litters, whereas pedigree II represents data from 10 complete litters (Table 1). There were 2 dogs that did not have a diagnosis of EPI prior to being tested for the purpose of our study. Because both of these dogs had no clinical signs of EPI, these dogs were classified as having subclinical disease. One dog was retested for confirmation and also had a severely low serum cTLI concentration (cTLI ≤ 2.0 μg/L) at the time of the second evaluation. The

other dog died before a second sample could be collected. The serum cTLI concentration for this dog was 1.4 μg/L, suggesting that this dog was affected.

When using a complex segregation analysis, dogs can either have the phenotype or not have the phenotype in question. Therefore, a cut-off point had to be established to distinguish affected dogs from unaffected dogs. The cut-off value chosen for our study was a serum cTLI concentration of ≤ 2.0 μg/L. Any dog with a serum cTLI concentration of ≤ 2.0 μg/L was considered affected, whereas all other dogs were considered unaffected.

Table 1. Number of affected dogs with exocrine pancreatic insufficiency (EPI) in litters of German Shepherd Dogs from 2 pedigrees

	# parents w/ EPI	# affected in litter	# dogs in litter
Pedigree I	0	0	5
	0	0	7
	0	1	4
	0	1	7
	1	0	6
	1	1	4
	1	5	10
Pedigree II	0	1	3
	0	1	4
	0	1	5
	0	1	5
	0	1	6
	0	1	6
	0	1	7
	0	1	8
	0	2	7
	1	0	7

Complex segregation analysis revealed that the most substantial contrast of likelihoods was between a model with no major locus, which included the possibility for polygenic inheritance, and a model with a single major locus exhibiting general Mendelian transmission. The model assuming general Mendelian transmission showed a significantly higher likelihood (p=0.046) than the model assuming no major locus (Table 2).

Table 2. Parameter estimates (± SE) from the logistic regression model in complex segregation analysis of pancreatic acinar atrophy (PAA) in German Shepherd Dogs

	No Major Locus		General Major Locus Arbitrary Transmission		General Major Locus Mendelian Transmission		Recessive Major Locus Mendelian Transmission	
Parameter	Estimate[d]	SE	Estimate[d]	SE	Estimate[d]	SE	Estimate[d]	SE
p(a)[a]	NA[d]		0.017	0.003	0.175	0.074	0.180	0.074
Pooled Base	-2.659	0.404	NA		NA		NA	
aa	NA		2.938	2.028	1.033	0.711	1.274	2.416
Aa	NA		33.688	20.319	-68.112	15.647	-11.132	4.643
AA	NA		-11.998	10.921	-9.130	2.421	-11.132	4.643
τ_{aa}	NA		0.499	0.891	1.00	Fixed	1.00	Fixed
τ_{Aa}	NA		0.298	0.524	0.50	Fixed	0.50	Fixed
τ_{AA}	NA		0.001	0.000	0.00	Fixed	0.00	Fixed
Parent Regr.[c]	-0.124	0.249	-4.794	0.28	-3.976	0.832	-4.330	0.822
ln(L)[b]	-75.022		-70.381		-71.024		-71.915	

A positive estimate value indicates an increased risk for PAA, whereas a negative estimate value indicates a reduced risk for PAA. The aa genotype, having a positive estimate, is likely to be affected. AA genotypes and heterozygotes, Aa, are least likely to develop PAA.

[a] Frequency of the putative major allele a. [b] Natural log of the likelihood. [c]Regression effect for parents. [d] Estimate for each parameter calculated for the specific regression model

A = Dominant allele for PAA. a = Recessive allele for PAA. NA = Not applicable. τ = Major locus transmission probabilities for transmission of putative major allele a.

Discussion

From a clinical standpoint, PAA in GSDs is a hereditary disease that can be readily diagnosed because identification of disease status can easily be accomplished by measuring a single serum cTLI concentration. The cTLI assay has been shown to be 100% sensitive and 100% specific for diagnosing EPI in dogs (Williams and Batt 1988). The almost even distribution of PAA between males and females in the 2 pedigrees indicates that PAA is not a sex-linked disease. If PAA were inherited as an X-linked disease, we would expect many more males to be affected than females. Also, Y-linked inheritance would produce only affected males, which is not the case for PAA.

Parents lacking clinical signs of EPI produced dogs with EPI. This provides further evidence that the putative trait for PAA is recessive and supports the findings in Finnish GSDs by Westermarck (Westermarck et al. 1989; Westermarck 1980). However, the rate of affected dogs is slightly lower than would be expected for a simple autosomal recessive inheritance (ie, 18/102 dogs belonging to complete litters; 17.8% compared to 25.0% expected for simple autosomal recessive inheritance). This maybe explained by a higher rate of stillbirths in affected dogs or by the fact that at the time of analysis, some dogs had not yet reached 4 to 5 years of age. These dogs may develop clinical signs and a low serum cTLI concentration at a later time in life. Finally, we used a cut-off value of 2.0 μg/L for serum cTLI concentration to ensure that all dogs with positive results truly were affected. In contrast, the cut-off value for serum cTLI concentration for EPI currently reported by our laboratory is ≤ 2.5 μg/L. This may have led to a small increase in false-negative results, decreasing the apparent prevalence of the disease.

Small intestinal disease is common in GSDs and may be associated with a slight decrease in serum cTLI concentrations. However, these low serum cTLI concentrations are > 2.5 μg/L. This clinical impression may account for dogs that had serum cTLI concentrations less than the lower limit of the reference range (5.0 μg/L) but still greater than the cut-off value for EPI (2.0 μg/L). For the purpose of our study, dogs with serum cTLI concentrations that fell into this range were considered not affected.

At the time of our study, 17 dogs were not yet 4 years of age when they were tested for EPI. However, 7 of these dogs were siblings of dogs that had already been determined to have EPI. None of these dogs had any signs of EPI. Additionally, none of the remaining 10 dogs that were not yet 4 years of age and did not have siblings that had previously been determined to have EPI, had any clinical signs of EPI. Although some of these dogs may develop EPI in the future, we consider this possibility unlikely. These dogs were considered unaffected for the purpose of our study.

Elston et al. outline criteria that must be satisfied before accepting a major gene model (Elston et al. 1975). The first model to fit is one with no major locus, which includes a term for polygenic inheritance. Alone, this model is uninformative, but it will serve as a baseline for future comparisons. The next model is one that includes a parameter for a major locus, an effect expected to pass from parent to offspring on the basis of Mendel's laws. The contrast of these 2 models is insufficient to establish a putative major gene or to have a reduced incidence of false positives. Additional models fitted to the data include a major locus effect but estimate the transmission from parent to offspring.

In our study, the most substantial difference of likelihood ratios was seen between a model with no major locus, which included the possibility for polygenic inheritance and a model with a single major locus exhibiting general Mendelian transmission. The latter model resulted in a higher likelihood, indicating a better fit, and was shown to be significantly (P=0.046) different from the first model. A recessive Mendelian model was then compared with the original model of PAA having no major gene. It reported the maximal likelihood ratio and a significant (P=0.044) difference. These results support the theory that a major gene is responsible for PAA in these populations.

Other models that were fitted to the data include a dominant Mendelian model and one with a term for sex differences. These models resulted in likelihood ratio statistics that were not significant (data not shown).

Likelihood ratios for the different models are reported (Table 2), including the "general" major locus model ("general" meaning the locus does behave in a strict dominant or recessive manner). Mendelian transmission of the putative alleles provides a significantly better fit than a "no major locus model". For this comparison, the log of the likelihood ratio is calculated as follows: -2(-75.022– (-71.024)) = 7.996, with 3 degrees of freedom ($P < 0.046$). However, a "general major locus model" where the transmission probabilities are estimated from the pattern of inheritance displayed within the data does not provide a significantly better fit than the "general model with fixed Mendelian transmission probabilities" (i.e., -2(-71.024 – (-70.381)) = 1.286, with 3 degrees of freedom $P < 0.732$). This contrast between the two models is suggested by

Elston et al. to reduce the probability of falsely declaring the presence of a major locus (Elston et al. 1975). Alleles of a genuine major locus would have to be transmitted from parent to offspring with probabilities that reflect Mendelian transmission. A test for equal transmission probabilities (not presented) also supports the 3 criteria of a major locus model as described by Elston et al. The recessive major locus model was not significantly different from the general major locus model (-2(-71.915– (-71.024)) = 1.782, with 1 degree of freedom, $P < 0.182$), though the recessive model is more parsimonious. Accordingly, we conclude that a major locus with an impact on PAA in GSDs in the United States can be established with the present data. This major locus apparently acts in a recessive, or close to completely recessive, fashion.

Statistical analysis supports the theory that a major gene is responsible for PAA in the pedigrees evaluated in our study. The single major locus model exhibiting general Mendelian transmission had a higher likelihood than the model assuming no major locus. This indicates that the single major locus model has a better fit to the data observed in these pedigrees. In addition, the recessive Mendelian model had the maximal likelihood ratio and significant difference from the model assuming no major gene. Because no other Mendelian models had significant likelihood ratio statistics, these data suggest that the mode of inheritance of PAA in GSDs in the United States is autosomal recessive.

One problem in our study is that for some litters, blood samples could not be collected from all the dogs. For instance, several dogs in pedigree II (Figure 2) were stillborn or died before the disease could have developed. There is no way to exclude the

possibility that some of these dogs would have developed PAA later in life. Additionally, other dogs died before follow-up samples could be collected. These losses may affect the observed incidence of PAA and may explain the lower than expected frequency observed in these 2 pedigrees. The only definitive way to determine whether a dog is affected with PAA would be to only include pedigrees that exclusively contain family members that live a full lifespan and in which a determination of disease status is being made shortly before a natural death. Unfortunately, such a study would not be feasible.

We conclude that there is evidence to suggest that PAA is inherited as an autosomal recessive trait in GSDs in the United States. Currently, linkage analysis is being performed by use of a set of 172 microsatellite markers (ie. MSS-1) that spans the entire canine genome (Richman et al. 2001). Since no candidate gene is available for PAA, the minimal screening set 1 is screened for a microsatellite marker that cosegregates with the disease.

CHAPTER III

MULTIPLEXING OF CANINE MICROSATELLITE MARKERS FOR WHOLE GENOME SCREENS*

Overview

A set of 172 canine microsatellite markers, termed Minimal Screening Set-1 (MSS-1), was recently characterized for use in whole-genome screens. We report here the multiplexing of 155 MSS-1 markers into 48 multiplex sets. Amplification of the multiplex sets is achieved using a single thermal cycling program. The markers are labeled with fluorescent dyes and optimized for resolution on an ABI 310 Genetic Analyzer or ABI 377 Sequencer. The multiplexing strategy involves amplifying combinations of markers so that no two markers with the same dye and product size overlap. Multiplexing the MSS-1 provides an efficient tool for the collection of genotypes and streamlines whole genome screens. Screening the canine genome for linkage of markers with various hereditary diseases facilitates identification of affected and carrier individuals, thereby providing researchers and clinicians with an additional diagnostic tool.

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* Reprinted with permission from Cargill EJ, Clark LA, Steiner JM, Murphy KE (2002) Multiplexing of canine microsatellite markers for whole-genome screens. Genomics 80(3), 250-253

Introduction

More than 400 hereditary diseases of the domestic dog have been described, and more than 200 of these have pathologies resembling specific human hereditary diseases (Ostrander et al. 2000). Furthermore, many canine and human hereditary diseases have common genetic etiologies. This fact, combined with the marked genetic homogeneity and the ease with which multigenerational pedigrees can be established, makes the dog an ideal model for the study of simple and complex human hereditary diseases. Even so, until very recently an impediment to the study of canine hereditary diseases and to the use of the dog as a model has been the lack of a high-density map of the canine genome. However, advancements towards development of such a resource have come from construction of radiation hybrid (RH) (Priat et al. 1998) and linkage maps (Werner et al. 1999). The subsequent integration of these maps provides coverage of approximately 99% of the canine genome (Mellersh et al. 2000). The most recent advance is an 1800-marker map replete with microsatellite and gene loci (Breen et al. 2001). This rapid development of the canine map allows for tools that are readily available for study of the human (Lapsys et al. 1997; Li et al. 2001; Beekman et al. 2001), murine (Rithidech et al. 1997; Devereux and Kaplan 1998) and bovine genomes (Womack et al. 1997; Konfortov et al. 1998; Grosse et al. 2000) to now be used in examination of the canine.

The MSS-1 is suitable for genome-wide linkage studies because it provides coverage of the canine genome with average spacing of 10 cM and an average polymorphic information content (PIC) value of 0.74 (Richman et al. 2001). The MSS-1 contains 64 dinucleotide repeats, three trinucleotide repeats, 104 tetranucleotide repeats

and one short interspersed nuclear element. Although the MSS-1 is an extremely useful tool, multiplexing will enhance its utility by allowing for more efficient genotyping. Multiplexing has already been utilized for linkage studies and verification of lineage in bison (Schanbel et al. 2000), cattle (Schanbel et al. 2000), humans (Beekman et al. 2001), and dogs (Koskinen and Bredbacka 1999; Altet et al. 2001). To this end, we report here the multiplexing of 155 MSS-1 markers into 48 sets of two to five markers with 151 co-amplified and four co-loaded. The remaining 17 MSS1 markers were amplified and resolved individually.

Materials and methods

DNA from a mixed breed dog was used for optimization of the multiplex sets. Genomic DNA was extracted from whole blood using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN). DNA was stored at 4^{o}C in 150 µl of rehydration buffer. The concentration of the DNA was determined by spectrophotometric analysis and a working solution of 50 ng/µl was maintained.

The microsatellite primers were synthesized using an ABI Expedite Nucleic Acid Synthesis System (PE Biosystems, Foster City, CA). The 5´-end of each forward primer was labeled with one of three fluorescent dyes: 6-FAM, HEX, or TET (PE Biosystems). Dyes were selected based on product size ranges (Richman et al. 2001) to evenly distribute dye type and to limit marker overlap.

The multiplex sets and individual markers were amplifiabled with stepdown thermal cycling conditions of 2 min 95^{o}C followed by 5 cycles of 30 seconds at 95^{o}C, 15

seconds at 58°C, 10 seconds at 72°C, and an additional 30 cycles of 20 seconds at 95°C, 15 seconds at 56°C, 10 seconds at 72°C, with a final extension of 5 minutes at 72°C. Concentrations for a 10 µl PCR volume were 0.5 mM of each dNTP, 5 ng/µl of genomic DNA, 3.0 mM of MgCl, 1x *Taq* DNA Polymerase Buffer B (Fisher Scientific, Pittsburgh, PA), 1x MasterAmp PCR Enhancer (Epicentre Technologies, Madison, WI), and 0.04 units/µl of *Taq* DNA Polymerase (Fisher Scientific, Pittsburgh, PA). Primer concentrations were varied for each marker (Table 4). Amplifications were carried out using an Eppendorf Mastercycler (Eppendorf Scientific, Inc., New York, NY). To co-load markers in multiplex sets M47 and M48, individual markers were amplified and equal parts of reaction products were mixed together.

Products of reactions were resolved using an ABI 310 capillary-based Genetic Analyzer or an ABI 377 Sequencer (PE Biosystems) and sized relative to an internal size standard (MAPMARKER HI, Bioventures). The ABI GENESCAN version 3.1 software package (PE Biosystems) was used for analysis of the multiplex sets.

Results

The MSS-1 markers have individual amplification conditions and observed product sizes (Richman et al. 2001). A complete list of references for each marker can be found on-line (http://www.cvm.tamu.edu/cgr/multiplex.html). For multiplexing, 53 markers were labeled with 6FAM, 53 markers were labeled with HEX, and 66 markers were labeled with TET. In an effort to further enhance multiplexing, one PCR mix and one thermal cycling program were developed for amplification of the markers. To

determine compatibility for multiplexing, markers were amplified in various combinations. Multiplex set optimization was completed by variation of primer concentrations to give approximately equal amplification of each product in a set.

The use of three fluorescent dyes permits overlap of similarly sized markers, and 48 multiplex sets of two to five markers were developed. More specifically, 151 markers can be co-amplified and 4 markers can be co-loaded after individual amplification. This reduces the number of reactions required to generate data for the MSS-1 by 60%, from 172 to 69. Table 3 lists each multiplex set as they correspond to each canine linkage group. The multiplex sets, primer concentrations, and fluorescent dyes are listed in Table 4.

A table sorted by canine linkage group listing the MSS-1 markers, primer sequences, PIC values, heterozygosity values, marker types, fluorescent dyes, observed allelic sizes, primer concentrations, multiplex sets, and marker references is available (http://www.cvm.tamu.edu/cgr/multiplex.html). Of the 172 markers, 155 are multiplexed and the remaining 17 are amplified individually because they are not compatible in any multiplex combinations. Of these 17, 8 amplify under the same conditions as the multiplex sets (C10.16, FH2200, FH2347, RVCE, FH2165, REN06C11, FH2538, CXX.390.2), whereas 9 do not amplify under the multiplex conditions (ZuBeCa6, FH2149, FH2279, FH2346, N41, FH2457, AHT006, REN51i12, FH2600). The multiplexed MSS-1 provides an expedient and cost-effective method for collection of genotype information by reducing the number of reactions, quantities of reagents, and time required for whole-genome screens of the dog.

Table 3. Multiplex sets as they correspond to each canine linkage group.

Linkage Group	Multiplex Sets	Linkage Group	Multiplex Sets
CFA1	M02 M08 M13 M17	CFY	M32
CFA2	M17 M21 M22 M23 M28 M46	S1/L2	M01 M22 M25 M28 M45
CFA3	M11 M12 M26 M38 M42 M48	S2/L13	M09 M37
CFA5	M14 M25 M27 M43	S3/L14	M09 M15 M44
CFA6	M13 M20 M38 M47	S4/L3	M01 M07 M13 M19 M29 M42 M43
CFA7/L1	M03 M11 M12 M27 M45	S5/L18	M07 M29 M44
CFA8	M03 M08 M21 M27	S6/L12	M24 M32 M39 M44
CFA9	M01 M08 M35	S7/L7	M08 M39 M41
CFA10	M18 M33 M37	S8/L8	M15 M41 M46
CFA12/L4	M32 M38	S9/L5	M08 M28 M40
CFA13,19/L17	M16 M35	S10/L6	M23 M31 M40 M42
CFA15	M14 M20 M21	S11/L9	M14 M29 M34 M36
CFA16	M01 M05 M18	S12/L11	M31 M37 M47
CFA18	M05 M12 M23 M26	S13/L10	M24 M33, M45
CFA20	M01 M40 M41 M43 M48	S14/L15	M30 M32 M35
CFA22	M14 M22 M30 M31 M48	S15/L20	M16 M25 M34 M36
CFA26	M02 M06 M15 M39	S16/L21	M33 M39
CFA29,35/L16	M06	S17/L22	M19 M34
CFA30/L19	M04 M06 M24 M26	L1/L23	M19
CFX	M04 M07	Unlinked	M10 M16 M20 M30 M36 M47

Table 4. Multiplex sets (M01 through M48) with marker primer concentrations [a] and fluorescent dyes [b].

Multiplex	Conc.	Dye	Multiplex	Conc.	Dye	Multiplex	Conc.	Dye	Multiplex	Conc.	Dye
M01						**M25**					
FH2263	0.8	T	**M13**			CPH18	0.8	F	**M37**		
FH2289	0.8	T	C01.673	1.0	H	FH2594	0.8	F	FH2339	1.0	F
CPH16	0.8	F	C06.636	1.0	T	FH2142	0.8	H	FH2312	1.0	H
AHT103	0.8	F	LEI001	0.6	F	CPH2	0.8	T	FH2155	1.0	H
AHT137	0.8	T									
M02			**M14**			**M26**			**M38**		
C01.246	0.8	F	C05.377	1.0	H	FH2531	0.8	F	FH2107	0.8	H
C01.424	0.8	F	CPH5	0.8	F	FH2429	0.8	H	FH2525	0.8	T
FH2016	0.8	T	FH2283	0.8	F	FH2305	0.4	T	FH2223	0.8	F
REN01O23	0.8	H	AHT133	0.6	T						
C26.733	0.8	F							**M39**		
M03			**M15**			**M27**			FH2171	1.0	H
FH2201	1.0	T	FH2130	1.0	H	GLUT4	0.8	T	REN49F22	0.8	H
FH2174	0.8	H	FH2385	1.2	T	C07.1000	0.8	F	CPH10	1.2	T
C08.618	0.8	F	CXX.391	0.6	H	FH2138	0.8	T	FH2566	0.8	F
			M16								
M04			AHT124	0.4	F	**M28**			**M40**		
1F11	0.8	T	AHT127	0.8	T	FH2062	0.8	H	AHTk209	0.8	H
F8C	1.0	T	PEZ2	2.0	H	AHT128	0.8	F	PEZ10	0.8	T
FH2584	1.0	H	AHT106	0.4	T	FH2547	0.6	T	CXX.213	0.8	T
M05			**M17**			**M29**			**M41**		
LEI002	0.8	F	FH2598	1.0	H	CXX.750	0.8	T	PRKCD	1.0	H
FH2356	1.0	T	FH2309	1.0	H	FH2159	0.8	H	REN49C08	1.4	F
FH3010	1.0	T	AHT132	0.8	F	FH2587	0.8	T	CXX.900	1.0	T
M06			**M18**			**M30**			**M42**		
REN48E01	1.0	H	FH2422	0.8	T	C22.745	0.8	T	C03.895	0.6	T
FH2507	0.6	T	FH2293	0.8	T	CXX.176	0.8	T	FH2018	0.4	H
FH2050	0.6	F	PEZ6	0.8	F	CXX.452	0.8	F	AHT140	0.6	F
M07			**M19**			**M31**			**M43**		
FH2548	0.8	H	CXX.873	0.8	T	FH2325	0.8	T	FH2383	1.0	F
FH2985	0.8	H	REN02C20	0.8	F	FH2141	0.8	H	FH2528	0.4	T
FH2096	0.8	F	CXX.672	0.8	F	FH2175	0.8	F	FH2319	0.8	H
FH2079	0.8	T	FH2516	0.8	T						
M08						**M32**					
FH2294	0.8	H	**M20**			C12.852	0.8	T	**M44**		
C08.410	0.8	F	CPH3	1.2	T	AHT139	0.8	T	FH2364	1.4	F
GALK1	0.8	T	FH2321	0.8	T	FH2585	0.8	F	FH2261	0.8	F
CXX.279	0.8	T	TAT	0.8	F	SRY	0.8	H	FH2278	1.0	H
FH2060	0.8	F									
M09			**M21**			**M33**			**M45**		
FH2441	0.8	H	C02.342	0.6	F	FH2537	0.8	H	FH2396	1.0	F
FH2233	1.0	F	FH2144	1.0	H	FH2199	0.8	F	FH2534	0.6	T
REN45F03	0.8	F	Cos15	1.0	T	PEZ8	0.8	H	FH2239	0.6	H
M10			**M22**			**M34**			**M46**		
FH2247	0.8	F	FH2132	1.0	T	CXX.172	0.4	T	C02.864.A	0.8	T
REN01G01	0.8	T	FH2001	0.8	F	REN41D20	0.8	F	FH2394	0.8	T
FH2377	0.8	T	FH2412	0.8	H	FH2244	0.6	T			

Table 4. Continued.

Multiplex	Conc.	Dye	Multiplex	Conc.	Dye	Multiplex	Conc.	Dye	Multiplex	Conc.	Dye
M11			**M23**			**M35**			**M47 c**		
FH2137	0.8	T	FH2087U	0.8	H	FH2186	1.0	T	FH2119	0.8	H
FH2301	0.8	F	WILMS-TF	0.8	H	FH2206	1.0	T	AHT131	0.8	T
FH2581	0.8	T	FH2526	0.8	T	FH2208	0.8	H	PEZ7 a	0.8	F
M12			**M24**			**M36**			**M48 d**		
FH2302	0.8	T	FH2290	0.8	H	FH2532	0.8	H	FH2316	0.8	H
FH2226	0.8	H	CXX.608	0.8	H	FH2238	0.6	T	REN55P21	0.8	H
AHT130	0.8	F	CXX.642	0.6	T	FH2550	0.8	F	FH2227	0.8	H

[a] Primer concentration in final reaction as μmol/primer, see http://www.cvm.tamu.edu/cgr/multiplex.html
[b] F = 6-FAM, H = HEX, T = TET.
[c] M47 co-amplify FH2119 and AHT131, individually amplify PEZ7, mix products 1:1 for co-loading.
[d] M48 individually amplify FH2316, REN55P21, and FH2227, mix products 1:1 for co-loading.

CHAPTER IV

EVALUATION OF A RAPID SINGLE MULTIPLEX MICROSATELLITE-BASED ASSAY FOR USE IN FORENSIC-GENETIC INVESTIGATIONS IN DOGS*

Overview

Our objective was to develop a set of microsatellite markers, composed of a minimal number of these markers, suitable for use in forensic genetic investigations in dogs. Blood, tissue, or buccal epithelial cells were collected from 364 dogs of 85 pure breeds and mixed breed dogs, and 19 animals from related species in the family *Canidae*. Sixty-one tetranucleotide microsatellite markers were characterized on the basis of number and size of alleles, ease of genotyping, chromosomal location, and ability to be co-amplified. The range in allele size, number of alleles, total heterozygosity, and fixation index for each marker were determined by use of genotype data from 383 dogs and related species. Polymorphism information content was calculated for several breeds of dogs. Seven microsatellite markers could be co-amplified. These markers were labeled with fluorescent dyes, multiplexed into a single reaction, and optimized for resolution in a commercial genetic analyzer. The utility of the multiplex set was shown by identifying sires for two mixed litters. This test was not

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* Reprinted with permission from Clark LA, Famula TR, Murphy KE (2004) Evaluation of a rapid single multiplex microsatellite-based assay for use in forensic-genetic investigations in dogs. Am J Vet Res, In Press.

species specific; genotype information collected for wolves, coyotes, jackals, New Guinea singing dogs, and an African wild dog could not distinguish between these relatives of the dog. This set of 7 microsatellite markers is useful for forensic applications (i.e., identification of dogs and determination of parentage) in closely related animals and is applicable to a wide range of species belonging to the family *Canidae.*

Introduction

Microsatellite markers are tandem repeats of 1 to 6 bp that are abundant and evenly distributed across vertebrate genomes. Errors during DNA replication (slippage) occur when the polymerase loses its place and causes loss or gain of tandem repeats, resulting in microsatellites that are highly polymorphic. This polymorphic nature, in conjunction with their strict Mendelian inheritance and the ease with which genotypes can be collected, has made microsatellites the markers of choice for use in forensic genetic investigations (i.e., determination of parentage and identification of individuals) (Leopoldino et al. 2002).

Studies of microsatellites in dogs reveal their variability within and among breeds as well as their utility in identifying individuals and determining parentage (Binns et al. 1995; Fredholm and Winterø 1996; Sutton et al. 1998; Zajc and Sampson 1998; Muller et al. 1999; Ichikawa et al. 2001). High sequence conservation within the family *Canidae* also allows comparative studies of canine microsatellites across species (Fredholm and Winterø 1995).

It is desirable to minimize the number of polymerase chain reactions (PCR) required to collect genotype information because the amount of available DNA is often limited; materials, reagents, and resolution of PCR products on a genetic analyzer are costly; and data can be generated more efficiently. Until recently, it has been difficult to assemble a panel of microsatellite markers suitable for use in canine forensic investigations because purebreed dogs have been highly inbred and line bred, resulting in decreased allelic diversity (Pihkanen et al. 1996).

In 2001, Richman et al. characterized a set of 172 microsatellite markers (MSS-1) that was suitable for use in canine whole genome screens. All markers of the MSS-1 were genotyped on panels of 17 reference families or radiation hybrid cell lines and were selected on the basis of high informativeness and inclusion in linkage groups (Mellersh et al. 1997; Neff et al. 1999; Vignaux et al. 1999; Richman et al. 2001). To enhance the utility of the MSS-1, our group multiplexed 155 markers into 48 multiplex sets (Cargill et al. 2002). Multiplexing is the simultaneous amplification and resolution of markers.

Much of the debate regarding the use of DNA for forensic investigations has focused on issues of statistics and population genetics. Part of the concern is about the accuracy and validity of the data collected and issues of population sampling. The focus of forensic genetics is the computation of a match probability, an expression that requires the knowledge of allelic frequencies and fixation index (F_{ST}), a measure of population diversity (Hartl and Clark 1997; Evett and Weir 1998). Implicit in the calculation of a match probability is the effect of population subdivision on the independence of alleles in the population as a whole.

Specifically, F_{ST} measures the amount of genetic variation in the entire population of dogs that can be attributed to differentiation among subpopulations (i.e., breeds), such that when $F_{ST} = 0$ there are no genetic differences among subpopulations. Algebraically, $F_{ST} = (H_T - H_S)/H_T$, where H_T is the measure of the total heterozygosity for a locus (i.e., the probability that 2 gametes chosen at random from the total population will carry different alleles) and H_S is the subpopulation heterozygosity (i.e., the mean heterozygosity among subpopulations).

Although PIC values are not involved in the calculation of match probabilities, many forensic geneticists use PIC values as a means of characterizing the diversity of allelic and genotypic frequencies for a given locus. This statistic is bound by 0.0 and 1.0 such that the closer the value is to 1.0, the greater the amount of polymorphism, and therefore linkage information, captured by this locus (Botstein et al. 1980; Liu 1998).

The purpose of the study reported here was to develop a set of microsatellite markers, composed of a minimal number of these markers, suitable for use in forensic genetic investigations in dogs and with advantages over multiplexed parentage tests already available (Koskinen and Bredbacka 1999; Altet et al. 2001).

Materials and methods

Selection of microsatellite markers

Sixty-one tetranucleotide microsatellite markers with high (> 0.5) PIC values (measurements of variability at a locus) were selected from the MSS-1 for analysis. To characterize these microsatellite markers, preliminary data, including number of alleles

and peak morphology, were collected from multigenerational pedigrees of German Shepherd Dogs, a family of mixed breed dogs, and several groups of purebreed dogs. A subset of 11 microsatellite markers was selected on the basis of the number and range of allelic sizes, ease of genotyping, and chromosomal location. Marker compatibility was determined via PCR amplification of various combinations of the 11 markers until co-amplification of a maximum number of markers was obtained.

Samples for genotyping

Blood, tissue, or buccal epithelial cell samples collected from dogs and related species were donated by owners, collaborating laboratories, and breeders and exhibitors at dog shows. Relatives of dogs that were collected include the gray wolf (*Canis lupus*), Mexican wolf (*Canis lupus baileyi*), red wolf (*Canis rufus*), maned wolf (*Chrysocyon brachyurus*), coyote (*Canis latrans*), jackal (*Canis adustus*, *Canis mesomelas*, and *Canis aureus*), African wild dog (*Lycaon pictus)*, and New Guinea singing dog (*Canis hallstromi*). Genotyping was also performed to determine the paternity of 2 potentially mixed litters. Genomic DNA was isolated from whole blood and buccal cells using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) and from tissue using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Working solutions of 50 ng of DNA/μL were prepared.

DNA amplification

The 5′ end of the forward primer for each microsatellite marker selected was labeled with 1 of 4 fluorescent dyes (6-carboxy-fluorescein [6FAM], VIC®, NED®, or PET®)[c] (Table 5). Labels were chosen on the basis of observed allelic sizes to prevent overlap of dye types. Concentrations for a 13.45 µl PCR volume were 0.37 mM total dNTPs, 3.7 ng/µl genomic DNA, 2.2 mM of MgCl, 0.74X Taq DNA Polymerase Buffer B (Fisher Scientific, Pittsburgh, PA), 0.74× MasterAmp PCR Enhancer (Epicentre Technologies, Madison, WI), and 0.06 units/µl of Taq DNA polymerase (Fisher Scientific). Concentrations of primers varied for each microsatellite marker. Thermal cycling conditions for DNA amplification were 2 minutes at 95˚C; 5 cycles of 30 seconds at 95˚C, 15 seconds at 58˚C, and 10 seconds at 72˚C; 30 cycles of 20 seconds at 95˚C, 15 seconds at 56˚C, and 10 seconds at 72˚C; and a final extension of 5 minutes at 72˚C using an Eppendorf Mastercycler (Eppendorf Scientific, Inc., New York, NY).

Products of reactions were resolved using an ABI 3100 capillary-based Genetic Analyzer (PE Biosystems, Foster City, CA) and were sized relative to an internal size standard (GeneScan 500 LIZ, PE Biosystems). Genotypes were assigned using Genotyper 2.0 (PE Biosystems).

Calculation of H_T, H_S, F_{ST}, and PIC values

Calculations of H_T, H_S, and F_{ST} were performed by use of public domain software GENEPOP (Version 3.3) (http://wbiomed.curtin.edu.au/genepop/). Total

heterozygosity and F_{ST} were calculated for each microsatellite marker by use of genotype data for all dogs and related species.

Calculation of PIC values from the estimated allelic frequencies was performed by use of software developed by our group. The PIC values and H_S were calculated for several groups of purebreed dogs.

Results

Eleven microsatellite markers were selected on the basis of ease of resolution of products, number and range in size of alleles, and chromosomal location. Seven markers could be co-amplified: FH2309, FH2263, FH2293, FH2321 (Mellersh et al. 1997) FH2132, FH2137, and FH2001 (Table1) (Francisco et al. 1996). All loci are on different chromosomes. Genotype data for the panel of markers were generated for 364 dogs that represent 82 breeds recognized by the American Kennel Club, including 16 breeds with the highest registration rates in 2002. Breeds not recognized by the American Kennel Club included the Boykin Spaniel, Blue Tick Hound, and Long Hair Weimeraner. Forty-eight of 364 dogs were mixed breed dogs. Genotype data were also collected for 7 gray wolves from Alaska, Canada, Sweden, Spain, and Oman, 1 Mexican wolf, 1 red wolf, 3 coyotes, representing 3 populations in the United States, 3 species of jackals from Africa, 2 New Guinea singing dogs, 1 maned wolf, and 1 African wild dog.

No 2 genotypes were identical for all 7 microsatellite markers, even among closely related individuals. Total heterozygosity and F_{ST} were calculated for each marker by use of all 383 genotypes. Total heterozygosity ranged from 0.83 to 0.96 with a mean

of 0.91 ± 0.04 for all 7 markers. Fixation index ranged from 0.16 to 0.27 with a mean of 0.19 ± 0.04 (Table 5).

Polymorphism information content and H_S values were calculated for 7 breeds of dogs and for the gray wolf (Table 6). Four purebreed groups (Miniature Schnauzer, Boxer, Golden Retriever, and Labrador Retriever) were comprised of unrelated, randomly selected dogs. Polymorphism information content and H_S values for 3 breeds (American Eskimo Dog, Dalmatian, and 3 unrelated families of German Shepherd Dog) for which extended pedigrees were available, were calculated in these multigenerational families. The Miniature Schnauzer, Golden Retriever, and Labrador Retriever groups had PIC values similar to published values, which are calculated using 17 reference pedigrees of purebred dogs (Mellersh et al. 1997). As expected, PIC values calculated within families were generally lower than published values. Among the unrelated populations analyzed, the Boxer had the lowest PIC values for five of the seven markers.

We tested the utility of our multiplexed set of microsatellite markers via 2 applications of the technique. A breeder of Blue Tick Hounds suspected that 2 males had bred a bitch and that the litter of 8 puppies was mixed. Genotype data for the panel of markers were collected for the dam, both potential sires, and all puppies. Genotypes for FH2137 (Figure 3) and other data revealed that each male sired 4 puppies. Puppies 4 and 7 had only 1 peak at allele 174 and were homozygous for this locus. Because each parent

Table 5. Microsatellite data

Marker name	Chromosomal Location	Fluorescent Label	Concentration of primer (μmol)	Range in size of alleles (bp)	No. of Alleles	H_T	F_{ST}
FH2309	CFA01	VIC	0.60	342 to 474	34	0.93	0.20
FH2132	CFA02	FAM	0.74	152 to 370	52	0.92	0.27
FH2137	CFA03	PET	0.30	154 to 316	27	0.90	0.16
FH2263	CFA09	NED	0.89	175 to 503	50	0.96	0.16
FH2293	CFA10	VIC	0.74	183 to 527	49	0.91	0.16
FH2321	CFA17	NED	0.60	276 to 396	42	0.93	0.20
FH2001	CFA23	FAM	0.45	115 to 163	13	0.83	0.21

H_T = Total heterozygosity
F_{ST} = Fixation index

must have contributed 1 of these alleles each, sire 1 can be excluded as the sire of these puppies. Puppies 3, 5, and 8 had allele 166, which could only have been contributed by the dam. Because the remaining alleles for puppies 3 and 5 were 164 and 174, respectively, sire 1 can again be excluded as the sire of these puppies. Puppies 1, 2, 6, and 8 had a copy of either allele 162 or 172, excluding sire 2 as the sire of these puppies.

We also used our set of microsatellite markers to determine the paternity of a litter of 4 Miniature Schnauzers. The intended breeding was a repeat breeding, but a male from the dam's first litter had also bred the dam. Genotype data were collected for the dam, both potential sires, and the 4 puppies. Polymorphism information content and H_S values were calculated within this population (Table 6). Two microsatellite markers,

Table 6. Breed data

Unrelated populations															
	Miniature Schnauzer (14*)			**Labrador Retriever** (13)			**Golden Retriever** (12)			**Boxer** (11)			**Canis Lupus** (7)		
Marker	PIC	No. alleles	Hs	PIC	No. alleles	Hs	PIC	No. alleles	Hs	PIC	No. alleles	Hs	PIC	No. alleles	Hs
FH2309 (0.82†)	0.78	7	0.84	0.85	10	0.90	0.67	7	0.84	0.38	2	0.52	0.82	8	0.92
FH2132 (0.76)	0.7	12	0.76	0.82	11	0.88	0.58	4	0.67	0.73	6	0.79	0.9	12	0.98
FH2137 (0.60)	0.79	10	0.85	0.82	10	0.87	0.75	6	0.83	0.63	4	0.72	0.87	10	0.95
FH2263 (0.92)	0.83	11	0.88	0.87	12	0.92	0.85	9	0.9	0.45	4	0.54	0.79	8	0.98
FH2293 (0.87)	0.72	10	0.78	0.82	9	0.87	0.81	9	0.87	0.7	6	0.79	0.89	11	0.98
FH2321 (0.84)	0.82	12	0.88	0.89	14	0.94	0.74	8	0.8	0.23	3	0.25	0.85	9	0.93
FH2001 (0.62)	0.49	5	0.56	0.73	7	0.8	0.67	6	0.75	0.52	3	0.64	0.69	5	0.79
Related populations															
	American Eskimo Dogs (45)			**Dalmatian** (44)			**German Shepherd Dog** (31)			**Miniature Schnauzer** (7)					
Marker	PIC	No. alleles	Hs	PIC	No. alleles	Hs	PIC	No. alleles	Hs	PIC	No. alleles	Hs			
FH2309 (0.82)	0.59	6	0.65	0.65	5	0.71	0.36	4	0.55	0.58	3	0.69			
FH2132 (0.76)	0.54	4	0.62	0.09	3	0.09	0.44	3	0.54	0.58	3	0.68			
FH2137 (0.60)	0.7	6	0.75	0.63	6	0.7	0.53	3	0.64	0.5	3	0.6			
FH2263 (0.92)	0.87	14	0.89	0.72	8	0.76	0.58	4	0.66	0.58	3	0.7			
FH2293 (0.87)	0.39	3	0.47	0.79	8	0.76	0.47	4	0.6	0.46	3	0.59			
FH2321 (0.84)	0.65	7	0.71	0.68	6	0.73	0.29	4	0.37	0.58	3	0.7			
FH2001 (0.62)	0.55	4	0.62	0.39	3	0.5	0.62	4	0.44	0.28	2	0.36			

*Number of dogs
† Published PIC value

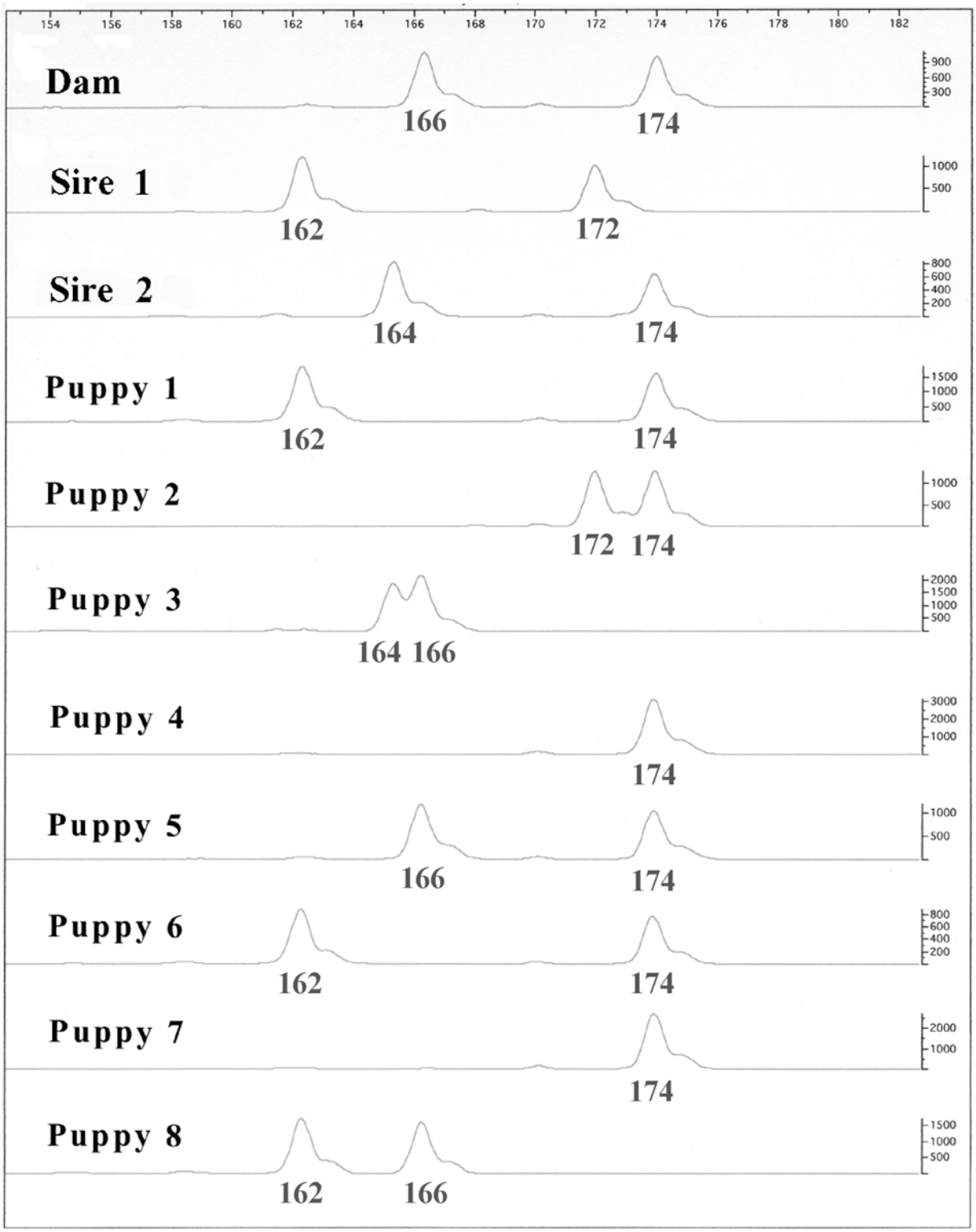


Figure 3. Genotype data for marker FH2137 for 11 Blue Tick Hounds (dam, 2 potential sires, and 8 puppies). The number below each peak is the size of the allele

FH2293 and FH2321, excluded the dam's previous offspring as the sire of 3 puppies and 1 marker, FH2263, excluded the intended sire as the sire of the fourth puppy.

Discussion

We developed a single multiplex set of 7 tetra-nucleotide microsatellite markers suitable for use in forensic genetic investigations in dogs. Only tetranucleotide microsatellite motifs were selected because they are more polymorphic than dinucleotide repeats and typically resolve with fewer stutter bands, extra peaks that can result from errors during replication and complicate analysis of products (Francisco et al. 1996). The polymorphic nature of tetranucleotides is thought to be the result of greater instability associated with longer repeat lengths (Francisco et al. 1996). Our data support this hypothesis. Of the 7 microsatellite markers, those with the smallest product sizes, FH2001 and FH2137, had the lowest number of alleles (13 and 27, respectively). The remaining markers had product sizes > 200 nucleotides (with the exception of the maned wolf, which has alleles 152 and 160 for FH2132) and a mean of 45 alleles across all breeds. FH2001, which had the fewest alleles overall and was the only marker to have all alleles differ by exactly 4 nucleotides, was the only (GATA) repeat microsatellite. The other 6 markers were (GAAA) repeats known to be markedly polymorphic microsatellites and to have variation within the repeat sequence (Francisco et al. 1996).

In general, all dogs and related species had similar ranges of allelic sizes. The widest range in size of alleles was in FH2293, for which the maned wolf had the smallest allele and the Black-backed jackal had the largest. Marker FH2263 also had a wide range

of allelic sizes. For example, many dogs in the American Eskimo Dog family and 7 unrelated dogs of other breeds had allelic sizes of approximately 500 nucleotides for FH2263, with the largest allelic size for this marker in the remaining breeds being 275 nucleotides. Sequence data confirmed that these large alleles were composed of (GAAA) repeats.

Many breeds were represented by a small number of dogs. Therefore, PIC values were not calculated for every breed. Total heterozygosity and F_{ST} were calculated for each marker across all dog breeds and species as measures of genetic variation. The mean F_{ST} among the microsatellite markers (0.19) lies between 0.15 and 0.25, indicating marked genetic differentiation among subpopulations (Liu 1998). Values for humans are in the range of 0.02 to 0.11 (Liu 1998).

The PIC values and allelic sizes in 7 unrelated wolves were similar to those in dogs. The similarity of alleles between species indicates that this panel of markers is not appropriate for use in evolutionary studies. However, the high PIC values in the wolf and the high H_S values for the markers indicate that the panel is suitable for forensics applications in this species.

Although the large number of alleles that exist for each marker indicates that the panel is highly polymorphic, it is not an indicator of how polymorphic the markers are within individual breeds. The PIC values calculated for the Miniature Schnauzers, Labrador Retrievers, and Golden Retrievers were similar to published PIC values and indicate that these markers are informative within these breeds (Mellersh et al. 1997). Only the PIC values for the Boxer were lower than published values (Mellersh et al.

1997). This was a factor in selecting the Boxer to be the first breed of dog to have its genome sequenced (http://www.genome.gov/11007358).

Empirically, genetic variability in populations of related dogs will be lower than in populations of unrelated dogs (Sutton et al. 1998). Overall, the PIC values calculated in the American Eskimo Dog, Dalmatian, and German Shepherd Dog families were lower than those of dogs in unrelated populations. Despite a lesser degree of polymorphism, sufficient markers are informative (i.e., those with PIC values ≥ 0.5) to identify every dog analyzed.

The 7 selected microsatellite markers were sufficient to determine parentage. In the mixed litter of Blue Tick Hounds, all markers supported the conclusion that each male sired 4 puppies. One marker (FH2137) was sufficiently polymorphic to reveal the paternity of each puppy. The testing involving the Miniature Schnauzers illustrated the ability of the 7 microsatellite markers to identify parentage in closely related individuals. Despite the lower PIC values in this family of Miniature Schnauzers, compared with values for unrelated Miniature Schnauzers, the panel of markers was able to detect the paternity of all 4 puppies.

CHAPTER V

CHROMOSOME-SPECIFIC MICROSATELLITE MULTIPLEX SETS FOR LINKAGE STUDIES IN THE DOMESTIC DOG

Overview

To expedite linkage studies and positional cloning efforts in the dog, the MSS-2 of 327 canine microsatellite markers has been multiplexed into chromosome-specific panels. MSS-2 provides 9 Mb coverage of the canine genome with no gaps larger than 17.1 Mb and is the most recent and comprehensive set of microsatellites available for whole genome scans. Markers were labeled with fluorescent dyes based on locations and expected product sizes to facilitate the multiplexing of a maximum number of markers for each chromosome. All markers are amplified using a single thermal cycling program and PCR mix and are optimized for resolution on an ABI 3100 Genetic Analyzer. Seventy chromosome-specific panels were created by co-amplification of a maximum number of markers and subsequent co-loading of the remaining markers.

Introduction

Studies of the dog offer insight into the genetic basis for many hereditary diseases affecting both canine and human populations (Ostrander and Kruglyak 2000; Ostrander et al. 2000; Patterson 2000). Linkage analyses and linkage disequilibrium studies are useful for eventual identification of mutated alleles in the dog, many breeds of which are characterized by small founding populations, high degrees of inbreeding,

and popular sire effects. The creation and maintenance of multigenerational pedigrees offers additional advantages as compared to studies using human populations. To facilitate linkage mapping and positional cloning studies in the dog, it is necessary to have a defined set of polymorphic markers that provides complete coverage of the genome.

Previously, we multiplexed MSS-1, a set of 172 microsatellite markers (Richman et al. 2001) selected from a map comprised of 600 markers (Mellersh et al. 2000) into 69 reactions, thereby reducing the time, expense, and DNA required for whole genome screens (Cargill et al. 2002). The most recent version of the canine map has 3,270 markers, including 1,596 microsatellite markers (Guyon et al. 2003). From this, a superior screening set of microsatellite markers providing increased density and a greater level of informativeness was developed. MSS-2 is comprised of 327 microsatellite markers that have an average spacing of 9 Mb with no gaps larger than 17.1 Mb (Guyon et al. 2003). The set includes 171 tetra-, 151 di-, and 3 tri-nucleotide repeats with an average heterozygosity value of 0.73 when analyzed on a panel of unrelated purebred dogs.

In the human, chromosome-specific multiplexed microsatellite sets have been developed to accelerate collection and analysis of data for linkage studies (Reed et al. 1994; Lindqvist et al. 1996). However, no such sets exist for the dog. The development of chromosome-specific multiplex panels will allow rapid screening of those chromosomes presumed to harbor genes of interest as determined through comparative mapping and genomic sequencing.

Materials and methods

Primer pairs were synthesized by Applied Biosystems (PE Biosystems, Foster City, CA) and forward primers were labeled with one of four fluorescent dyes: 6FAM, NED, PET, VIC. Previously multiplexed microsatellites (Cargill et al. 2002) that were labeled with 6FAM were not relabeled, and those labeled with TET and HEX were relabeled with VIC and NED, respectively, to retain their original dye colors. Dye types for new markers were chosen for even distribution across each chromosome and size range. Primers were diluted to 10 μmol. The primer sequences and intermarker distances are available at www.fhcrc.org/science/dog_genome/guyon2003/guyon_data/mss2.html and http://www-recomgen.univ-rennes1.fr/doggy.html.

All multiplex sets were amplified with a single stepdown thermal cycling program: 5 minutes at 95°C followed by 5 cycles of 30 seconds at 95°C, 15 seconds at 58°C, and 10 seconds at 72°C, and an additional 30 cycles of 20 seconds at 95°C, 15 seconds at 56°C, and 10 sec at 72°C, with a final extension of 5 minutes at 72°C. A single mastermix, excluding primers, was used for all multiplex and individual reactions. Concentrations are 0.0045 units/μl *Taq* DNA polymerase with 0.67X Buffer B (Fisher Scientific, Pittsburgh, PA), 4.5 mM $MgCl_2$, 0.67X MasterAmp PCR Enhancer (Epicentre Technologies, Madison, WI), 0.75 mM total dNTPs, and 2.8 μl water to bring the final mastermix volume to 6.65 μl. One μl of 50 ng/μl genomic DNA was used in each reaction. Primer volumes vary by multiplex (Table 7) resulting in different total reaction volumes and concentrations.

Table 7: Chromosome-specific panels for the MSS-2, listed by multiplex name[a] followed by marker name[b], primer amount[d], and fluorescent label[e]

1.1	FH3413	0.8	P	REN112I02	0.8	V	C01.424	0.8	F	C00901	0.8	P	FH2793	0.6	P	FH2326	1.0	N	
1.2	FH3325	0.8	P	FH3300	0.8	N	C01.251	0.8	N	FH2309	0.8	V	REN143K19	0.6	V				
1.3	FH2663	1.1	F	FH3603	1.5	F	FH3922	1.1	F	FH2294	0.3	N							
2.1	FH3210	0.8	P	REN303H07	0.8	V	REN70M14	0.8	V	FH3965	0.8	F							
2.2	FH2890	0.4	N	C02.609	0.6	P	FH2613	0.6	V	FH2132	0.6	F							
2.3	FH2274	0.8	N	FH2608	0.8	P	C02.342	0.8	F										
3.1	REN161A12	0.6	F	FH3252	0.6	P	FH3464	0.6	V	FH2316[c]	0.8	N	FH3377	0.6	N				
3.2	FH3115	0.8	N	C03.629	1.2	V	FH2145	0.8	P	REN260I04	0.6	F							
3.3	FH3396	1	F	FH2302	0.6	V													
4.1	REN298N18	0.4	P	REN303C04	0.4	V	FH2732	0.8	F	FH3310	0.8	F	REN74B13	0.6	V	AHT103	0.8	F	
4.2	FH2776	1.0	P	REN195B08	0.8	N	FH2097	0.6	N	G07704	0.6	V							
5.1	FH3004	0.8	F	DTR05.8	0.8	N	FH3978	1.0	P	REN175P10	0.6	V	CPH14[c]	0.8	N				
5.2	FH3928	0.8	P	FH3320	0.6	N	FH3702	0.8	F	FH3089	0.8	V							
5.3	FH2140	0.8	P	REN285I23[c]	0.8	V	FH3278	0.8	P	C05.771	0.6	V							
6.1	FH2525	0.8	V	FH2561	1.4	N	FH2734	0.8	V	FH2164[c]	0.8	V	FH3303	0.5	P				
6.2	FH2576	0.6	F	FH3933	0.8	P	FH2370	0.8	N										
6.3	REN285H12	0.8	F	FH2119	0.8	N	REN111L07	0.8	P										
7.1	REN97M11	0.8	P	FH3972	0.6	N	REN162C04	0.8	V	REN143L20	0.6	P	FH2860	0.4	V				
7.2	FH2226	0.8	N	VIASD10	0.8	P	FH2973	0.8	P										
8.1	FH3241	0.8	P	REN204K13	0.8	N	FH3316	0.8	V	C08.618	0.8	F							

Table 7: Continued

8.2	FH3425	0.8	N	C08.410	0.8	F	REN178J05	0.6	F	FH2989	1.0	V							
9.1	GALK1	0.6	V	FH2263	0.8	N	C09.173	0.4	N	REN54L20	0.4	F	G06401	0.6	P	REN287G01	0.6	N	
9.2	FH2186	1.4	V	REN145P07	0.6	P	FH3835	0.3	F	REN73K24	0.3	V	FH2885	0.4	N				
10.1	FH2537	0.8	N	FH4081	0.8	P	C10.781	0.6	V	ZUBECA1	0.4	N	DTR10.5	0.8	F	FH3381[C]	0.8	P	
10.2	REN06H21	0.8	P	FH2293	0.8	V	C10.16	0.8	F	FH2422	0.8	N							
11.1	FH3203	0.8	V	REN242K04	0.8	F	FH2004	0.6	F	C11.868	0.8	P	C11.873	0.8	V	DGN13	0.8	V	
11.2	AHT137	0.3	V	FH4031	0.8	P	FH2319	1.0	N	FH2019	0.2	N							
12.1	REN153O12	0.6	F	FH2401	0.6	V	FH3591	0.6	N	G01811	0.6	P	REN94K11	0.6	N				
12.2	REN258L11	0.8	P	REN213F01	0.8	F	FH3711[C]	0.8	N	FH1040	0.6	V	FH3748	0.8	P				
13.1	C13.391	0.8	N	REN120P21	0.6	F	FH3619	0.6	P	DTR13.6	0.6	F	FH2348	1.2	V	FH3800	0.6	N	
13.2	FH3494	0.8	V	REN227M12	0.8	P													
14.1	FH3951	1	F	FH3725	0.6	P	FH2658	1	P	FH2763	0.4	N							
14.2	C14.866	0.8	F	FH3285	0.8	P	PEZ10	0.8	V										
15.1	FH4012	0.6	P	FH3813	0.8	V	FH2171	0.6	N	CPH4	0.8	N	REN230G12	0.6	F				
15.2	FH3802	0.6	V	REN06C11	0.6	F	FH2360	0.8	P										
16.1	REN214L11	0.4	V	FH2670	0.6	F	REN73O19	0.6	P	REN85N14	0.8	P	FH3592	0.8	V				
16.2	FH2155	1.0	N	REN275L19	0.4	N	FH2175	1.2	F										
17.1	REN240A05	0.6	F	FH3369	0.6	P	REN294E18	0.6	V	FH3995	0.8	F							
17.2	FH3047	0.8	P	FH4023	0.8	P	PEZ8	1.0	N	FH2869	0.6	V							

Table 7: Continued

18.1	FH4060	1.0	N	FH3944[C]	0.8	P	FH3824	0.4	V	FH3815	0.4	F	REN54P11[C]	0.8	P	FH2834	0.4	N	REN47J11[C]	0.8	V
	AHT130	0.6	F																		
19.1	REN213G21	0.6	V	FH3491	0.4	F	FH3313	1.0	P	FH2206[C]	0.8	P	FH2380	0.6	N						
19.2	FH3299	0.6	V	FH3834	0.6	F	FH3969	0.6	N												
20.1	PEZ19	0.8	N	FH2951	0.6	F	FH2158	1.0	P	REN114M19	0.4	F									
20.2	REN55P21[C]	0.8	N	REN100J13	0.8	P	REN93E07	0.2	V	AHTk209	1.0	N									
21.1	FH3803	1.0	P	FH2233[CA]	0.8	F	REN118B15	0.3	V	FH2441	0.8	N	REN37A15	0.3	V	FH3398	0.8	P	FH2312[CA]	0.8	N
22.1	REN42F10	0.8	V	FH3355	0.8	V	FH3411	0.8	N	FH3853	0.8	P									
22.2	REN49F22	1.0	N	REN128E21	1.0	P	C22.279	0.4	V	REN78I16	0.6	F									
23.1	FH3078	0.8	P	FH2508	1.0	P	FH2626	0.8	F	REN113M13	0.6	V	REN02P03	0.8	N	REN181K04	0.8	P			
24.1	FH3023	0.8	P	FH2261[C]	0.8	F	AHT125	0.8	P	FH3287	0.8	F	REN228J19	0.8	V						
24.2	FH3750	0.8	P	FH2159	0.8	N	REN106I06	0.6	V	REN272I16	0.4	F									
25.1	REN54E19	0.8	F	FH3245[C]	0.8	P	FH2324	0.3	N	FH2141	1.0	N	FH3627	0.3	V	FH4027	0.7	F			
26.1	REN62M06	0.4	N	DTR26.9	0.4	V	FH3426	0.4	V	DGN10[C]	0.8	P	FH2130	1.0	N	C26.733	0.6	F			
27.1	FH3221	0.8	P	PEZ6	0.6	F	REN181L14	0.4	N	REN72K15	0.4	V									
27.2	FH2289	0.8	P	PEZ16	0.3	N	LEI002	0.3	F	FH3924	0.6	V									
28.1	C28.176	0.8	V	FH3963	0.8	P	FH2585	0.8	F	REN146G17	0.8	V	FH2208[C]	0.8	N						
29.1	FH2952	0.8	P	FH2364[C]	0.8	F	REN52D08	0.4	P	REN45F03	0.8	F	FH2385	1.0	V	FH1007	0.3	V			
30.1	FH3489	0.4	F	REN51C16	0.4	P	REN248F14[CA]	0.8	V	FH2290	1.0	N	FH3632[CA]	0.8	P	FH3053	0.4	F			
31.1	FH2189	0.8	N	RVC11	0.6	V	REN43H24	0.6	N	REN109B10	1.2	P	REN110K04[C]	0.8	P	FH2712	0.4	F			

Table 7: Continued

32.1	REN244E04	0.3	F	CPH2	0.4	V	FH2875	0.6	N	FH3635	1.0	F	FH3236	0.8	N	AHT127	0.3	V	FH3294[C]	0.8	P
33.1	FH2790	0.4	F	FH3608	0.8	F	FH2361	0.3	V	REN186B12	0.6	V	FH2165[C]	0.8	N						
34.1	FH3721	0.8	P	REN174M24	0.6	F	REN243O23	0.8	F	REN314H10	0.4	V									
34.2	REN109L16	0.8	N	FH2377	0.8	V	FH3836	0.8	N												
35.1	FH3570	0.8	F	REN282I22	0.4	V	REN94K23	0.6	N	REN112C08	0.6	P									
36.1	REN106I07	0.8	V	FH2611	0.8	P	REN179H15	0.8	P	FH3865	0.8	V	DTR36.3[C]	0.8	N						
37.1	FH3272	0.8	F	H10101	0.8	V	REN67C18	0.8	P	FH3449	0.8	F	FH2532	0.8	N						
38.1	FH2766	0.8	P	REN02C20	0.8	F	REN164E17	0.8	N												
X.1	FH2916	0.8	F	REN101G16	1.0	N	D04614	0.8	F	REN144O22	0.6	V									
X.2	FH3027	0.8	N	FH1020	0.8	F	FH2985	0.6	N	REN230I20	0.6	V									
X.3	REN130F03	0.8	F	FH2584	0.8	N	REN75A05	0.8	P												
Y.1	REN197E16	0.8	V	REN44K10[CA]	0.6	F	DTRY.13[CA]	0.4	F	REN75H09	0.2	V	REN173O16	0.2	P						

[a]Denoted by chromosome and then multiplex number within chromosome
[b]Marker names; [C] indicates that the marker is amplified individually and co-loaded into the panel; [CA] indicates that the markers are co-amplified then co-loaded into the panel.
[d]Total amount (µl) of primer (forward and reverse) in the multiplex.
[e]F=6FAM, P=PET, V=VIC, N=NED

Multiplex sets were first established by amplifying markers for each chromosome in various combinations. Once the maximum number of co-amplified markers was achieved, the remaining markers were amplified individually and co-loaded into a multiplex set representing individual chromosomes. Duplex sets were co-loaded into other multiplexes on the chromosome, if possible. For ease of genotyping, no markers having the same dye type and product sizes less than 50 bp apart were multiplexed in a chromosome panel.

PCR products were diluted 1:20 with water and resolved with an internal size standard (GeneScan 500 LIZ, PE Biosystems) using an ABI 3100 capillary-based Genetic Analyzer (PE Biosystems). For co-loading, dilutions of 1:10 were made for the multiplex and combined with dilutions of 1:20 for the co-loaded marker. Analysis of multiplex sets was done using ABI GENESCAN version 3.7 (PE Biosystems).

Results

Three hundred sixteen microsatellite markers from MSS-2 are resolved in 70 chromosome-specific panels, providing an average of 1.75 multiplex sets per chromosome (Table 7). Two hundred ninety six markers can be co-amplified within the chromosome panels. The remaining 20 markers are amplified individually and co-loaded into designated panels for resolution in a single capillary. Six markers are co-amplified in pairs and are then co-loaded with the appropriate panel. Three markers, FH3245, REN51i12, and, FH2239, could not be co-amplified or co-loaded in chromosome-specific fashion and must be resolved individually.

All multiplexed markers are amplified using a single PCR mix and thermal cycling program. For multiplexing, 78 primers were labeled with 6-FAM, 82 with VIC, 82 with NED, and 85 with PET. In multiplexes 7.1, 9.1, 12.2, 23.1, and 37.1, two markers with the same dye-type have similar product sizes. Eight markers, REN262G24, REN286O18, FH3970, FH2200, REN297D17, FH3939, REN89K14, and FH3399, did not amplify or were not able to be genotyped using these parameters and, hence, were not incorporated into the chromosome-specific panels described here.

Discussion

The most comprehensive screening set currently defined for linkage studies in the dog is MSS-2, which offers 9 Mb coverage and highly polymorphic markers, including 64 markers from the MSS-1. To enhance the utility of MSS-2, we have developed chromosome-specific multiplex sets, which expedite whole genome scans in the dog and have the potential to exclude candidate genes on a given chromosome. Collection of data by chromosome also allows for statistical analysis for individual chromosomes to be conducted before the whole genome scan is complete and will facilitate confirmation of linkage studies, as well as positional cloning efforts.

The multiplex sets minimize the expense, time, and genetic material necessary to collect genotype information for MSS-2. The number of reactions and runs on a Genetic Analyzer are reduced by 68% and 75%, respectively. Collection of data is further streamlined through use of a single thermal cycling program and PCR mix. To increase the ease with which alleles can be determined, markers having the same dye type and

product sizes separated by less than 50 bp were not combined in the same panel. However, similar product sizes from markers having the same dye type were observed in multiplexes 7.1, 9.1, 12.2, 23.1, and 37.1 and alternate dye-types could be selected to eliminate possible genotype error.

CHAPTER VI

LINKAGE ANALYSIS FOR PANCREATIC ACINAR ATROPHY IN THE GERMAN SHEPHERD DOG

Overview

PAA is a degenerative disease of the exocrine pancreas that occurs in the GSD and leads to EPI. We have previously shown that PAA segregates in an autosomal recessive fashion in two multigenerational families of GSDs. In an effort to facilitate the identification of affected and carrier dogs, we hope to identify a genetic marker that co-segregates with the disease. To do this, linkage analysis using the MSS-1, a set of 172 microsatellite markers that provides 10 cM coverage of the canine genome and has been multiplexed into 69 reactions, was carried out.

Using the aforementioned multigenerational pedigrees and the multiplexed MSS-1, 30 GSDs were genotyped for 163 microsatellite markers. The markers were labeled with fluorescent dyes and were resolved on an ABI 377 Sequencer. LOD (Logarithm of the Odds) scores for these markers were generated using the SOLAR (Sequential Oligogenic Linkage Analysis Routines) software package. Those markers for which there were provocative LOD scores were analyzed on additional pedigree members. Unfortunately, there were no LOD scores higher than 3.0, which is considered to be the minimum score required to infer linkage.

These results suggest that no marker from the MSS-1 is in close enough proximity to the causative gene such that linkage could be detected. Further studies

using markers providing more complete coverage of the canine genome will be required in order to identify a marker that co-segregates with PAA. To that end, future work will include use of the MSS-2, which is comprised of 327 microsatellite markers and provides 9Mb coverage of the canine genome.

Introduction

PAA is a disease characterized by the degeneration of acinar cells of the exocrine pancreas that leads to EPI and occurs primarily in the GSD (Westermarck et al. 1993). Ninety-six percent of affected dogs present with symptoms of EPI by five years of age, although many dogs show signs as early as 6 months (Westermarck et al. 1993; Raiha and Westermarck 1989). Clinical signs include a ravenous appetite, weight loss, and voluminous soft stools (Westermarck et al. 1989). Steatorrhea, borborygmus, coprophagia, and polydipsia are also associated with EPI (Raiha and Westermarck 1989). Diagnosis of PAA is accomplished by the measurement of serum canine trypsin-like immunoreactivity (cTLI) using a radioimmunoassay, which determines the amount of trypsinogen released into the bloodstream from the pancreas (Williams and Batt 1988). The reference range for this assay is 5.0 μg/L to 35.0 μg/L, and values below 2.5 μg/L are considered diagnostic for EPI (Williams and Batt 1988). This assay has been reported to be 100% sensitive and specific for EPI and thus is an effective diagnostic tool (Williams and Batt 1988).

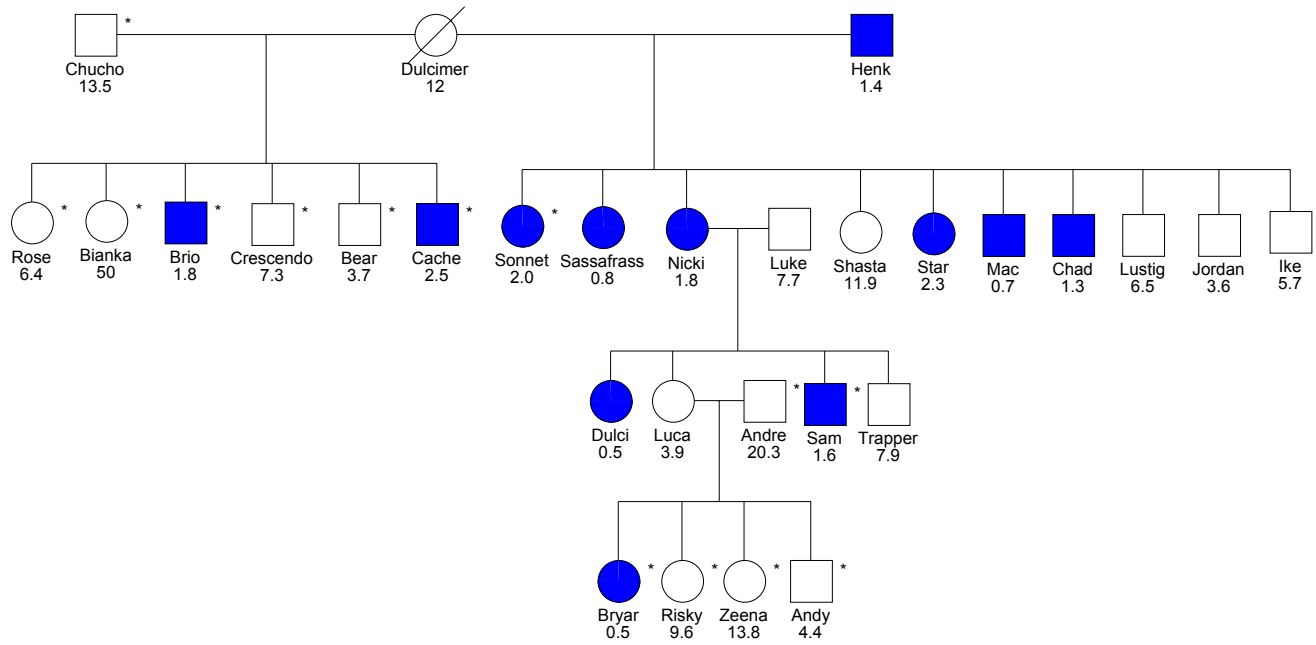
The prognosis for dogs having PAA is typically good with treatment (Wiberg et al. 1998). Expensive enzyme supplements must be administered with each meal for the

duration of the animal's life (Wiberg et al. 1998). However, many dogs with EPI are euthanized because owners are unable to afford the enzyme supplements (Hall et al. 1991, Wiberg et al. 2002).

To understand inheritance of PAA, we previously assembled two multigenerational pedigrees of GSDs segregating the disease (Moeller et al. 2002). Clinical data for 75 dogs spanning four generations was collected from the first family, and for 40 dogs spanning four generations from the second family. Statistical analyses conducted using these two families suggest that a single locus segregating in an autosomal recessive fashion causes PAA (Moeller et al. 2002).

It is difficult for breeders to eliminate PAA from their lines because there are no means to identify carrier dogs or affected dogs before the onset of clinical signs. Identification of a marker linked with the gene causative for PAA would allow for both the early detection of affected dogs, and the identification of carrier dogs. To conduct linkage analysis, it is necessary to have a set of markers that are evenly distributed across all chromosomes. The MSS-1 is a set of 172 microsatellite markers and is the first screening set providing 10 cM coverage of the canine genome (Richman et al. 2001). Our group previously multiplexed the MSS-1 into 69 reactions (Cargill et al. 2002).

Pedigree I



White = normal
Blue = affected
/ = deceased
* = genotypes from informative markers

Pedigree II

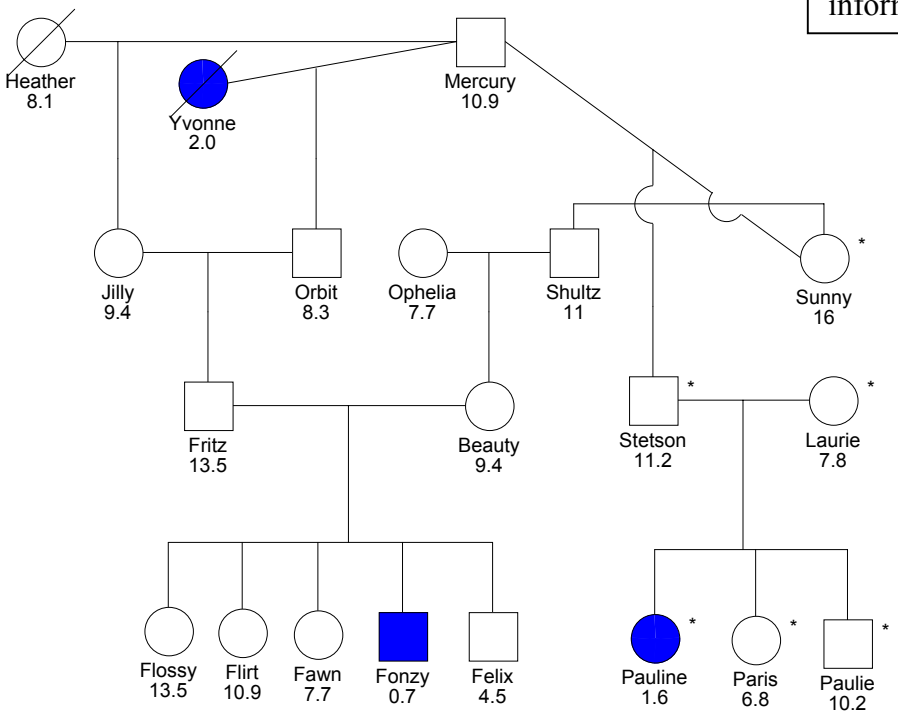


Figure 4. Pedigrees I and II: Subsets from 2 unrelated families of GSDs segregating PAA.

Materials and methods

For each dog included in the analysis, blood and serum were collected by veterinarians and submitted for use in the study. Serum samples were submitted to the Gastrointestinal Laboratory at Texas A&M University for measurement of cTLI to determine the clinical status of each dog. DNA was extracted from 3 ml of whole blood using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) and rehydrated in 250 ul of DNA rehydration solution. To ascertain the quality and quantity of DNA, 5 ul of each sample were analyzed by gel electrophoresis and 5 ul were used for spectrophotometric analysis.

Only litters having affected members were included in the screen. For our initial linkage screen, 14 dogs from Pedigree I and 12 dogs from Pedigree II were selected (Figure 4). Also included were 4 dogs from a smaller, unrelated pedigree. Primers that amplify the MSS-1 markers were synthesized and the forward primers were labeled with one of three fluorescent dyes, 6FAM, TET, or HEX (PE Biosystems, Foster City, CA). Amplification and resolution of microsatellite markers were achieved exactly as described in Chapter III. Genotypes were determined using Genotyper 2.0 software and each peak was given an integer value (PE Biosystems).

Once genotypes for all pedigree members were determined for the MSS-1 markers, a two-point LOD (Logarithm of the Odds) score was calculated for each marker. A LOD score is the measurement of the likelihood that the marker is linked with the disease. The minimum LOD score necessary to infer linkage is 3.0, which corresponds to odds of 1000:1 in favor of linkage. Calculation of LOD scores was

completed using the SOLAR (Sequential Oligogenic Linkage Analysis Routines) software package. To reduce false negative results, analyses were conducted using both binary and quantitative traits, based on the clinical status and the TLI result, respectively.

Because population size is critical in linkage analyses, genotypes for additional pedigree members were collected for markers that were informative in the two families. Genotype data for these markers were generated for twenty additional dogs from Pedigrees I and II (Figure 4) and LOD scores were recalculated. Markers of interest were further analyzed using genotype data for all members of the three pedigrees.

Results

Genotype data for 163 microsatellite markers from the MSS-1 were collected for 30 GSDs. The remaining nine markers (ZuBeCa6, FH2149, FH2279, FH2346, N41, FH2457, AHT006, REN51i12, FH2600) could not be amplified using the reported conditions and data were not generated for them. No LOD scores from the initial screen indicated linkage. The highest LOD score was 1.3 for REN49C08, a marker on CFA11. Further analysis using additional pedigree members eliminated this marker as a candidate marker.

Eighty of the 172 markers had four or more alleles in our families. For these markers, PIC values were calculated. The PIC value is a measurement of how informative a marker is. A subset of 63 MSS-1 markers, that had four or more alleles

Table 8. Chromosomal location and LOD score for 63 MSS-1 markers determined to be informative in our families

Chromosome	Marker	LOD	Chromosome	Marker	LOD
CFA01	C01.424	0.00	CFA16	FH2175	0.00
CFA01	FH2016	0.00	CFA17	PEZ8	0.00
CFA01	FH2598	0.00	CFA18	AHT130	0.00
CFA02	FH2087U	0.00	CFA18	WILMS-TF	0.10
CFA03	FH2137	0.90	CFA19	FH2206	0.00
CFA03	FH2531	0.43	CFA20	CPH16	0.00
CFA03	FH2145	0.01	CFA20	FH2528	0.00
CFA03	FH2107	0.53	CFA21	FH2233	0.00
CFA03	FH2302	0.32	CFA22	CXX.279	0.00
CFA04	FH2142	0.00	CFA23	FH2283	0.00
CFA04	FH2534	0.13	CFA23	FH2227	0.00
CFA05	FH2594	0.00	CFA24	FH2261	0.00
CFA05	FH2383	0.00	CFA25	FH2526	0.00
CFA06	FH2525	0.00	CFA25	FH2141	0.00
CFA07	FH2301	0.00	CFA27	PEZ6	0.09
CFA07	FH2581	0.00	CFA28	FH2585	0.00
CFA07	FH2226	0.00	CFA29	FH2364	0.00
CFA07	FH2396	0.00	CFA29	REN45F03	0.00
CFA08	C08.410	0.00	CFA30	1F11	0.00
CFA09	FH2263	0.00	CFA30	FH2305	0.14
CFA09	FH2186	0.00	CFA30	FH2290	0.00
CFA10	FH2293	0.10	CFA31	FH2199	0.41
CFA10	FH2422	0.04	CFA31	CXX.642	0.03
CFA10	FH2339	0.14	CFA31	FH2239	0.03
CFA11	FH2018	0.09	CFA32	FH2238	0.20
CFA11	FH2319	0.00	CFA33	FH2507	0.00
CFA12	FH2223	0.14	CFA37	FH2587	0.00
CFA13	FH2394	0.00	CFA37	FH2532	0.00
CFA14	FH2547	0.00	CFA38	FH2244	0.00
CFA15	FH2278	0.00	Unlinked	FH2247	0.00
CFA15	FH2171	0.00	Unlinked	PEZ7	0.00
CFA16	FH2155	0.00			

and a PIC ≥ 0.55, was determined to be informative in our families.

No LOD scores calculated for the 63 markers using the additional pedigree members were ≥ 3 (Table 8). Four of the five highest LOD scores were from markers located on CFA03: FH2137 (0.9), FH2107 (0.53) FH2531 (0.43), and FH2302 (0.32). Further analyses of these markers resulted in a reduced LOD score for FH2137 (0.7), but an increased score for FH2107 (1.14), FH2531 (0.44), and FH2302 (0.6) (Figure 5).

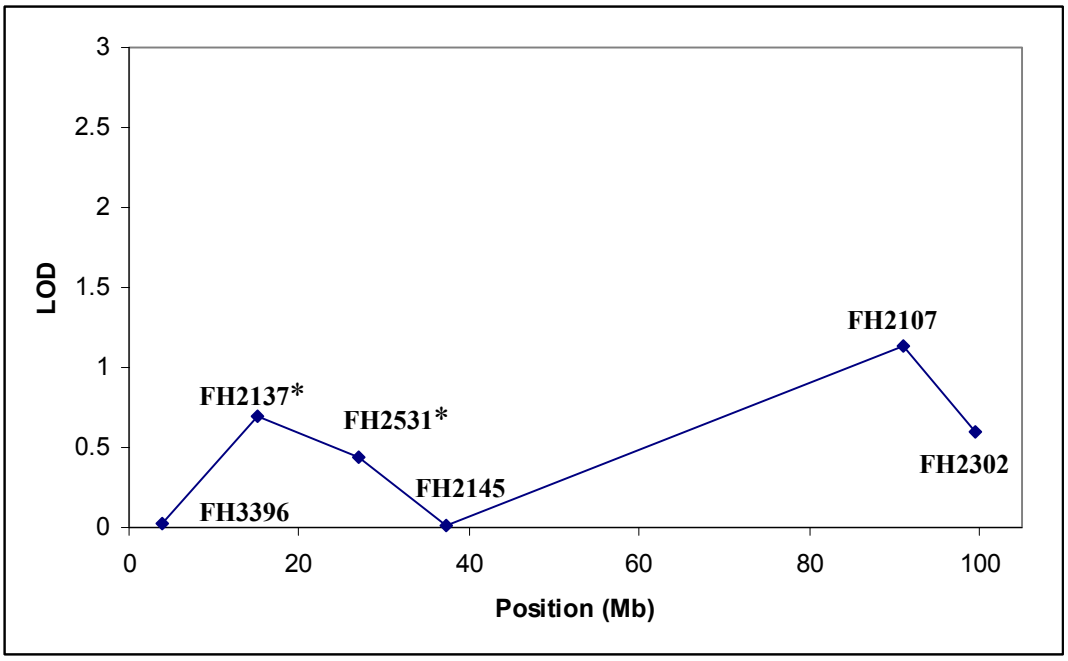


Figure 5. CFA 03: LOD scores for markers which were informative (≥ 4 alleles) in our families.
* marker positions are estimated based on linkage map

Nine additional markers from CFA03 (FH3396, FH3115, REN161A12, C03.629, FH3252, FH2145, FH3464, REN260I04, and FH3377) were genotyped for the 50 GSDs.

Only two of these markers were informative in our pedigrees and no LOD scores were ≥ 3 (Figure 5).

Discussion

Our results indicate that none of the 163 microsatellite markers analyzed are located in close enough proximity to the causative gene for PAA to be useful as a genetic marker. The highest LOD scores were identified for markers located on CFA03. Analyses will be conducted for additional markers in this region as they are identified.

Based on the number of alleles and PIC values, less than 40% of the markers analyzed were informative in our families. Although the MSS-1 provides 10cM coverage of the canine genome, the limited number of informative markers diminishes the coverage actually achieved with our screen. The MSS-2, a screening set of 327 microsatellite markers providing 9Mb coverage of the canine genome, has recently been characterized (Guyon et al. 2003). Now that multiplexing of the MSS-2 has been completed, we intend to use this set in a second screen for linkage. We also continue to collect pedigree members of the families of GSDs examined here in order to increase the power of future screens.

CHAPTER VII

CONCLUSIONS

Our laboratory's interest in canine genetics stems from the importance of the dog to our society. For thousands of years, dogs have been our protectors, workers, aides, and companions. Today, studies in dogs also contribute to our understanding of mammalian hereditary diseases (Ostrander and Kruglyak 2000). The goals of the work presented here were to 1) develop tools that can accelerate the characterization of genes that are involved in hereditary diseases affecting the dog, and 2) determine the mode of transmission of PAA in the GSD and identify possible causative genes.

PAA is one of many diseases of the dog that are inherited in an autosomal recessive manner. Roughly two-thirds of hereditary diseases of the dog exhibit autosomal recessive inheritance (Ostrander and Kruglyak 2000), and for many of these, symptoms are not evident until later in life. Such diseases are particularly frustrating to breeders because it is not possible to recognize affected dogs before the onset of symptoms or to identify which dogs carry mutations.

Microsatellites are widely used for the identification of individuals, evolutionary studies, and as marker-based tests for hereditary diseases (Leopoldino et al. 2002). The identification of microsatellite markers linked with various hereditary diseases provides breeders with the necessary tools to identify carrier and affected dogs and reduce the incidences of diseases in their breed. Two sets of microsatellite markers providing complete coverage of the genome have been described for linkage analysis in the dog

(Richman et al. 2001; Guyon et al. 2003). The utility of these sets can be enhanced through multiplexing.

Chapter II reports the assembly of two extended pedigrees of GSDs that segregate PAA and the results of statistical analyses conducted using these families (Moeller et al. 2002). Other research efforts to understand the genetics of PAA have suggested an autosomal recessive mode of inheritance (Weber and Freudiger 1977; Westermarck 1980), but no statistical data were collected in these studies. Complex segregation analysis revealed that in the two pedigrees described, PAA is caused by a single gene segregating in autosomal recessive fashion.

The development of a set of multiplexed microsatellite markers suitable for linkage studies in the dog (Cargill et al. 2002) is described in Chapter III. The MSS-1, comprised of 172 microsatellites providing 10cM coverage, is the first set of polymorphic markers defined for whole genome screens in the dog (Richman et al. 2001). The MSS-1 was enhanced by the development of 48 multiplex sets, which minimize expenses and time required to collect genotype information by reducing the number of reactions by 60%. Primers were labeled with fluorescent dyes (TET, HEX, 6FAM) and sets of markers that could be co-amplified were identified. More specifically, 151 markers could be co-amplified, 4 markers could be co-loaded after individual amplification, and 17 were amplified individually. Reactions were optimized for resolution on an ABI 377 Genetic Analyzer.

Chapter IV describes the development and evaluation of a specialized, multiplexed set of microsatellite markers for use in canine forensics (Clark et al. 2004).

MSS-1 markers with high PIC values were analyzed for significant polymorphism and ease of genotyping. A subset of 7 markers that could be co-amplified in a single reaction was identified. Genotypes were collected for 85 breeds and 19 other members of family *Canidae*. This marker set is used to determine parentage in closely related dogs.

Chapter V reports the development of chromosome-specific multiplex panels of microsatellite markers for more comprehensive linkage studies in the dog (Clark et al. 2004). The MSS-2, comprised of 327 microsatellites, is the latest screening set defined for linkage analysis in the dog and offers greater coverage (9Mb) and more polymorphic markers (Guyon et al. 2003). Primers were labeled with fluorescent dyes (6FAM, VIC®, NED®, PET®) based on chromosomal locations, so that multiplex sets could be created for each chromosome. Reactions were optimized for resolution on an ABI 3100 Genetic Analyzer. Seventy chromosome-specific panels for the MSS-2 were developed, reducing the number of reactions by 68% and number of runs on the ABI 3100 by 75%.

Results from linkage analyses for PAA using the aforementioned pedigrees of GSDs are reported in Chapter VI. Genotypes for 30 GSDs were generated for 163 markers using the multiplexed MSS-1. LOD scores and PIC values were calculated for each marker. Twenty additional GSDs were genotyped for 63 markers that were informative in our pedigrees. The highest LOD scores were found for markers located on CFA03, though none of the scores approached statistical significance. It will be necessary to analyze additional GSDs using a more comprehensive set of markers in order to identify linkage with PAA.

In conclusion, this work provides new tools that will facilitate study of canine hereditary diseases and adds to the knowledge concerning transmission genetics of PAA in the GSD. To date, linkage of markers with PAA has not been identified. Now that multiplexing of the MSS-2 is complete, we intend to use this set to conduct a second screen for linkage with PAA using Pedigrees I and II. We also continue to collect members of these families in order to increase the power of this screen. Another method that we are pursuing to identify candidate genes for PAA is microarrays. We are collecting tissue from affected and normal GSDs and will use cDNA isolated from these tissues to probe a canine oligonucleotide array to assess differences in gene expression in an attempt to identify (1) biomarkers that may help in early diagnosis and (2) potential candidate genes that may be causative for PAA.

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VITA

Name: Leigh Anne Clark

Permanent Address: 607 Meadow View Court
College Station, Texas
77845

Education: Texas A&M University
College Station, Texas
B.S., 2000, Biomedical Science
Ph.D., 2004, Veterinary Microbiology

Selected publications:

Cargill EJ, Clark LA, Steiner JM, Murphy KE (2002) Multiplexing of canine microsatellite markers for whole-genome screens. Genomics 80, 250-253

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