Canine hip dysplasia is a heritable developmental disease resulting, in part, from increased laxity in hip joints and is a precursor to degenerative joint disease. Identification of genetic markers linked to joint laxity would foster development of more accurate diagnostic methods, facilitate identification of the disease gene(s), and supplement efforts to establish physical/genetic maps of the canine genome. Work presented here describes analysis of randomly amplified polymorphic DNA in the search for markers which cosegregate with increased joint laxity in Canis familiaris, the domestic dog. The Boykin spaniel, a highly inbred breed afflicted with an extremely high incidence of hip dysplasia, served as a model for study of canine hip dysplasia. Only 5% of 200 random primers revealed significant polymorphisms within this breed. However, polymorphisms were detected in seemingly nonpolymorphic amplification products when digested with restriction enzymes. Restriction digestion revealed polymorphisms in 15% of the monomorphic amplification products. Among the primers that revealed polymorphisms, one primer correctly identified 9 of 12 dogs with regard to joint laxity. However, extensive evaluation is required before any assertion can be made regarding linkage of this marker to joint laxity. Of interest, another primer amplified a genomic segment unique to the canine Y chromosome.
importance of mapping the canine genome is underscored by recent work to identify linkage groups (Lingass et al. 1997; Mellersh et al. 1997) and establishment of hybrid cell lines for study of specific canine chromosomes (Langston et al. 1997).

DNA fingerprinting techniques rely on detection of polymorphisms. Polymorphisms have been detected by analyses of randomly amplified polymorphic DNA (RAPD) (Gu et al. 1997a; Rothuizen and Van Wolferen 1994; Williams et al. 1990) and of microsatellite DNA (Ostrander et al. 1993). RAPD is especially useful since allelic polymorphisms can be quickly identified. The utility of the RAPD approach is exemplified by its use in identification of markers in insect (Garner and Slavicek 1996), barley (Poulsen et al. 1995), canine (Gu et al. 1997a), and sheep (Cushwa et al. 1996) genomes. The real advantages of using RAPD techniques in any "genome poor" species are (1) the set of RAPD primers may be purchased commercially and can be used in any organism, (2) genome screening may be faster since RAPD, unlike microsatellite markers, simultaneously screens several loci in the genome. As our interests are in canine genetics, it is important to note that RAPD has been used to identify a marker linked to canine progressive rod-cone degeneration (Gu et al. 1997b). Reported here are some interesting polymorphisms revealed by RAPD analysis of the highly inbred Boykin spaniel, although it is not clear at present whether any of these polymorphisms are related to the CHD phenotype.

Materials and Methods

Dogs and Genomic DNA

The Boykin spaniel was used as a model for study of clinical CHD because this breed has a high incidence of hip dysplasia and high DI. The average DI for this breed is 0.66, which is significantly higher than 0.38, the average of all dogs (more than 12,000) examined using the PennHIP method. The Boykin spaniel has a small breed population, which certainly contributed to its high incidence of CHD. The dogs reported here represented a family of 12 members spanning three generations.

Blood was collected from dogs into buffered sodium citrate tubes. Genomic DNA was isolated using the Puregene DNA Isolation Kit (Gentra Systems Inc., NC).

RAPD-PCR

Random decanucleotide primers were obtained from Genosys (TX) and Operon Technologies (CA). The GC content of these primers varies from 50 to 70%. Other PCR reagents were from Perkin-Elmer (CT). PCRs were carried out in a total volume of 25 µl and contained the following: 100 ng of genomic DNA, 1 mM MgCl₂, 1× PCR buffer, 0.1 mM of each dNTP, 0.5 units of AmpliTaq DNA polymerase, and 0.4 µM of random primer. The reaction mixtures were incubated in a PCR thermal cycler (Perkin-Elmer 2400) using the following parameters: a holding step at 94°C for 3 min; 3 cycles at 94°C for 2 min, 33°C for 1 min, and 72°C for 2 min; and 42 cycles at 94°C for 1 min, 33°C for 1 min, and 72°C for 2 min. After cycling, the elongation reaction was extended 7 min at 72°C.

After PCR, 5 µl of the reaction mixtures along with 2 µl of loading dye were loaded on a 1.5% agarose gel prestained with ethidium bromide (0.5 µg/ml). Electrophoresis proceeded at 60 V for 2.5 h in 1× TAE buffer. Sometimes restriction digestion was carried out on nonpolymorphic amplification products. Briefly, 5 µl of the PCR product was digested with 1 unit of restriction enzyme (HaeIII, HinfI, Alul, Sau3AI, MspI, or Msel) in a total volume of 10 µl at 37°C for 1–2 h. The digestion products were then resolved by electrophoresis.

Results

In the Boykin spaniel family tested (Figure 1), the parent dog 11 has loose hips (DIs of 0.48 and 0.60) and dog 12 has tight hips (DI = 0.32 and 0.37). All offspring of these dogs have loose hips, with DIs ranging from 0.50 to 0.71, but at least one hip presents with a DI greater than 0.60. When one of the offspring was bred to a tightly hipped dog, dog 5 (DI = 0.24 and 0.24), the resultant offspring had loose hips (DI = 0.65 and 0.75). Therefore it seems that the high distraction index was found in all dogs related to dog 11. Most offspring of dog 11 have been clinically confirmed to have CHD.

A total of 200 primers have been used to amplify genomic DNA from selected Boykin spaniels. About 95% of these primers amplified nonpolymorphic DNA fragments. The lack of polymorphisms within the family is a result of the breed’s high level of inbreeding, as suggested by the breed’s narrow national distribution (mainly in South Carolina).

The failure of the primers to generate a significant number of polymorphisms led to incorporation of the restriction fragment length polymorphism (RFLP) technique. RFLPs arise from sequence differences at restriction sites. While a powerful and widely used method, the need for large amounts of DNA for analysis has limited its use in DNA fingerprinting. However, the amount of DNA generated from PCR amplification is sufficient for RFLP analysis. Thus it was theorized that restriction digestion of amplification products would reveal polymorphisms within the study family. Among 39 restriction digestion experiments performed on nonpolymorphic PCR products, 25 experiments showed restriction on these products, but only 6 revealed internal polymorphisms. Hence the chance for restriction digestion to reveal internal polymorphisms was about 15% (6/39), much higher than the RAPD tech-
nique alone (5%). For example, primer r37 (GAGTCACTCG) amplified a single identical DNA fragment from all 12 dogs. Digestion with HaeIII revealed that there exist internal polymorphisms in the individual fragments (Figure 2A). The observed polymorphisms follow the Mendelian pattern of inheritance. Sequencing results confirmed the internal polymorphisms, for example, dogs 1 and 2 have different numbers of HaeIII restriction sites (Figure 2B).

While certainly the majority of random primers are not informative, about 5% of random primers have revealed polymorphisms within this Boykin spaniel family. For example, amplification using primer r55 (CGCATTCGCG) yielded a 1,600 bp band in dogs 6-10 and 12, but not in the other dogs (data not shown). Apparently the DNA region corresponding to this band is only present in dog 12 and its offspring. Interestingly polymorphisms were observed with primer OPW9 (GTGACCGAGT). An extra band (800 bp) was observed with primer OPW9 (GTGACCGAGT). Polymorphism is indicated by the presence/absence of specific fragments, polymorphisms are also evident as differences in band intensity of similarly sized fragments. As shown in Figure 4, primer r105 (GACCGAAGAC) generated bands of about 1,000 bp in all family members, but two distinct intensities existed, suggesting that bands of different intensities are unique. These obvious amplification differences allowed segregation of family members into two groups. One group includes dogs 2, 3, 6-9, and 11. The high-intensity band was present in amplification products of these dogs. All dogs except dogs 3 and 6 in this group were confirmed to have hip dysplasia. In this group, a band of identical size but of lower intensity was present. Dogs 4 and 10 in this group were diagnosed with hip dysplasia. Overall the correctness of this primer in predicting the presence and absence of CHD in this family is 75% (9/12). Similarly the correctness of this primer in predicting high joint laxity (DI ≥ 0.6) in this family is 75% (9/12). The average DI for the first and second groups are 0.60 and 0.44, respectively. The DNA fragments in these bands were sequenced. The sequence of the bands of dogs 11 and 12 exhibit very low similarity (data not shown). This indicates that although the bands of dogs 11 and 12 are the same size, they represent different regions of the genome or radically different sequences from the same loci. It must be noted that this analysis is preliminary and that further testing of this primer on additional Boykin spaniels and other breeds is needed to find out whether this 1,000 bp fragment has potential as a marker for elevated joint laxity.

Discussion

CHD occurs in all breeds, with drastic differences in prevalence varying from 3 to 70% among breeds. The working assumption is that the same genes are responsible for CHD in all breeds. The Boykin spaniel was chosen as a model for studying molecular genetics of CHD based on two considerations. First, for genetic study of any heritable disease it is imperative that a small population with well-defined pedigrees and a higher than normal incidence of the disease be used. For example, in small or isolated populations, consanguineous mating increases the incidences of recessive diseases. In certain of these small populations, the founder effect is a primary factor responsible for abnormally high incidences of various genetic diseases. For example, the high incidence of Ellis–van Creveld syndrome (a form of dwarfism) (Brueton et al. 1990).
and several other diseases has been described within the Amish community of Lancaster County, Pennsylvania. It follows that efforts to identify the genetic cause(s) of canine hip dysplasia will have a higher probability of success if research focuses on a breed that has (1) a high incidence of CHD and (2) a relatively small population in which breeding practices and events have resulted in an increased frequency of disease alleles as compared with open-breeding populations. The Boykin spaniel meets these criteria as it is among the breeds with the highest incidence of hip dysplasia and/or joint laxity. Of importance, its population is quite small (restricted mostly to South Carolina) and there exist extensive pedigree data. Second, since identification of genetic markers using the RAPD technique is a random search and there are likely copious polymorphisms among individual dogs, we tried to minimize the noise polymorphisms by selecting the highly inbred Boykin spaniel. Specifically, initial molecular analyses were carried out on dogs within one Boykin spaniel family in order to test the possibility that identification of markers in such a pedigree would be enhanced as compared with other pedigrees with greater diversity. Unfortunately this approach can actually work against marker identification since too great a reduction in availability of polymorphic markers may hinder identification of a marker associated with CHD. Within the selected family of 12 Boykin spaniels, 7 dogs were confirmed by veterinary radiographic and physical examinations to suffer from hip dysplasia. This family showed a great degree of genetic homo- geneity since only 5% of arbitrary primers tested in arbitrarily primed-PCR revealed DNA polymorphisms. Among the primers able to reveal polymorphism, OPW9 distinguished male dogs from female dogs with 100% accuracy; r105 identified five of seven dogs that were diagnosed with hip dysplasia. While this latter polymorphism is intriguing, any conclusion regarding its linkage to increased joint laxity would be premature. That being understood, it is important to realize that based on the work of Gu et al. (1997a,b), it is possible that testing of 200 primers may be insufficient to draw definite conclusions regarding the utility of the RAPD approach for identification of markers linked to CHD.

Analysis of dinucleotide microsatellites has also been performed as a tool for identifying markers linked to increased joint laxity but not shown to be useful since there were hardly any polymorphisms detected. However, it is likely that even in such an inbred population many polymorphisms are present and will be more readily found through examination of tetrancleotide microsatellites (Ostrander E, personal communication).

While the high level of genetic homogeneity reduced the number of polymorphisms irrelevant to CHD, and thus facilitated the search progress, it is clear from these results that the utilization of additional DNA fingerprinting techniques in the search for markers for CHD is warranted. These techniques include restriction digestion of RAPD and analyses of amplified fragment length polymorphisms (AFLP). Tests of RFLP on seemingly non-polymorphic DNA fragments generated by RAPD-PCR using primer r37 indicated that RFLP, when combined with RAPD-PCR, can reveal polymorphisms that cannot be revealed by RAPD-PCR alone. AFLP (Vos et al. 1995) is based on the selective amplification of fragments from complete restriction digestion of genomic DNA. In this technique, genomic DNA is first digested by two restriction enzymes followed by selective amplification of the digests using selective primers. The AFLP approach is a variation of the RFLP approach, but requires less genomic DNA and allows specific amplification of a large number (50–100) of restriction fragments, whereas RAPD-PCR generates 3–10 amplified fragments.

Finally, the search for markers of canine hip dysplasia is not restricted to the Boykin spaniel, but has been extended to other breeds such as the golden retriever, the Labrador retriever, and the samoyed. Furthermore, humans have been incorporated into this work because developmental dislocation of the hip (DDH), a disease that has a very similar pathology to canine hip dysplasia, exists in humans. The incidence of DDH is 1–1.5 per 1000 live births (Kim and Weinstein 1998) and 75% of all cases of secondary osteoarthritis of the hip result from uncorrected DDH (Michae-li et al. 1997). The rationale for studying DDH is that more is known about the human genome with respect to chromosome markers and specific genes. Therefore it may be advantageous to try to identify markers for DDH and use these to probe for their canine homologues.

Should polymorphisms tightly linked to increased joint laxity be identified, fragments containing these will be cloned and sequenced. A pair of primers of more than 20 bases each could be designed so that they selectively amplify the DNA region corresponding to the polymorphic fragment. Diagnostic tests can then be developed using this information. In addition, work can be directed toward identification of the genes responsible for the disease.

References
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