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The incidence of mini- and micro-satellite repetitive DNA in the canine genome

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Abstract We have estimated the incidence of micro- and mini-satellites in the dog genome. A genomic phage library from canine liver, with an average insert size of 16 kb, was screened to detect potentially polymorphic micro- and mini-satellite sequences, which may be useful for the development of markers of inherited diseases, for fingerprinting, or for population genetics. Synthetic oligonucleotide probes were used to search for microsatellite sequences, and minisatellites were investigated with eight heterologous VNTR probes. $(CA)_n.(GT)_n$ sequences were by far the most frequent, with a calculated average distance between consecutive loci of 42 kb. The average distance between loci of tri- or tetra-nucleotide repeats was about 330 kb. Mean inter-locus distances were 320 kb for $(GGC)_n$, 205 kb for $(GTG)_n$, 563 kb for $(AGG)_n$, 320 kb for $(TCG)_n$, 233 kb for $(TTA)_n$, 384 kb for $(CCTA)_n$, 368 kb for $(CTGT)_n$, 122 kb for $(TTCC)_n$, 565 kb for $(TCTA)_n$, and 229 kb for $(TAGG)_n$. Cross-hybridization with eight human minisatellite probes was found at average distances of 1 400 kb; only one did not hybridize at all. We conclude that the di-, tri and tetra-nucleotide short tandem repeats, as well as some minisatellite sequences, are potentially useful as genetic markers, for mapping of the canine genome, and also for paternity testing and the analysis of population characteristics.

Key words Dog · short tandem repeats
Minisatellites · Genetic markers

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Introduction

The genetic variability of purebred dogs has been progressively reduced over the past decades. Successive generations in each breed have been derived from the few animals that have most fully met the required external standards at exhibitions. Cross-breeding between individuals of different breeds is prohibited so that dog breeds are strictly closed populations. As a result, most breeds have accumulated one or more breed-related diseases (for a review see Willis 1989). The incidence of most of these inherited diseases is not known exactly, but in a few systematically-investigated populations 2–50% of the animals were phenotypically affected (Rothuizen, unpublished). Apart from the so-far-neglected impact on veterinary medicine in the future, dogs may be expected to harbour many diseases that are interesting models for comparative medicine or for the elucidation of gene functions, as exemplified by several recent publications (Cooper et al. 1988; Fyfe et al. 1991; Giger et al. 1992; Sharp et al. 1992).

The vast majority of inherited canine diseases have not been characterized biochemically and hence the approach of positional cloning for the detection, mapping, and analysis at the level of DNA, of diseases of interest is required. Moreover, DNA techniques may permit an analysis of the genetic variability of (sub)populations and the identification of heterozygous carriers. All of these applications demand that highly variable sequences can be detected at the multi-locus and single-locus level, for which purpose tandemly-repeated polymorphic structures seem very suitable. The classical variable number of tandem repeats (VNTRs) are useful for multilocus fingerprinting using heterologous probes (Jeffreys and Morton 1987; Vassart et al. 1987; Georges et al. 1988; Schelling et al. 1991). Sequence elucidation of highly-variable VNTR loci could make them applicable as single-locus genetic disease markers, although due to their length they may be slightly less appropriate for PCR amplification (Jeffreys et al. 1988), and they are probably not evenly distributed over the chromosomes (Royle et al. 1988). The more-recently-described short tandem repeat (STR) sequences (Schäfer et al. 1988; Edwards et al. 1991; Stallings et al. 1991;

Turner et al. 1991, 1992; Ellegren et al. 1992; Hundrieser et al. 1992) may also be used in PCR-based linkage analysis, for fingerprinting, and for the mapping of genetic traits. Of these, the $(CA)_n.(TG)_n$ microsatellites occur very frequently and are evenly distributed in mammalian genomes (Weber 1990; Stallings et al. 1991), which makes them suitable for high-density genome mapping (Fain 1991; Hazan et al. 1992; Wilkie et al. 1992). Polymorphic tri- and tetra-nucleotide repeats are potentially equally suitable; however, in the human genome they seem to be less abundant than $(CA)_n$ repeats (see Table 8 in Human gene mapping 1991), and their frequency in genomes of other species is not well known.

The incidence within a genome of minisatellites and STRs of sufficient total length determines whether they are suitable candidate genetic markers, and useful for DNA fingerprinting and for gene mapping. We have investigated the incidence of minisatellite sequences and of di-, tri-, and tetra-nucleotide microsatellites in a genomic library of canine DNA as a basic tool for further investigations of inherited diseases in dogs.

Materials and methods

Canine genomic library

The library was purchased from Clontech Laboratories (Palo Alto, California, USA). Genomic DNA was isolated from dog liver and partially digested with *Sau*3AI. DNA fragments were separated on a sucrose gradient and fragments of 9–22 kb were used for cloning. DNA was cloned into the *Bam*HI site of phage EMBL-3 Sp6/T7. The average size of the inserts was measured by releasing them by digestion with *Xho*I from 25 randomly-chosen plaques of recombinant bacteriophage selectively grown on NM539. The size of the inserts was then determined by agarose-gel electrophoresis using an appropriate molecular size marker. In order to evaluate whether the inserts of the library were indeed from canine DNA, five plaque lifts were hybridized with labelled total dog DNA, and the fraction of positively-hybridizing plaques was counted. The specificity of the latter hybridization was tested by hybridizing a zoo blot with the probe under identical conditions. This was a Southern blot of genomic DNA digested with *Pst*I and electrophoresed in 1.2% agarose; the species tested were dog, pig, man, cow, cat, elephant, and horse.

Screening of the genomic λ-library for satellite-containing recombinants.

After determining the titre of the library, a dilution in lambda buffer was made to obtain about 600-plaque forming units per 90-mm agar plate. Appropriate aliquots of diluted bacteriophage were incubated at 37 °C with 200 µl of an overnight culture from a single isolated colony of the *E. coli* strain. The cell suspension was then mixed with 3 ml of fluid LB agar containing 0.2% (w/v) maltose and 10 nM $MgSO_4$, and poured onto 90 mm LB agar plates. The plates were incubated overnight at 37 °C, giving clearly-visible, well-isolated plaques. From each plate the plaques were lifted onto two positively-charged nylon membranes (Hybond-N^+, Amersham, Den Bosch, The Netherlands). Membrane-bound DNA was then denaturated with a solution containing 1.5 M NaCl and 0.5 M NaOH, neutralized with a buffer (pH 8.0) of 1.5 M NaCl and 0.5 M tris-HCl, and rinsed in 3 × SSC. The membranes were dried and baked for 2 h at 80 °C.

Hybridization with the probes

Oligonucleotide probes for microsatellite sequences were assembled by Pharmacia LKB Biotechnology, Woerden, The Netherlands. The oligonucleotides were $(CA)_{15}$, $(CCG)_7$, $(AGC)_7$, $(CAC)_7$, $(AAT)_7$, $(TCC)_7$, $(AGAT)_6$, $(ATCC)_6$, $(AAGG)_6$, $(GGAT)_6$, and $(GACA)_6$.

Heterologous minisatellite probe D4S139 was previously described by Milner et al. (1989), probe D4S163 was described by Mills et al. (1992), probe D17S308 was published by Borrow et al. (1991), and D17S74 by Nakamura et al. (1988). Minisatellite probes 33.6 and 33.15 (Jeffreys et al. 1985), and M13 (Vassart et al. 1987) were used, and plasmid pUCJ containing a multimer of Jeffreys' core sequence (Georges et al. 1988) was obtained from Prof. Vassart (Brussels). The non-recombinant λ vector was labelled for control hybridization of all plaques.

The probes were labelled with digoxigenin for chemiluminescence detection (Boehringer, Mannheim, Germany). The oligonucleotides were 3′-end-labelled with digoxigenin-labelled ddUTP using terminal transferase, and the minisatellite probes, the λ vector and total genomic dog DNA were randomly labelled with digoxigenin-dUTP using Klenow polymerase.

Hybridization of the membranes was carried out at 40 °C for $(AAT)_7$, at 50 °C for $(AGAT)_6$, at 54 °C for $(CA)_{15}$, at 62 °C for $(AGC)_7$, $(CAC)_7$, $(TCC)_7$, $(ATCC)_6$, $(AAGG)_6$, $(GGAT)_6$, and $(GACA)_6$, and at 75 °C for $(CCG)_7$. The membranes were prehybridized for 1.5 h at 68 °C and hybridized for 5 h. The hybridization solution for STR probes consisted of 5 × SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, and 1% of the manufacturer's blocking reagent. Membranes were then washed at hybridization temperature; twice for 5 min in 2 × SSC, 0.1% (w/v) SDS, followed by two-fold washing for 5 min in 0.1 × SSC, 0.1% SDS. Hybridization with the minisatellite probes and with the vector was performed at 42 °C for 6 h after prehybridization for 1.5 h at the hybridization temperature. The hybridization buffer consisted of 5 × SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, 50% (v/v) formamide, and 1% (w/v) of the manufacturer's blocking reagent. The membranes were then washed in the same solutions as those for STR hybridization, but the second wash was performed at 68 °C, twice for 15 min. Hybridization of the zoo blot and plaque lifts with labelled total dog DNA was carried out as for the minisatellite probes. Detection of the hybridized probes was by chemiluminescence (DIG luminescent detection kit, Boehringer, Mannheim, Germany). The membranes were exposed to an X-ray film for 2–3 h, giving a clearly visible signal with low background. Some of the membranes were rehybridized with another probe after stripping off the hybridized probe with 0.2 nM NaOH, 0.1% (w/v) SDS, and rinsing of the membrane in 2 × SSC. The detection limit of hybridization with the digoxigenin-labelled probes was measured with dot blots of dilutions from 10^{-1}–10^{-4} of each probe, in comparison with three standards provided by the manufacturer.

For each probe, at least four membranes were hybridized to allow evaluation of about 2 500 plaques for the presence of the satellite of interest. The total number of plaques lifted onto each filter was determined, as was the number of hybridizations for each probe. Using the average size of the inserts in the genomic library and the fraction of hybridizing plaques, the average incidence of the satellite sequences in the canine genome was calculated.

Results

The detection limit for all of the probes was in the range of 5–10 fmol of DNA. Measurement of the size of the inserts from 25 plaques revealed an average length of 16 kb. The insert sizes were evenly distributed over a range of 10–21 kb. Hybridization of the library with labelled total dog DNA gave positive signals in 96% of the plaques. The genomic DNA probe hybridized only to canine DNA in the zoo blot. The non-recombinant λ vector hybridized to 99% of the plaques.

Positive hybridizations were recorded for all of the microsatellite sequences and for seven of the eight heterolo-

Table 1 Comparative frequencies of minisatellite and di-, tri-, and tetra-nucleotide sequences in the canine genome. Minisatellite probe D17S74 did not cross-hybridize to the genomic DNA of dogs

Probe	Average distance between loci (kb)
$(CA)_{15}$	42
$(CCG)_7$	320
$(AGC)_7$	320
$(CAC)_7$	205
$(AAT)_7$	233
$(TCC)_7$	563
$(AGAT)_6$	565
$(ATCC)_6$	229
$(AAGG)_6$	122
$(GGAT)_6$	384
$(GACA)_6$	368
D4S139	420
D4S163	734
D17S74	–
D17S308	2400
33.15	1645
33.61	2130
pUCJ	1620
M13	845

gous minisatellite probes. The frequencies of the investigated sequences in the canine genome were variable. In general, sequences were more common when the basal unit was shorter. The fraction of recombinants hybridizing with $(CA)_{15}$ by far exceeded that of the other repeats and corresponded to an incidence of once every 42 kb. Tri- and tetra-nucleotide microsatellites generally had similar distances between different loci (330 and 340 kb, respectively). Among the latter, elements hybridizing with $(AAGG)_6$ were by far the most common, with an average distance between loci of 122 kb, thus more or less approaching that between the dinucleotide repeats. The average distances between consecutive loci of the short tandem repeats were 320 kb for $(GGC)_n$, 205 kb for $(GTG)_n$, 563 kb for $(AGG)_n$, 320 kb for $(TCG)_n$, 233 kb for $(TTA)_n$, 384 kb for $(CCTA)_n$, 368 kb for $(CTGT)_n$, 122 kb for $(TTCC)_n$, 565 kb for $(TCTA)_n$, and 229 kb for $(TAGG)_n$.

Minisatellite sequences were detected with seven of the heterologous probes; only probe D17S74 did not hybridize to canine genomic DNA. Minisatellite sequences were less common than the short tandem repeats, with a mean incidence of one locus for every 1400 kb. However, the frequencies of different minisatellite sequences varied greatly, some being almost as common as short tandem repeats. Comparative figures for individual minisatellite and short tandem repeat sequences in the canine genome are listed in Table 1.

Discussion

These findings indicate that short tandem repeat sequences occur quite frequently in the canine genome. Dinucleotide $(CA)_n$ microsatellites were most abundant; in fact the incidence of one dinucleotide repeat per 42000 bases may well be an underestimation, since more than one $(CA)_n$ locus may have been present in one recombinant phage. By comparison, the average distance between these loci in rats and mice is about twice as short (20000 and 18000 bases, respectively) (Stallings et al. 1991). The frequency of tri- and tetra-nucleotide STRs and of the minisatellite loci reported here is more accurate because inclusion of several of these less-abundant structures in one 16-kb recombinant is very unlikely. It is to be noted that these figures are calculated averages assuming an even distribution of the investigated sequences over the genome. This assumption is likely to be valid for CA repeats in comparison with other mammalian genomes (Stallings et al. 1991) but it remains to be verified for the other STRs and it does not hold for minisatellites. The calculated frequencies depend largely on the quality of the library. Labelled genomic dog DNA, which was shown to hybridize specifically to dog DNA in the zoo blot, identified 96% of the recombinants as carrying dog DNA. Assuming that there was no hybridization in some recombinants containing dog DNA without repetitive sequences it may be concluded that the library was of high quality and suitable for the pupose of this study.

We have used a number of VNTR probes which have proved to be useful in studies of human DNA . Of these, sequences related to Jeffreys classical probes, i.e., 33.6, 33.15, and PucJ, as well as phage M13, were all recovered in our genomic library, in agreement with earlier reports (Jeffreys and Morton 1987; Vassart et al. 1987; Georges et al. 1988; Schelling et al. 1991). In addition, probes D4S139, D4S163 and D17S308 hybridized to sequences in the canine genome. All of these probes are therefore potentially useful for DNA fingerprinting in dogs. The minisatellite sequences that hybridized to a heterologous probe were relatively infrequent in dogs, as in other species (Armour et al. 1990). Although minisatellites are suitable for personal identification of dogs (Jeffreys and Morton 1987; Georges et al. 1988), their low incidence and asymmetric distribution in the genome (Royle et al. 1988) make them less suitable for high-density mapping or as genetic markers.

Regarding incidence, distribution in the genome, and applicability in the PCR, short tandem repeats are superior to minisatellites. $(CA)_n.(GT)_n$ repeats have proved applicable in humans (Weber, 1990; Fain et al. 1991; Hazan et al. 1992; Wilkie et al. 1992), sheep (Buchanan et al. 1991), cows (Brezinsky et al. 1992; Vaiman et al. 1992), horses (Ellegren et al. 1992), and swine (Winterø et al. 1992) genomes. Very recently, Ostrander et al. (1993) have demonstrated them to be equally applicable to dogs. It is of interest to compare the frequency of CA repeats with that of more complex STRs. Like dinucleotide repeats, tri- and tetra-nucleotide repeats seem to occur quite frequently in mammalian genomes (Epplen et al. 1991; Edwards et al. 1992; Wilkie et al. 1992); in human DNA once every 300–500 kb (Edwards et al. 1991). Hence, their here-reported frequency in the canine genome is well comparable with that in humans, albeit slightly higher. This implies that the potentially highly-polymorphic tri- and tetra-meric repeats with basic units comparable to those in the human genome (Human gene mapping 1991 Edwards et al. 1992; Hundrieser et al. 1992; Zischler et al. 1991) have an inci-

dence in the DNA of dogs that makes them very suitable candidate DNA markers. A considerable advantage over dinucleotide sequences is that length polymorphisms due to a variable number of the larger basal units will be much easier to detect in routine analysis. In addition, these short tandem repeats, as well as the minisatellites, may serve to estimate the average degree of kinship and the family structure (Capy and Brookfield 1991) of dog populations.

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