ESTABLISHMENT OF AN INBREEDING INDEX IN HOLSTEIN DAIRY CATTLE USING DNA FINGERPRINTING

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In memory of my dearest grandparents :

Baoyan Ding and Yulin Sun Ding

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Establishment of an Inbreeding Index in Holstein Dairy Cattle Using DNA Fingerprinting

Abstract

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In order to establish a method of assessing the degree of inbreeding within herds of cattle, we constructed a calibration index relating kinship and the degree of DNA band sharing in DNA fingerprints. Firstly, chickens were used as a model system to test the possibility of using microsatellite DNA as a probe for DNA fingerprinting in inbreeding analysis. Six genetic groups of chickens with estimated coefficients of inbreeding ranging from 0.026 to > 0.98 (pedigree analysis) were fingerprinted using the minisatellite probe derived from M13 and the microsatellite probe $(CAC)_5$. The degree of band sharing using either probe increased in concert with the known amount of inbreeding and was described by the equation $Y = 0.56X\ (\pm\ 0.06) + 0.42\ (\pm\ 0.03)$; $\underline{r} = 0.998$. Since in-gel hybridization using the microsatellite probes was faster and less labour intensive than using the minisatellite probe, it was used in the subsequent studies. Pedigree analysis in Holstein dairy cattle allowed for the empirical calibration of the association of band sharing with the coefficient of relatedness, (r), defined as the expected

proportion of genes in 2 individuals that are identical by descent (i.e. for monozygous twins r = 1; for first order relatives r = 0.5; for half sibs r = 0.25 etc.). The average band sharing between pairs (6 pairs at each r value) of individuals within each class formed the basis for calibration. DNA was digested using RsaI. The relationship between band sharing and relatedness was well represented by a linear approximation $Y = 0.51X (\pm 0.09) + 0.50 (\pm 0.04)$; $\underline{r} = 0.992$. Using this calibration curve, random samples of animals within herds can be tested to establish the herd variability and to minimize inbreeding.

Création d'un Index de consanguinité de bovins Holstein à l'aide d'empreintes d'ADN

Résumé

Afin d'établir une méthode de détection du degré de consanguinité à l'intérieur de troupeaux bovins, un index de calibration a été construit reliant parenté et degré de bandes partagées, à même l'empreinte d'ADN. Dans un premier temps, le poulet a servi de modèle afin de vérifier la possibilité d'utiliser un microsatellite (ADN) comme sonde d'empreinte d'ADN dans l'analyse de consanguinité. Les empreintes de six groupes génétiques de poulets ayant un coefficient de consanguinité estimé entre 0,026 et 70,98 (analyse de pedigree) ont été prises à l'aide d'une sonde minisatellite obtenue à partir de M13 et de la sonde microsatellite $(CAC)_5$. Quelque soit la sonde utilisée, le degré de bandes partagées a augmenté de concert avec la quantité connue de consanguinité et peut être décrit par l'équation suivante; $Y = 0{,}56X\ (\pm\ 0{,}06) + 0{,}42\ (\pm\ 0{,}03)$; $\underline{r} = 0.998$. Etant donné que l'hybridation en présence de gel à l'aide de sondes microsatellites s'est effectuée plus rapidement et moins laborieusement qu'avec la sonde minisatellite, c'est cette première qui a servi par la suite. L'analyse de pedigree de bovins Holstein a permis la calibration empirique entre l'association du degré de bandes partagées et le coefficient de parenté (r). Ce dernier est

défini comme étant la proportion attendue de gènes identiques par descendance entre 2 individus (i.e. jumeaux monozygotes r = 1; parents de première génération r = 0.5; demi-frères (sœurs) r = 0.25, etc.). La moyenne des bandes partagées entre chaque paire (6 paires pour chaque valeur r) d'individus à l'intérieur de chaque classe a servi de base pour la calibration. L'ADN a été digérée à l'aide de RsaI. La relation entre le degré de bandes partagées et le coefficient de parenté fut bien représenté par l'approximation linéaire suivante: $Y = 0.51X (\pm 0.09) + 0.50 (\pm 0.04)$; $r = 0.992$. Ainsi, à l'aide de cette courbe de calibration, des échantillons d'animaux pris au hasard à l'intérieur de mêmes troupeaux peuvent être mis à l'épreuve pour établir la variabilité du troupeau et pour minimiser la consanguinité.

用DNA指纹图对Holstein乳牛近交相关指数的构建

文摘
Wen-Zhai

为了寻找一种在乳牛群体之间或内部近交水平的计算方法，我们构建了一个个体间的亲缘关系与DNA指纹图的条纹共有率之间的相关指数。首先，用鸡作为模型来测验使用微卫星DNA作探针并依个体的DNA指纹图来分析亲缘关系的可能性。六组遗传关系分别为0.026至0.98以上（依亲谱分析），和同种系的鸡的指纹图谱被进行分析。使用分离自噬菌体M13的小卫星DNA作为和微卫星DNA：$(CAC)_5$分别作为探针。在这两种情况下，系统发育和个体间的亲缘关系都呈正相关，并可以表达为公式：$Y=0.56X(\pm 0.02)+0.42(\pm 0.05)$；$r=0.95$。此时使用微卫星DNA作为探针直接在凝胶中杂交的使用小卫星DNA作探针更快速和节省劳力。微卫星DNA作为随后的实验中作为探针使用。

亲缘系数（r）是指两个个体之间具有来自共同祖先的基因的比例程度（比如，在同卵双胞胎中，r=1；在兄弟姐妹之间，r=0.5；在表兄弟姐妹之间，r=0.25；等）。依据对Holstein乳牛家谱及其DNA指纹图的分析，以由具有相同亲缘系数的个体对个体为一组中的平均条纹共有率作为相关计算的基础，从而建立了条纹共有率和亲缘系数的相关指数。此相关指数可以很好地用直线相关公式：

$y=0.51x(\pm0.09)+0.50(\pm0.04)$；$r=0.992$ 所表示。使用这个相关曲线，可以从乳牛群体中随机抽取个体，对群体的遗传结构进行检测，以便控制乳牛群体中近交指数的水平。

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1. Introduction

At present, milk and its products are consumed by people in many parts of the world. Cow's milk contains about 86.9% water, 4% fat, 3.5% protein, 4.9% lactose, as well as about 0.7% inorganic components (Russoff, 1955), and is considered to be of high nutritional value due to its high protein content and also as an important source of calcium and phosphorus in the food supply. The latter are needed in the diet for building bones and maintaining bone strength. Milk is a good source of many required vitamins as well.

Under natural conditions, wild mammals produce only enough milk for their offspring. However, since domesticated animals (e.g. cows) were selected particularly for milk production traits, the yield of milk per animal has dramatically increased. In 1920, the average milk yield per cow in the United States was 1,421 kg per year, whereas 40 years later, in 1960, it had almost doubled, and was 2,391 kg per year. During the next 20 years, however, the milk yield per cow in the United States was doubled again, to 5,350 kg per year (Ensminger, 1983). This increase in milk yield from dairy cows has been achieved through a combination of improvements in the genetics of the animals as well as improvements in the environment in which the animals are maintained. During the past twenty years in Canada, improvement in the environment of the animals (Holstein dairy cattle) such as advances in nutrition, prevention of diseases and milking practices are estimated to be responsible for about 46% of the improvement in milk yield. Animal selection accounts for about 54% of the

improvement in milk production (Gavora, 1989).

The main improvements in selection have arisen as a result of the synergistic actions of milk recording programmes, artificial insemination programmes, as well as sire and cow evaluation programmes. In the future, it is likely that genetic engineering, in vitro maturation and fertilization may further contribute to the improvement of dairy cattle.

Pedigree analysis shows that 704 of 1203 Holstein bulls used as sires in the artificial insemination (AI) industry are inbred (Su, 1990). Inbreeding and inter se relationship of bulls born in 1981-1982 are 0.92% and 1.44%, and that of those born in 1987-1988, 1.58% and 3.42%, respectively (Su, 1990). Both inbreeding and inter se relationship show a tendency of increasing with time, reflecting inter-mating within the same families. To avoid inbreeding, accurate pedigree information is of paramount importance. However, records are not always available.

During the past twenty years, protein markers based on electrophoretic separation of protein isoforms such as blood groups (Cawley, 1969) have been used for the study of genetic relationships, population differentiation and evolution. However, this technique is not always sensitive enough since the number of known protein variants is too small to determine the degree of relationship between animals.

The search for more sensitive techniques for detecting genetic relationships has continued and technical advances in molecular biology have resulted in new methods. Currently, the analysis of DNA is considered to be much more powerful than the detection of protein markers in assessing genetic variation. In particular, DNA

fingerprinting, a technique based on the simultaneous detection of Variable Number of Tandem Repeats (VNTRs), is a powerful tool for the analysis of genetic variation .

In 1985, Jeffreys et al. noted that there are many regions dispersed throughout the human genome which consist of short tandemly repeated DNA sequences, termed minisatellite loci. The number of the repeat units at locus is highly variable from individual to individual, and gives rise to a substantial degree of polymorphisms. These polymorphisms are detected by the hybridizing DNA which has been cut by a restriction enzyme, with the repeat unit as a probe. The resultant complex pattern of DNA banding is called a DNA fingerprint.

DNA fingerprints are completely individual specific (Jeffreys et al., 1985c). Comparison of the band pattern of offspring with their parents revealed that the bands are inherited in a Mendelian fashion. DNA fingerprinting has provided a powerful tool for detecting genetic variations in humans and animals. It has been used for paternity analysis (Jeffreys et al., 1985a; Quinn et al., 1987), linkage analysis (Jeffreys et al., 1986; Nakamura et al., 1987), gene introgression in breeding programs (Hillel et al., 1990), population genetic studies in birds (Wetton et al., 1987; Kuhnlein et al., 1989, 1990) and forensic studies (Gill et al., 1985; Wong et al., 1987; Neufeld and Colman, 1990).

The assessment of inbreeding, as a further application of DNA fingerprinting in population genetics, was recently reported (Kuhnlein et al., 1990). In the latter study, lines of chickens were used to establish a calibration curve relating the inbreeding coefficient to band variability and band sharing.

A new class of polymorphic genetic loci, termed microsatellite, have recently been

utilized for DNA fingerprinting (Ali et al., 1986; Nurnberg et al., 1989). The repeat sequences of these loci are much simpler than those of minisatellite loci and consist of di-, tri-, or tetra-nucleotide repeats, such as $(AT)_n$, $(CAC)_n$ and $(GATA)_n$. They have proved to be advantageous for routine applications of DNA fingerprinting (Nurnberg et al., 1989).

The objectives of the research presented here are:

1. To compare the use of microsatellite and minisatellite loci in the analysis of inbreeding in chickens.

2. To investigate the possibility of using microsatellite loci for DNA fingerprinting in dairy cattle.

3. To establish an inbreeding index in Holstein dairy cattle using DNA fingerprinting at microsatellite loci.

2. Literature Review

2.1 History of Domesticated Animals

Domestication of animals and plants forced the early nomadic tribal life to gradually evolve to a more stationary culture (Legates and Warwick, 1990). In animals, the dog was firstly domesticated by human beings as a hunting companion. Later, goats, sheep, chickens and cattle were domesticated basically to provide sources of meat and milk for food. Nowadays, cows provide the largest proportion of milk which is used by humans.

Cattle were probably domesticated during the New Stone Age in both Europe and Asia (Legates and Warwick, 1990). Bos longifrons cattle were domesticated about 6,000 B.C. probably in the Zagros Mountains in Asia, where cereal farming and village settlements had begun. It is likely that farmers from the beginning of agriculture have been concerned with the maintenance and improvement of their domesticated herds. Animals which were more productive than others were likely to be favoured, hence animal selection and domestication are linked.

2.2 Domesticated Animal Breeding

2.2.1 Overview

As domestication progressed, animals were selected for special purposes. The desire of humans for improved food quantity and quality, or the working ability of the animals began to suggest characteristics which could be propagated and inherited by

deliberate mating of pairs of animals. For example, it is possible that animals that were more efficient at working were kept in closer proximity to each other than other animals and were therefore more likely to mate. Thus, selection for human requirements was gradually imposed upon natural selection for reproductive fitness and led to a modification of the genotype of the animal. Modern animal breeding is a systematic science that incorporates genetics, reproductive physiology, statistics, computer science and animal husbandry in a highly interactive fashion to maximize genetic improvement.

Robert Bakewell was among the first prominent improvers of cattle, sheep and horses in the British Isles. His four main guidelines for animal breeding were (Ensminger, 1983):

1. Establish goals for a program of selection and breeding with respect to the type of animals which will be bred, e.g. either dairy type, beef type, or dual-purpose type in cows.

2. Carefully preserve the best stock animals which are available for the purpose of reproducing the herd.

3. Inbreed, so as to accumulate the desirable traits into the following generations.

4. Eliminate the individuals which do not have the desirable traits from the breeding herd.

Many prominent breeders since have followed Bakewell's practices of endeavouring to breed the best to the best. Most of his methods are still applied to large extent today.

2.2.2 Types of Animal Breeding

Inbreeding and outbreeding are two basic methods employed in animal breeding. Inbreeding is the mating of individuals which are related. It leads to the establishment of uniform families in a population, since members of the same family are more likely to inherit the same genes and gene combinations because their parents were related.

Outbreeding which refers to the mating of unrelated individuals is the very opposite to inbreeding. It is the standard method of increasing both phenotypic and genetic variation in a population (Legates and Warwick, 1990). Heterozygosity of the population is generally increased by outbreeding and as a result, general fitness and adaptation of the animal to its environment are usually observed.

2.2.3 Inbreeding and Artificial Insemination (AI)

In the breeding of domestic animals, consanguineous matings are frequently made (Wright, 1917). Bakewell gained prominence by breeding "the best to the best" regardless of the degree of relationship between mating pairs in order to propagate and improve the phenotypic characteristics of the offspring.

The main value of inbreeding is to concentrate genes in the population which are associated with superior phenotypic characteristics. At the same time however, the increased level of genetic homozygosity associated with the mating of animals with common ancestors may result in "inbreeding depression". "Inbreeding depression" may be the result of the "unmasking" of deleterious alleles which may lower the performance of observed phenotypic traits, such as fertility, survival or size of the offspring (Dalton,

1985). Inbreeding also imposes selection limits since the degree of genetic variation present in the population decreases from generation to generation.

Artificial insemination (AI) offers tremendous possibilities for breed improvement if it is properly managed and safeguarded. The major advantage of AI is that the mating pairs can be controlled and that semen can be easily transported over wide distances. At the same time, this method potentially offers great risk if careless methods are used. For example, properly safeguarded, AI may prevent the spread of disease which might occur as a result of transportation of the sire from herd to herd, but carelessly handled it may spread disease in every herd in the mating group. It is also essential to avoid the undesirable consequences of inbreeding which might ensue from the use of too few male parents in the breeding strategy. This is already a problem in some of the smaller breeds of cattle in which one male can provide all the semen necessary to reproduce the population of each generation (Bowman, 1976). It is therefore important to monitor the degree of inbreeding in domestic animals, especially in dairy cattle and swine, where artificial insemination is practised and a single male can give rise to thousands of offspring.

2.2.4 Inbreeding Coefficient and Coefficient of Relationship

In 1917, Wright first described a method of calculating the coefficient of inbreeding and the coefficient of relationship.

Inbreeding coefficient: The formula for calculating the inbreeding coefficient of an individual X is (Legates and Warwick, 1990):

$$F_x = \sum(1/2)^{n+1}(1 + F_A)$$

Where n is the number of segregations in a specific path between the parents of X, and F_A represents the probability that the parents of X are identical homozygotes through a previous generation.

Coefficient of relationship: The formula for calculating the coefficient of relationship of individuals X and Y is (Legates and Warwick, 1990):

$$R_{xy} = \frac{\sum(1/2)^n(1 + F_A)}{((1 + F_x)(1 + F_y))^{1/2}}$$

where n is the number of segregations in a specific path between individuals X and Y. F_A, F_x and F_y represent the inbreeding coefficients of common ancestor A of X and Y, respectively.

Comparison of the coefficients of inbreeding and relationship: Mating of related individuals produces an inbred offspring. The rate of increase in homozygosity with inbreeding is dependent on the closeness of the relationship of the individuals which are mated. The inbreeding coefficient is one half the numerator of the relationship coefficient for the sire and dam of an individual, or one half the relationship coefficient of the sire and dam when the two related individuals are not inbred. Therefore, knowledge of relationship can be helpful in selecting animals or in making mating choices to avoid high levels of inbreeding.

Originally, calculations of these coefficients were very time-consuming, but recent developments in computer technology have greatly simplified the process. However, accurate pedigree records are still required and these are sometimes not available.

2.3 Detection of Genetic Variations

2.3.1 Overview

Proteins are composed of various amino acids some of which have different electrical charges. Electrophoresis, (i.e. the migration under the influence of an applied electric field), has been the main tool in the detection of protein polymorphisms.

Isoenzymes, which have similar substrate-specific enzymic activity but differ in some of their physical/chemical properties have been shown to have many applications in disease diagnosis and clinical chemistry (Wilkinson, 1965). Enzymatic activities can be separated by electrophoresis and their location detected by substrate specific reactions in the gel. For example, 5 major isoenzymes of lactate dehydrogenase (LDH) were detected by electrophoresis and abbreviated as LDH1, LDH2, LDH3, LDH4 and LDH5 (Markert and Moller, 1959). They were different in the proportions of subunits H and M in their constitution (Cahn et al., 1962). The relative distribution of LDH isoenzymes remain constant in specific tissues (e.g. serum), but differ from tissue to tissue and also from species to species unless in the presence of disease (Wilkinson, 1965). Detection of polymorphic patterns of serum LDH isoenzymes by electrophoresis provided diagnostic information by comparing the serum samples of healthy and diseased individuals (Cawley, 1969). Therefore, isoenzymes which reflect the genetic makeup of

the species could be used as genetic markers.

However, genetic markers based on isoenzyme polymorphisms are of limited value. Firstly there are only a limited number of isoenzymes in isoenzyme families, which means that there are limited polymorphisms which can be detected. Secondly, amino acid sequence of proteins are only correspondent to a small proportion of genome, therefore a large proportion of genome which is responsible for the regulation of expression of genes and systematic development of plants and animals could not be detected. Thirdly, an important factor which affects the reproducibility of the electrophoresis technique is that protein samples, especially enzymes, are susceptible to physical changes if they have not been handled or stored properly.

Currently, the degree of polymorphism at the DNA level has been found to be considerably higher than that observed in protein markers (Cooper and Schmidtke, 1984).

2.3.2 Genetic Variations at the DNA Level

A key discovery in the evolution of molecular biology techniques was the discovery of restriction enzymes. This class of enzymes was capable of cleaving DNA at very specific sites (cleavage recognition sequence usually 4 - 8 nucleotides). Cleavage of DNA by restriction enzymes followed by electrophoretic separation of the fragments and the hybridization of labelled DNA probes (Southern blotting) led to the discovery of a new class of polymorphisms, restriction fragment length polymorphisms (RFLP) (Botstein et al., 1980).

Two main categories of RFLPs have been described. The first occurs as a result of a change in DNA sequence within the restriction recognition site resulting in the gain or loss of a cutting site. The second type of RFLP results from an insertion or deletion of a DNA segment that changes the size of the fragment produced by a restriction enzyme.

2.3.3 DNA Fingerprinting

2.3.3.1 Satellite DNA and Tandem Repetitive Fragments

In 1957, calf thymus DNA was first analyzed by cesium chloride density gradient ultracentrifugation and shown to be heterogenous in density (Meselson et al., 1957). Later, two bands with buoyant densities of 1.699 g/cm^3 and 1.713 g/cm^3 were detected. This latter band was called the satellite band (Kit, 1961). When this band was further characterized, it was discovered that satellite DNA consisted of repeated fragments (Waring and Britten, 1966), and these repeated elements exist in the genomes of a wide variety of higher organisms. For example, repetitive DNA comprises 13% and 40% of the chicken (Eden and Hendrick, 1978) and the bovine genome (Britten and Kohne, 1968), respectively.

Since satellite DNA was first described by Kit et al. (1961), the term has evolved from describing the minor fraction of nuclear DNA resolved by CsCl equilibrium density gradient centrifugation to more generally referring to the part of the DNA of higher organisms which renatures very rapidly due to the high degree repetition in the genome (Waring and Britten, 1966; Britten and Kohne, 1968). Eventually, the term, satellite DNA, was used to refer to a highly repetitive sequence which was tandemly arranged in

the genome regardless if they could be separated from the principal DNA by gradient ultracentrifugation or not (Pech et al., 1979; Lee and Singer, 1982; Meyerhof et al., 1983).

Studies have shown that these tandem repetitions do not code for proteins, but are thought to be important for the mechanism of gene amplification through an DNA intermediate. They are mainly located in the centromeric heterochromatin region of all chromosomes (i.e. in the genetically inert and compact parts of chromosomes), with the exception of the Y-chromosome (Jones, 1970; Pardue and Gall, 1970).

2.3.3.2 VNTR Probes and DNA Fingerprinting

Recently a new class of tandem repetitive elements have been recognized in a number of human genes including insulin (Bell et al., 1982); alpha - globin gene (Goodbourn et al., 1983), the oncogene c - Ha - ras - 1 (Capon et al., 1983) and myoglobin (Weller et al., 1984). Analysis of the nucleotide sequences in these genes showed that short sequences (16 to 64 bp, Jeffreys et al., 1987b) were repeated in a tandem fashion. For example, the sequence 5'-ACAGGGGTGTGGGG-3', upstream of the human insulin gene, is repeated 26 to 63 times (Bell et al., 1982). Such repetitive sequences have been termed minisatellites, and a large number of such sequences have now been described. A common motif in many minisatellites is 5'-GGGCAGGAXG-3', (Nakamura et al., 1987).

The number of repeat units in individuals is highly variable and the most informative locus described to date (MS1) shows 98% heterozygosity in human (Wong

et al., 1987). It is the extensive variability of repeat units between individuals which is exploited in DNA fingerprints. Jeffreys et al. (1985c) has shown that the probability of any 2 unrelated individuals sharing the same DNA fingerprinting pattern is 3×10^{-11}, thus the variable number of tandem repeat (VNTR) loci provides a rich source of markers for genetic analysis.

To produce DNA fingerprints, genomic DNA is digested by restriction enzymes which cleave outside of repeat units, and the DNA fragments are then separated in agarose gels by electrophoresis. The DNA fragments are in situ transferred to a nylon membrane and then permanently fixed onto a nitrocellulose or nylon membrane. The labelled repeat is then hybridized to the DNA fragments to produce individual-specific DNA banding patterns termed DNA fingerprints (Jeffreys et al., 1985c).

As a further refinement of DNA fingerprinting, Jeffreys et al. (1988, 1990) has shown that minisatellite repeat units at alleles are themselves hypervariable. Using the polymerase chain reaction (PCR) to amplify a single minisatellite allele followed by restriction analysis, Jeffreys showed that alleles of identical length (Southern blot analysis - Southern, 1975) had a considerable number of differences between repeat units (i.e. the absolute level of allelic variability at a minisatellite allele is much greater than detected by Southern blot analysis). For example, for the minisatellite locus MS32 (Wong et al., 1987), greater than 10^{70} allelic states could be discriminated compared to about 300 which could be resolved by Southern blot analysis. This elegant method has allowed the coding of DNA bands by a binary code, thus considerably simplifying interpretation and analysis of genetic relationships.

A third class of repetitive DNA has been described. This class has been called simple sequence or microsatellite DNA on the basis of the size of the repeat unit. Microsatellites typically have repeat motifs of 2 - 4 bases such as AT, CAC or GACA (Epplen, 1988).

The number of repeat units at an allele have a comparable level of variability to minisatellite loci and can also be used to produce complex DNA fingerprinting patterns (Ali et al., 1986; Nurnberg et al., 1989). An additional difference between micro- and minisatellite loci appears to involve the distribution in the genome. Whereas some evidence suggests that minisatellite regions tend to be clustered towards the proterminal regions of chromosomes (Royle et al., 1988), microsatellite loci appear to be relatively evenly distributed throughout the genome (Nurnberg et al., 1989).

2.3.3.3 Origin of VNTR Regions

The mechanism by which the high degree of variability at these loci arises has yet to be resolved. Originally, Jeffreys et al. (1985b) noted that the core sequence of minisatellites contained a high degree of homology to the recombination hotspot, chi, of Escherichia coli. He proposed that a high frequency of crossing-over and unequal exchange at meiosis could explain the high degree of variation of repeat unit lengths. However, Wolff et al. (1989) showed that new alleles can be generated by the loss of a repeat unit without exchange of flanking DNA as predicted from a simple crossing-over event. Jeffreys et al. (1987b, 1990) has also excluded crossing-over mechanisms as the main source of allelic diversity and concluded that gene conversion or DNA polymerase

slippage at replication forks are likely to be responsible.

2.3.4 Practical Applications of DNA Fingerprinting

DNA fingerprinting using either minisatellite or microsatellite probes has proved to be a powerful method to identify genetic markers for individual identification and genetic relationship studies in a large number of plant and animal species (Gill et al., 1985; Quinn et al., 1987; Wetton et al., 1987; Wong et al., 1987; Kuhnlein et al., 1989, 1990; Gilbert et al., 1990; Hillel et al., 1990; Reeve et al., 1990).

Application of DNA fingerprinting, particularly in the context of forensic and legal medicine has resulted in a very high public awareness of this methodology. Cases involving legal evidence obtained through DNA fingerprinting are regularly described in newspapers and have even resulted in a best selling novel. Of particular note to this thesis are the uses of DNA fingerprinting to quantity genetic relationships among animals.

Kuhnlein et al. (1989) demonstrated that DNA fingerprinting band patterns could be used to calculate genetic distances between different chicken lines in which levels of intra-population variability were similar. The estimated genetic distance correctly reflected the well documented breeding history of these lines.

The analysis of DNA fingerprints has further indicated that the similarity of band patterns in DNA fingerprints increases in concert with an increase of the inbreeding level of the strain (i.e. variability of the DNA fingerprint band patterns were reduced in inbred strains). Consequently, a calibration curve has been derived which relates the

inbreeding coefficient in these strains to the genetic variability, using closed breeding populations of chicken as a model system (Kuhnlein et al., 1990).

3. General Materials and Methods of DNA Fingerprinting with Microsatellite $(CAC)_5$ Probes

3.1 DNA Preparation

A blood sample of about 5 ml from cows was taken from the caudal artery or vein using a Vacutainer tube containing sodium heparin (Becton Dickinson) and stored on ice. The fresh blood sample was centrifuged at 1,300 x g for 15 minutes. The buffy coat which contained the white blood cells was carefully aspirated and transferred to another tube with a pasteur pipet, and 7 ml extraction buffer containing 10 mM Tris.HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 20 ug/ml RNAase and 0.5% SDS were added. The solution was gently mixed and then incubated at 37 °C for 1 hour. Subsequently, proteinase K was added to a final concentration of 100 ug/ml, and the tube was incubated in a waterbath at 50 °C overnight. The samples were extracted twice with phenol-chloroform prior to the addition of 0.2 volume of 10 M ammonium acetate. Genomic DNA was precipitated with 2 volumes of 100% ethanol. The DNA was then fished out with a glass rod, washed once with 70% ethanol and dried by vacuum for 15 minutes. DNA was dissolved in TE buffer containing 10 mM Tris.HCl (pH 7.5) and 1 mM EDTA (pH 8.0).

The concentration of DNA was estimated using spectrophotometry (Beckman, Model DU-20), at a wave-length of 260 nm. Subsequently, DNA samples were stored at -20 °C until needed.

3.2 Restriction Digestion

A 30 ul mixture containing 5 ug genomic DNA, 15 units restriction enzyme and 1 x One-Phor-All buffer was incubated at 37 °C for 3 hours to overnight according to the recommendations of the supplier (Pharmacia). Reactions were placed into a waterbath at 65 °C for 10 minutes to inactivate the enzymes prior to the addition of 5 ul 6 x gel-loading buffer (15% Ficoll and 0.01% bromophenol blue).

3.3 Electrophoresis

Preparation of agarose gels: 1.4 g agarose powder (electrophoresis grade, ICN) was melted and dissolved in 200 ml 1 x TPE buffer containing 90 mM Tris-phosphate and 2 mM EDTA (pH 8.0) in a microwave oven. Subsequently, the agarose solution was cooled to 60 °C and then poured carefully into a casting tray (20 x 15 cm).

When the agarose had gelled, the slab gel was placed into the electrophoresis tank (Bio-Rad) supplied with sufficient 1 x TPE buffer to cover the gel to a depth of about 1 - 2 mm.

Electrophoresis: DNA samples digested by restriction enzymes were loaded into the wells of the submerged gel, and electrophoresis was carried out at a voltage of 1.8 v/cm for 21 - 24 hours.

Completeness of digestion and migration of DNA samples was visualized using ultraviolet light following staining of the gel for 30 minutes in a solution of 0.5 ug/ml ethidium bromide. Excess gel was trimmed to the size of approximately 17 x 15 cm.

3.4 Slab Gel Drying

The gel was placed on two sheets of 3M filter paper, overlaid with saran wrap, and placed into a gel dryer (Bio-Rad, Model 483).

The gel was dried without heating under vacuum for 30 - 60 minutes until it was almost dry. Subsequently, heat was applied to 60 °C, and the gel was dried until completeness for another 30 minutes. The dried gel was stored at room temperature until required for hybridization.

3.5 Radioactive Labelling of Probes

Labelling reaction: Oligonucleotide probes used for DNA fingerprinting were labelled by phosphorylation of 5' termini with gamma-^{32}P-ATP using T_4 polynucleotide kinase (Pharmacia).

One ul of oligonucleotide $(CAC)_5$ (0.3 pM), 1 ul of 10 x One-Phor-All buffer (Pharmacia) and 2 ul distilled water were mixed well in an Eppendorf tube. Five ul gamma-^{32}P-ATP (ICN) and 1 ul T_4 Polynucleotide kinase (10 units/ul) were then added. Reactions were kept in a small lead container and incubated at 37 °C for 1 hour.

Ninety ul of column buffer containing 10 mM Tris.HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 100 mM NaCl were used to stop the reaction, and the 100 ul reaction solution was then applied to G-25 column.

Purification of probe: A G-25 column was prepared in a sterile pasteur pipette. The pipette was plugged using sterile glass wood and Sephadex G-25 (Pharmacia) which was suspended in water containing 0.2% sodium azide was added until the column

height reached the constriction near the top of the pipette (about 2 cm). The column was washed with 4 ml column buffer containing 200 mM NaCl, 10 mM Tris.HCl (pH 7.5) and 1 mM EDTA (pH 8.0).

The labelling reaction was applied to the top of the column and eluted using column buffer. Fractions were collected in Eppendorf tubes and the elution profile of radiolabelled oligonucleotide was monitored using a Geiger counter (Technical Association, Model PVG 1). The lead fraction of the elution peak (1 ml) was retained for hybridization, and radioactivity of the probe was measured by liquid scintillation counting (LKB, 1209 Rackabeta).

Although the unincorporated radionucleotides were separated from the oligonucleotide, this method does not separate labelled from unlabelled oligonucleotides.

3.6 In-gel Hybridization

Before hybridization, the dried gel was immersed in distilled water to float off the plastic wrap and filter paper.

Denaturation: Gels were denatured in 200 ml of 0.5 M NaOH, 0.15 M NaCl for 30 minutes at room temperature with gentle agitation.

Neutralization: The gel was rinsed with distilled water, then neutralized in 100 ml of 0.5 M Tris.HCl (pH 7.5), 0.15 M NaCl for 30 minutes at room temperature with gentle agitation.

Equilibration: The gel was then equilibrated in 200 ml of 6 x SSC (20 x : 175.3 g

NaCl, 88.2 g sodium acetate per liter).

In-gel hybridization: Hybridization was carried out using a rotisserie style hybridization incubator (Robbins Scientific), in 20 ml of hybridization buffer (for a 18 x 15 cm gel) containing 10 ug/ml denatured herring sperm DNA, 5 x Denhardt's solution (Sambrook et al., 1989), 5 x SSPE (900 mM NaCl, 50 mM NaH_2PO_4 and 5 mM EDTA ph8.0) and 1 - 2 x 10^6 cpm/ml of the labelled $(CAC)_5$. Hybridization was at 42 °C for 2 to 3 hours.

Prehybridization steps were eliminated without affecting the result.

Washes: After hybridization, the gel was washed 3 times in 200 ml of 6 x SSC at room temperature for 30 minutes with gentle agitation. The gel was then washed for 0.5 - 1 minutes with 500 ml of 6 x SSC prewarmed to 42 °C, and then immediately transferred to 6 x SSC at room temperature. Gels were then blotted dry and wrapped in plastic wrap for autoradiography.

The efficiency of the wash steps was monitored using a Geiger counter. In order to minimize background, additional washes were performed until background radioactivity was equal or less to 200 cpm.

3.7 Autoradiography

Gels were exposed to Kodak XAR-5 films at -70 °C for 24 - 72 hours. A single Cronex intensifying screen was used.

Sharper bands were obtained without the intensifying screen. However, as a rough guideline, exposure without a screen had to be 2 - 3 times longer to obtain the same

signal intensity as with a screen.

X-ray film was developed manually as following: the film was bathed in X-ray developer (Kodak) for 5 minutes, rinsed with deionized water for 1 minute, bathed in rapid fixer for 5 minutes, and then washed in running water for 15 minutes. The temperature of all solutions were 18 - 20 °C. The autoradiograph was air dried.

3.8 Rehybridization

Dried gels are suitable for repeated rehybridizations with the same or different probes. Oligonucleotides can easily be eluted from the gels. After the correct exposure was obtained, the probe was removed by a denaturation-neutralizing step as described in chapter 3.6. Complete removal of the probe was monitored by Geiger counting and/or autoradiography. Gels were equilibrated in 5 mM EDTA (pH 8.0) at 60 °C with gentle agitation. Subsequently, the gel was stored at 4 °C until required for rehybridization.

4. Experiment I: Analysis of Inbreeding in Poultry using DNA Fingerprinting with a Microsatellite Probe

4.1 Introduction

Kuhnlein et al. (1989, 1990) have successfully used DNA fingerprinting as a tool for determining distances between strains of chicken, and developed a calibration curve for inbreeding using closed breeding populations of chicken as a model system. In the latter study, the known coefficient of inbreeding (pedigree analysis) in these strains was related to the genetic variability assessed by DNA fingerprinting. Since the level of homozygosity increased in concert with the level of inbreeding, a linear relationship ($r = 0.996$) was observed.

The probe used for DNA fingerprinting was derived from bacteriophage M13 DNA which recognized hypervariable minisatellite loci (Vassart et al., 1987) and the measure for genetic variability was the average band frequency. In consideration of the advantages of microsatellite probes over minisatellite probes for DNA fingerprinting, we attempted to use a microsatellite probe to detect inbreeding in Holstein cattle. Since minisatellite and microsatellite loci have different distributions in the genome (Nurnberg, 1989; Litt and Luty, 1989), we first compared the concordance of results obtained with microsatellite and minisatellite probes using the same chickens that were used by Kuhnlein et al. (1990) to establish an index of inbreeding.

4.2 Materials and Methods

4.2.1 Genetic Groups

In order to facilitate comparison between probes, the same genetic groups of White Leghorn chicken (except strain 6_3) were used as by Kuhnlein et al. (1990). The origin and characteristics of these chickens are described in Table 4.1 and their inbreeding coefficients are listed in Table 4.3. The chickens (n = 6 per genetic group) identical to those which were used previously (Kuhnlein et al., 1990) were used in the analysis.

All these strains are currently maintained at the Animal Research Centre of Agriculture Canada in Ottawa, Canada, except line 7_2 which is kept at the Regional Poultry Research Laboratory of the U.S. Department of Agriculture, East Lansing, Michigan.

The inbreeding coefficients of strains 7, 8, 9, S and WG were computed on individual pedigree basis up to 1980 and subsequently estimated from the size of mating populations and type of matings (Falconer, 1960).

The contribution of the initial inbreeding coefficient has been estimated to be < 0.001. The highly inbred line 7_2 was derived by brother-sister matings (Stone, 1975).

4.2.2 DNA Fingerprinting

DNA samples were digested with MspI, AluI or RsaI and separated in 0.7% agarose gels. DNA fingerprinting using the microsatellite probe, $(CAC)_5$ was carried out using the methods described previously (Chapter 3). DNA fingerprinting analysis using the minisatellite probe was as described by Kuhnlein et al. (1990).

4.3 Results and Discussion

4.3.1 Evaluation of DNA Fingerprints

Representative DNA fingerprints of chickens from 6 strains with different degrees of inbreeding digested by AluI are shown in Figure 4.1 and 4.2.

DNA fingerprints of 6 randomly selected chickens per genetic group were scanned with a computer-linked densitometer and the 6 most intensive bands were marked on each DNA fingerprint. Representative scans of 2 individuals of strain 8 (F = 0.103) are shown in Figure 4.3. Bands which had the same apparent molecular weight and relative intensities differing by less than a factor of 2 (homozygote versus heterozygote), were scored as identical. Analysis was restricted to molecular weights between 2.5 kb to 21 kb (Figure 4.3 and 4.4). For each of these enzymes, the average band frequencies were computed by analyzing 6 chickens per strain.

The average band frequency (U) was computed according to the equation:

$$U = (1/n) \sum_{i=1}^{n} v_i \qquad (1)$$

where n is the number of different bands scored and v_i is the frequency of bands in the breeding population (Kuhnlein et al., 1990). A representative evaluation of band frequencies in strain 7 (digested by AluI) is shown in Table 4.2.

Assuming that each band represents an allele at a VNTR locus, this index describes the average frequency of genotypes which have a particular allele in common. Since the greater the level of inbreeding, the greater the frequency that alleles become fixed in the population, it was expected that in strain 7_2, U would approach a value of 1.

Indeed this was the case since in strain 7_2 ($F \geq 0.98$), U = 1, whereas in less inbred strains such as strain S (F = 0.39), U = 0.62 (Table 4.3). These values were not significantly different ($\alpha = 0.05$) from those observed using a minisatellite probe (Kuhnlein et al., 1990). Thus, both minisatellite and microsatellite probes produced similar values of U at each level of inbreeding.

4.3.2 Comparison of Calibration Curves Produced by Different Restriction Enzymes

Linear regression analysis between inbreeding and band frequencies determined using $(CAC)_5$ yielded correlation coefficients of 0.995, 0.997 and 0.999 for MspI, AluI and RsaI, respectively (Figure 4.5, 4.6 and 4.7), indicating that the relationship between band frequency and level of inbreeding in strains is well represented by a linear approximation.

The slopes of the linear approximation were 0.61 (±0.09) for MspI, 0.56 (±0.06) for AluI and 0.53 (±0.05) for RsaI, and the intercepts at no inbreeding were 0.40 (±0.05), 0.42 (±0.03) and 0.47 (±0.03), respectively. At the significance level of 0.05, there were no differences between these values.

There are several factors which might cause the observed variability in the number of alleles detected using the different restriction enzymes. Firstly, it was possible that the number of the cutting sites for RsaI may be less than those for MspI and AluI, respectively, which would reduce the number of bands produced. Since all 3 enzymes have 4 base recognition sequences, it is unlikely that the total number of restriction sites

is substantially different amongst the enzymes. However, it is possible that RsaI sites could have a less polymorphic distribution around the microsatellite repeat units than either MspI or AluI. In this regard, differences in DNA methylation within the restriction recognition sequence are least likely to affect RsaI digestion (GTAC). Therefore, polymorphisms associated with differences in DNA methylation patterns would be detected and hence fewer bands would be detected. Secondly, it should also be noted that multiallelic banding patterns associated with repeat unit probes produce DNA phenotypes and not genotypes (Jeffreys et al., 1991). This means that bands representing the same size class, may or may not represent the same gene locus between animals. Single locus probes would be required in order to more closely estimate true allelic variability. Thirdly, we also observed artifacts associated with the technique. Not all lanes of DNA ran perfectly straight in the gel, and it was sometimes difficult to decide if 2 bands were identical or not. There are several ways for the correction of this effect, including using monomorphic probes to calculate band shift correction factors (Norman, 1989), estimating the degree of match by using probability functions (Gjertson et al., 1988) and running a mixed sample of standard DNA with the test DNA (Lander, 1989). None of these factors was applied in the current study and we observed no differences in the frequency of banding artifacts associated with the different enzymes.

4.3.3 Comparison of Minisatellite and Microsatellite Probes

The relationship between average band frequency and known degree of inbreeding

in these lines of chickens using the minisatellite probe M13 and MspI as restriction enzyme was previously determined to be Y = 0.57X (±0.06) + 0.42 (±0.04) (Kuhnlein et al. 1990). Using the same enzyme and the microsatellite probe, $(CAC)_5$, the relationship was described by Y = 0.61X (±0.09) + 0.40 (±0.05). At the significant level of 0.05, statistically there was no difference between these two equations, which may indicate that the distribution of VNTR loci determined by the 2 probes (Figure 4.5) were not different. This is somewhat surprising since minisatellite and microsatellite loci have very different distributions in the genome. Minisatellite loci are thought to be clustered towards the proterminal regions of chromosomes (Royle et al., 1988), whereas microsatellite loci are considered to be evenly distributed throughout the genome (Nurnberg et al., 1989).

The cutting sites of each of the enzymes used in the current study produced a slightly different distribution of loci. Each enzyme however, was shown to produce a DNA banding pattern that was linearly related to the known degree of inbreeding, thus microsatellite loci can be used to predict the degree of inbreeding in animals. Since either type of probe accurately reflected the degree of relationship between animals but the microsatellite DNA fingerprints were technically easier to produce, the latter is the method of choice.

Table 4.1 Description of genetic groups of chickens which were used for DNA fingerprinting analysis *

S	White Leghorn strain selected for susceptibility to Marek's disease at Cornell University until 1971. Maintained since at Ottawa without selection.
7	Formed from 4 commercial White Leghorn strains in 1958 and maintained since without selection.
8	Derived from strain 7 in 1969 and selected since for high egg number and related traits.
9	Derived from strain 7 in 1969 and selected for high egg production rate and related traits.
WG	Inbred line derived from strain 9.
7_2	Highly inbred White Leghorn line derived at the Regional Poultry Research Station of the USDA, East Lansing, Michigan, USA. Susceptible to Marek's disease.

*. Adapted from Kuhnlein et al. (1990)

Table 4.2 Evaluation of band frequencies of DNA fingerprints in chickens of strain 7 (DNA was digested by AluI).

Allele	#1	#2	#3	#4	#5	#6	Band frequency[a]
1	+[b]	+[b]	+	+[b]			0.667
2	+[b]		+[b]		+[b]		0.500
3	+[b]		+			+[b]	0.500
4	+[b]	+[b]	+[b]	+[b]	+[b]	+[b]	1.000
5	+[b]		+				0.333
6	+[b]		+[b]		+[b]		0.500
7		+[b]	+[b]			+	0.500
8		+[b]	+[b]		+[b]	+[b]	0.667
9		+[b]	+[b]		+		0.500
10		+[b]				+[b]	0.333
11				+[b]			0.167
12				+[b]			0.167
13	+			+[b]	+		0.500
14				+[b]			0.167
15					+[b]	+	0.333
16					+[b]		0.167
17		+				+[b]	0.333
18	+	+	+	+	+	+[b]	1.000

Average band frequency = 0.46 ± 0.06

a. Average and standard error .

b. Six bands which had the highest intensity in the particular lane.

Table 4.3 Average band frequencies in six genetic groups of chickens with different degrees of inbreeding using AluI digestion.

Genetic group[a]	Inbreeding coefficient	No. of different bands scored	Average no. of bands scored per chicken	Band frequency[b]
7	0.026	18	8.3 ± 0.9	0.46 ± 0.06
8	0.103	15	7.0 ± 0.8	0.47 ± 0.06
9	0.126	15	7.2 ± 0.6	0.48 ± 0.05
S	0.390	13	8.0 ± 1.6	0.62 ± 0.10
WG	0.762	8	6.7 ± 0.6	0.83 ± 0.05
7_2	> 0.980	8	8.0 ± 0.0	1.00 ± 0.00

a. Strains were previously described in Table 4.1.

b. Average and standard error.

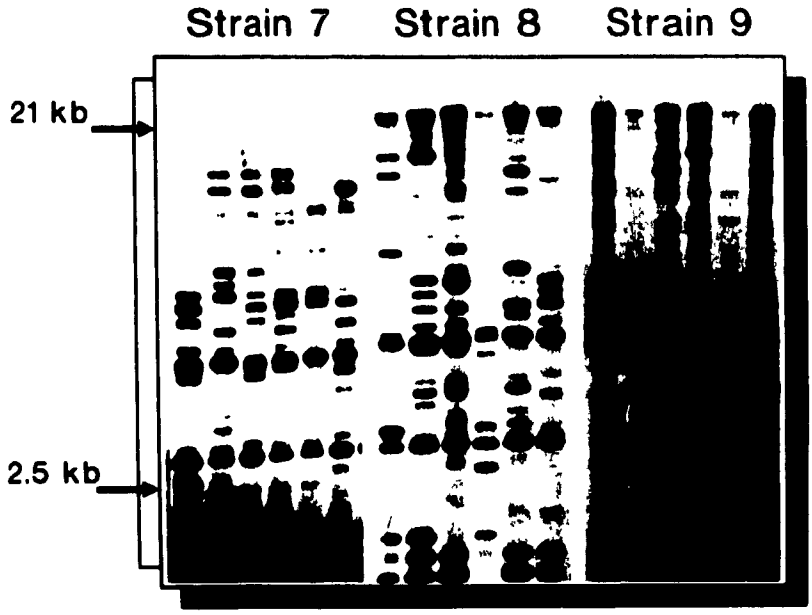

Figure 4.1 DNA fingerprints of chickens from strain 7, 8 and 9 produced by hybridization with $(CAC)_5$ and following digestion with AluI. Average inbreeding in these strains were 0.026, 0.103 and 0.126, respectively. Only bands with apparent molecular weights between 2.5 kb and 21 kb (indicated by arrows) were considered in the analysis.

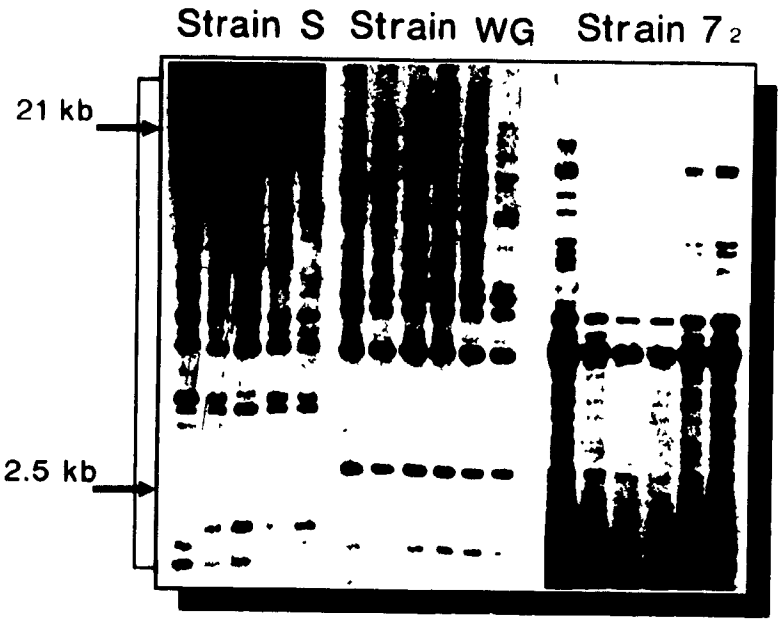

Figure 4.2 DNA fingerprints of chickens from strain S, WG and 7_2 produced by hybridization with $(CAC)_5$ and following digestion with AluI. Average inbreeding in these strains was 0.39, 0.762 and > 0.98, respectively. Only bands with apparent molecular weights between 2.5 kb and 21 kb (indicated by arrows) were considered in the analysis.

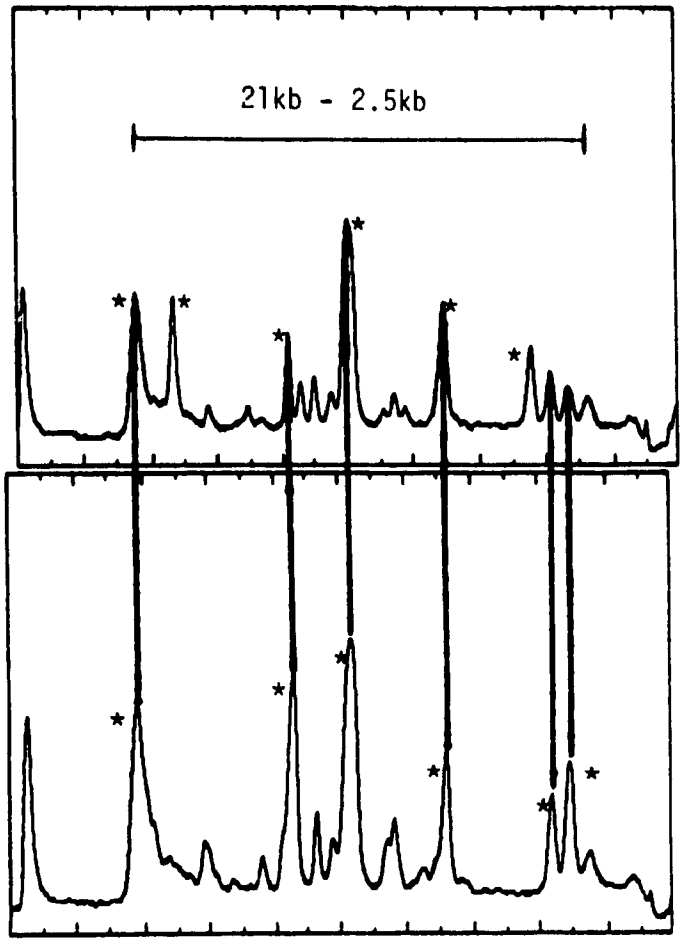

Figure 4.3 Representative DNA fingerprints scanned with a computer-linked densitometer (two individuals of chickens from strain 8). The six most intensive bands per individual are indicated by an (*) of the dominant bands are not shared by individuals.

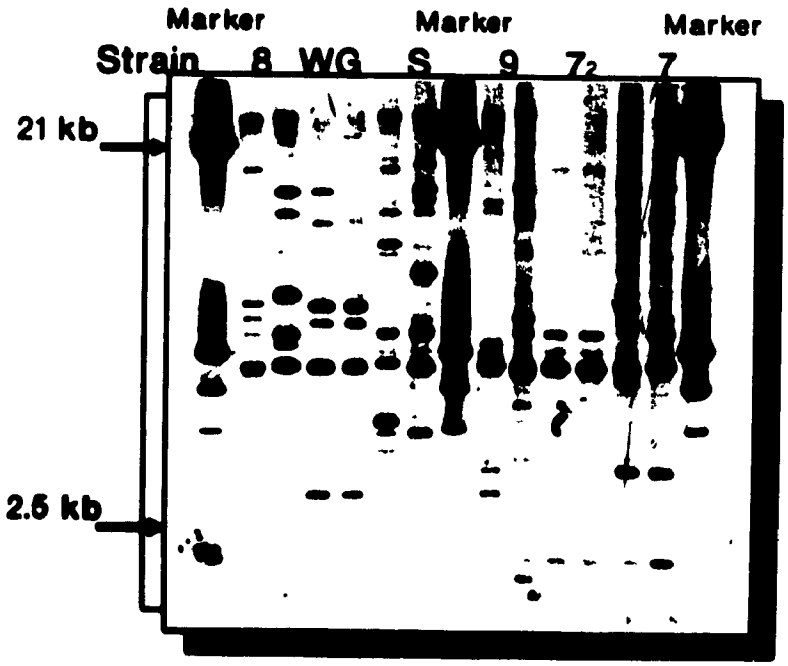

Figure 4.4 Molecular weight of the bands scored in 6 strains of chickens following digestion of DNA with AluI and hybridization with $(CAC)_5$. The molecular size marker is lambda DNA digested by EcoRI and HindIII.

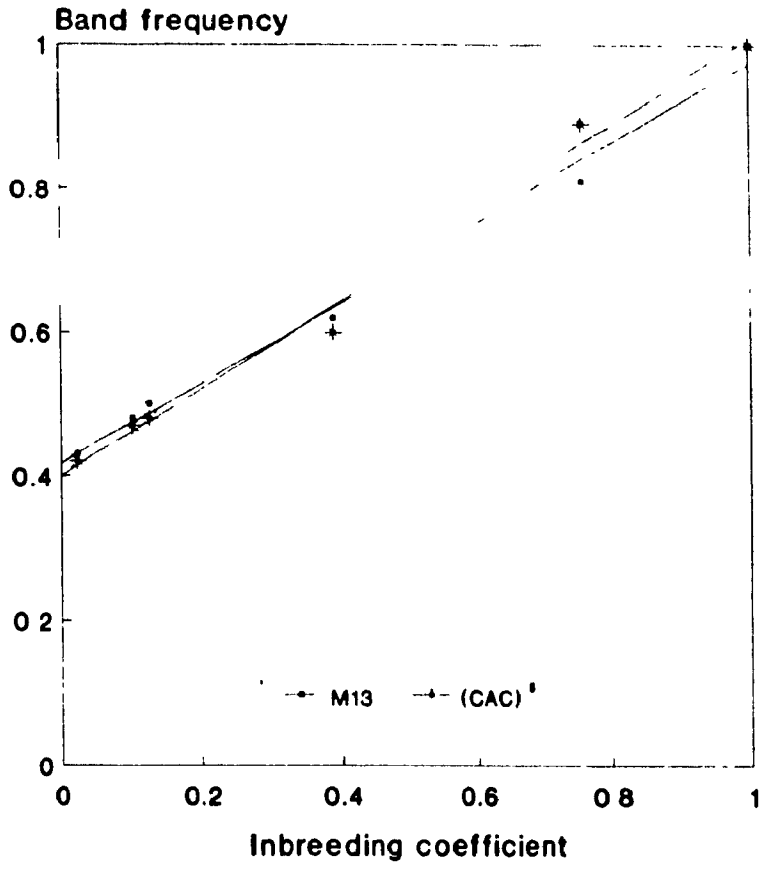

Figure 4.5 Dependence of band variability on inbreeding coefficient with different VNTR probes. Both are derived from MspI digestion, and the DNA fingerprints used to derive these relationships were based on the analysis of 6 chickens per inbreeding level and the minisatellite probe, M13 (■) or the microsatellite probe, $(CAC)_5$ (*).

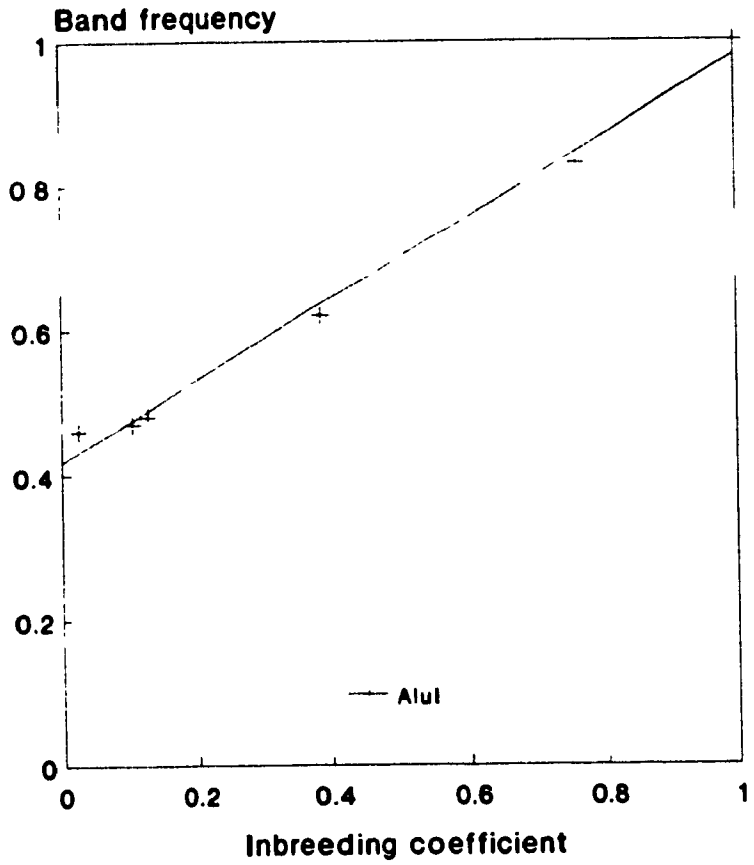

Figure 4.6 Dependence of band variability on inbreeding coefficient. DNA from 6 chickens per group of inbred individuals was digested with AluI and DNA fingerprints were derived from microsatellite probe, $(CAC)_5$.

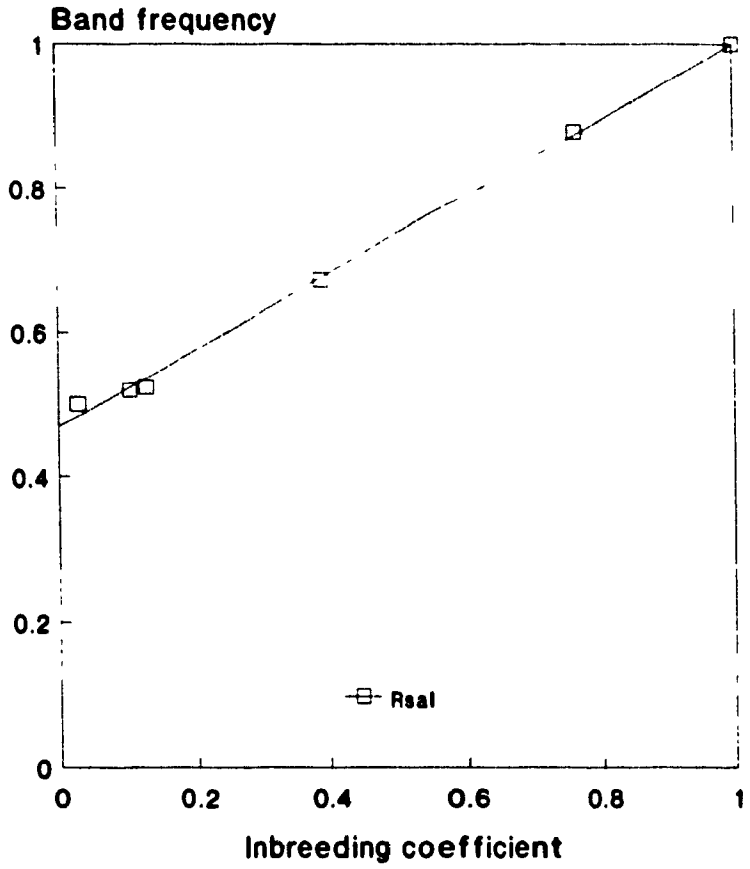

Figure 4.7 Dependence of band variability on inbreeding coefficient. DNA from 6 chickens per group of inbred individuals was digested with RsaI and DNA fingerprints were derived from microsatellite probe, $(CAC)_5$.

5. Experiment II: Establishment of an Inbreeding Index in Holstein Dairy Cattle

5.1 Introduction

Increased levels of inbreeding in cattle have been shown to be associated with reduced milk and fat yield, as well as a reduced birth weight and increased mortality. Mature body size and weight were also reduced (Dalton, 1985).

Since the rate of increase in homozygosity with inbreeding is dependent on the closeness of the relationship of the individuals which are mated, it is important to detect the closeness of two individuals. Knowledge of relationships can be helpful in selecting animals to keep in the herd or in selecting matings to avoid high levels of inbreeding.

Using the chicken as a model system, the oligonucleotide $(CAC)_5$ was shown to be useful as a microsatellite probe to assess inbreeding in domesticated animals (see Chapter 4). In the previous study, the average degree of inbreeding within the flock was known. In cattle, experimental herds with known degrees of inbreeding are not maintained in adequate numbers to test the possibility of detecting inbreeding levels using DNA fingerprinting. Hence, in this experiment, a calibration curve between inbreeding and band sharing in DNA fingerprints was established by analyzing individuals of known genetic relationships. DNA fingerprinting could provide a useful adjunct to pedigree records for the detection of inbreeding in dairy cattle.

5.2 Materials and Methods

5.2.1 Genetic Groups

DNA samples of animals were obtained from a Holstein dairy cattle population (82 individuals, 1991) maintained at the Macdonald Campus of McGill University, Montreal, Canada. This population was kept and developed since 1907 with low selection pressure. Most of the animals were born on this farm, but some were bought. All of the dams were mated with sires which came from the Centre d'insemination artificielle du Quebec (CIAQ) Inc. Pedigree records extending more than 28 generations for this herd have been recorded.

Six pairs of animals which had the same genetic relationship were qualified as a genetic group. Coefficients of relationship r, defined as the expected proportion of genes in two individuals that were identical by descent, were determined directly from pedigree analysis according to Wright (1921). For instance, for monozygous twins, r = 1; for first order relatives, r = 0.5; or for half sibs, r = 0.25 etc. (Table 5). Pedigree records extending over 5 generations were used for each individual to determine the degree of relationship between pairs of individuals.

5.2.2 DNA Fingerprinting

In a preliminary experiment to determine the restriction enzyme which produced the optimal distribution of DNA bands, genomic DNA of 3 unrelated cows were digested with HaeIII, BamHI, MboI, MspI or RsaI and DNA fingerprint analysis was carried out as previously described. Each of the tested enzymes produced variable

DNA banding patterns (Figure 5.1 and 5.2). MboI and RsaI each produced DNA banding patterns which were easy to score. Digestion of DNA with RsaI however, produced higher molecular weight bands and more bands than digestion with MboI. For these reasons, RsaI was used in the subsequent studies.

Genomic DNA of cattle digested with RsaI restriction enzymes, were separated in 0.8% agarose gels, and then DNA fingerprinting was carried out using the methods described previously.

5.3 Results and Discussion

5.3.1 Evaluation of DNA Fingerprints

DNA fingerprints of 6 pairs of cattle per genetic group were scanned with a computer-linked densitometer and the 6 most intensive bands were marked on each DNA fingerprint. Those bands having the same apparent molecular weight and relative intensities differing by less than a factor of 2 (homozygote versus heterozygote) were scored as identical. The molecular weights of the bands scored were between 3.3 kb to 12.6 kb (Figure 5.3 and 5.4).

Band sharing (BS) between 2 individuals were computed according to the equation:

$$BS = 2N_{ab} / (N_a + N_b) \qquad (2)$$

where N_{ab} is the number of bands that are shared between 2 individuals, N_a and N_b are the number of bands scored in individuals a and b, respectively. As expected, the average band sharing for the 6 genetic groups increased in concert with the degree of relationship (Table 5).

Representative DNA fingerprints from 6 groups of animals with different relationship coefficients are shown in Figure 5.3 and 5.4.

Linear regression analysis between relationship and band sharing yielded a correlation coefficient of 0.992, indicating that the relationship between them is well represented by a linear approximation (Figure 5.5). The slope of the linear approximation was 0.51 (± 0.09), and the intercept at 0 relationship was 0.50 (± 0.04).

5.3.2 Average Band Frequency and Average Band Sharing

Kuhnlein et al. (1990) introduced the average band frequency (see Chapter 4.3.1) as a measure of genetic uniformity of a population. In this equation, the values v_i were approximated by frequency of band i among the individuals tested and hence U is identical to

$$U = N / (kn) \qquad (3)$$

where N is the total number of bands scored, k is the number of individuals scored and n is the number of different bands scored. The accuracy of the estimate of the band frequencies in the population is dependent on the number of individuals tested and the actual band frequencies. Thus, when 2 individuals are compared, the lowest estimate will be 0.5 while actual band frequencies are much lower. It simply reflects that only bands which are present in either one of the individuals are scored.

A more common measure for genetic uniformity is the band sharing index (BSI) (see Chapter 5.3.1). The BSI between 2 individuals is the probability to draw identical bands from the two individuals in two successive trials.

Based on the population band frequencies, the expected number of bands in each individual is

$$N_A = N_B = \sum_{i=1}^{n} v_i$$

and the number of bands shared is

$$N_{AB} = \sum_{i=1}^{n} v_i^2$$

. Thus,

$$BSI = \sum_{i=1}^{n} v_i^2 \Big/ \sum_{i=1}^{n} v_i \qquad (4)$$

The variance of the band frequencies of the population is given by

$$\delta_v^2 = (1/n)\sum_{i=1}^{n}\left[v_i - (1/n)\sum_{i=1}^{n} v_i\right]^2$$

$$= (1/n)\left[\sum_{i=1}^{n} v_i^2 - (1/n)(\sum_{i=1}^{n} v_i)^2\right] \qquad (5)$$

and thus

$$\sum_{i=1}^{n} (v_i)^2 = (1/n)(\sum_{i=1}^{n} v_i)^2 + n\,(\delta_v)^2 \qquad (6)$$

Substituting this value into equation (4) yields

$$BSI = \left[2(1/n)(\sum_{i=1}^{n} v_i)^2 + n(\delta_v)^2 \right] / (2\sum_{i=1}^{n} v_i)$$

$$= (1/n)\sum_{i=1}^{n} v_i + n\delta^2 / (2\sum_{i=1}^{n} v_i)$$

and since $\sum_{i=1}^{n} v_i = N / k$

$$BSI = N / (kn) + (\delta^2 / 2)(kn / N)$$

$$= U + (\delta_v^2 / 2)(1 / U)$$

Thus, the band sharing index gives an overestimate of the average band frequency. The overestimate is proportional to the population variance and to the reciprocal value of the average band frequency. Thus, if the number of alleles per locus is relatively constant or the average band frequency is large, the two measures of relatedness are equivalent.

In the example of strain S probed with the minisatellite probe M13 (Kuhnlein et al., 1990) the band frequency was 0.663 and the sample variance was 0.016 yielding a corrective term of only 0.012. It is not to be expected that other minisatellite or microsatellite probes yield higher variances. If this is true, the average BSI can be considered as a good estimator for the band frequency.

5.3.3 Factors that Might Influence the Shape of the Calibration Curve

There are several factors that might influence the shape of calibration curves and the allelic variability detected at VNTR loci include the choice of restriction enzyme and the errors in determination of band sharing.

Allelic variability at VNTR loci: The intercept is a measure for the average number of alleles scored per locus. Calibration curves derived from different species are likely to be different. Indeed, neither the function nor the mechanism by which variation at these loci arise are known. Originally Jeffreys et al. (1985b) noted that VNTR loci contained a core sequence which had a high degree of homology to χ (chi), the recombinational hotspot of E. coli. He proposed that VNTR loci could be involved in unequal crossovers at meiosis and thus be involved in generating genetic diversity. This hypothesis has been largely discounted since Wolff et al. (1989) proved that new alleles do not arise as a result of a simple crossing over event. Unequal exchange between sister chromatids and/or DNA polymerase slippage at replication forks have been proposed as mechanism of generating allelic diversity.

Restriction enzymes: Restriction enzymes specifically bind to doubled-stranded DNA and cleave at specific sites within or adjacent to a particular sequence, known as the recognition sequence. The frequency of the cutting sites of restriction enzymes in the genome are determined by the length of the recognition sequence. Therefore, allelic variability changes with different restriction enzymes. Furthermore, the characteristics of the restriction recognition sequences which is partly homologous to the sequences of the probe may influence the variability of bands as well, which can be

detected by the probe. For example, all of the restriction enzymes used had 4 base recognition sequences. Of these, RsaI had the nucleotide C in the fourth position, whereas the microsatellite probe recognizes the repeat unit CAC. It is possible that RsaI might cut with a greater frequency immediately adjacent to the start of a repeat unit (i.e. C in fourth position of recognition sequence) and thus produce more bands of lower molecular weight. Relatively few bands in cattle with estimated molecular weights greater than 3.3 kb were observed (Figure 5.3) and in the chicken, less band sharing was detected using MspI (0.40) than RsaI (0.47). Clearly, enzyme and probe combinations which produce a lower proportion of shared bands between unrelated individuals would be more desirable. In this study, the enzymes HaeIII, BamHI, MboI, MspI and RsaI were tested in combination with the microsatellite probe CAC and RsaI was observed to produce the best pattern of bands for analysis. It would be informative to rehybridize the gels using additional microsatellite probes (e.g. AT, GA, GACA, GGGCA or TTAGGG). It is likely that each probe would produce a different degree of average band sharing. The latter has not been tested as yet.

Errors in determination of band sharing : Band shifting is a technical problem which involves a number of variables such as the concentration of the gel and the running conditions (Lander, 1989). For a host of reasons, not all gels run perfectly straight and it is sometimes extremely difficult to decide if 2 bands are identical or not.

Normally, small size differences between relatively large DNA fragments are difficult to detect (Baird et al., 1986; Boerwinkle et al., 1989; Gill et al., 1990). For hypervariable loci, some alleles may remain undetected because their size difference is

too small to be separated by the agarose gel. In addition, analysis is complicated by possibility of band shifting. This could cause a heterozygous individual with alleles that differ by only a small number of repeat units to be typed as a homozygote.

A number of procedures for the correction of this effect have been proposed including the use of monomorphic probes to calculate band shift correction factors (Norman, 1989), estimating the degree of match by using probability functions (Baird et al., 1986; Gjertson et al., 1988; Gill et al., 1990), by running a control DNA sample on every gel (Lander, 1989) or by including a molecular weight standard in every lane. In the current study, all animals within a single genetic group were compared in a single gel in order to minimize within group analysis artifacts. However, no additional procedures to reduce band shift artifacts were applied.

5.3.4 Comparison of Linear Approximations between Different Species

Unrelated individuals would be expected to have a proportion of unshared bands described by (1 - X) where X represents average band sharing within the species. Among related individuals, a proportion of these bands is expected to be shared by descent from a common ancestor. The coefficient of relatedness, r, can be used to estimate this fraction, r(1 - X). Observed band sharing would therefore be expected to be described by the linear function X + r(1 - X) which in unrelated individuals (r = 0) has the value of X and in monozygous twins (r = 1) has the value of 1. Although this relationship is theoretically linear, empirical measurements have shown evidence of non-linearity in some species (African lions: Gilbert et al., 1991; naked mole rat: M. Bruford,

personal communication).

In our analysis of cattle (Figure 5.5), departure from linearity was tested by fitting a quadratic term which was not significant. This suggests that a linear relationship adequately describes the data ($r = 0.992$). However, it should be noted that the extrapolated intercept at $r = 1$ passes above the theoretical point (1, 1). This suggests that the relationship cannot be linear over the entire range of r, and that accurate predictions of relatedness from band sharing will be difficult. Similar deviation from linearity have been observed in other species (Kuhnlein et al., 1990; Gilbert et al., 1991) and cannot be explained. Most notably, in a study of geographically isolated African lions which related band sharing to relatedness, 2 completely different empirical relationships were determined (Gilbert et al., 1991). One population was described by a linear model, whereas the other required fitting of curvilinear model.

Table 5. Average band sharing in 6 genetic groups of Holstein dairy cattle[a] with different degrees of genetic relationship.

Genetic group[b]	Coefficient of relationship[c]	Average no. of bands scored per cow	Average band sharing[d]
1.	0.000	6.25 ± 0.43	0.47 ± 0.17
2.	0.063	6.25 ± 0.43	0.56 ± 0.12
3.	0.125	6.25 ± 0.43	0.56 ± 0.20
4.	0.250	6.75 ± 0.83	0.63 ± 0.11
5.	0.500	6.75 ± 0.43	0.79 ± 0.10
6.	1.000	6.00 ± 0.00	1.00 ± 0.00[e]

a. Maintained in the farm of Macdonald Campus of McGill University
b. Six pairs of animals in each group except group 6
c. Calculated by pedigree analysis
d. Average and standard error
e. One pair of identical twins

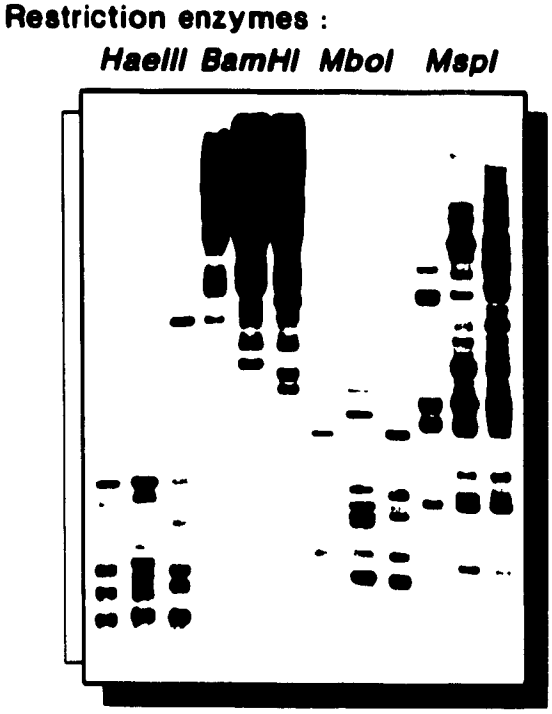

Figure 5.1 DNA fingerprints of Holstein dairy cattle, comparing the restriction enzymes HaeIII, BamHI, MboI and MspI. HaeIII and MboI gave clear-cut band patterns. Digestion with BamHI was incomplete.

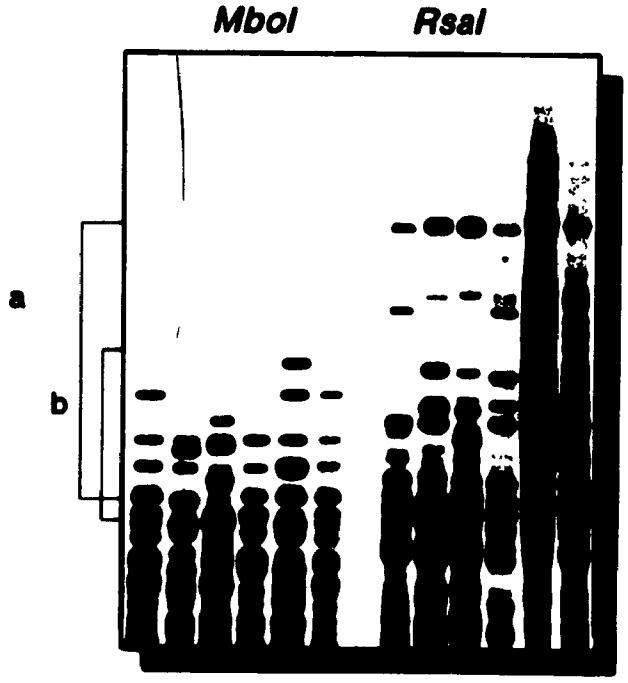

Figure 5.2 DNA fingerprints of Holstein dairy cattle, derived from MboI and RsaI digestion, respectively. Both enzymes produced clear-cut DNA fingerprinting band patterns. However, RsaI produced higher molecular weight bands (a) than MboI (b). In addition, more variable bands could be scored per individual with RsaI digestion than with MboI digestion.

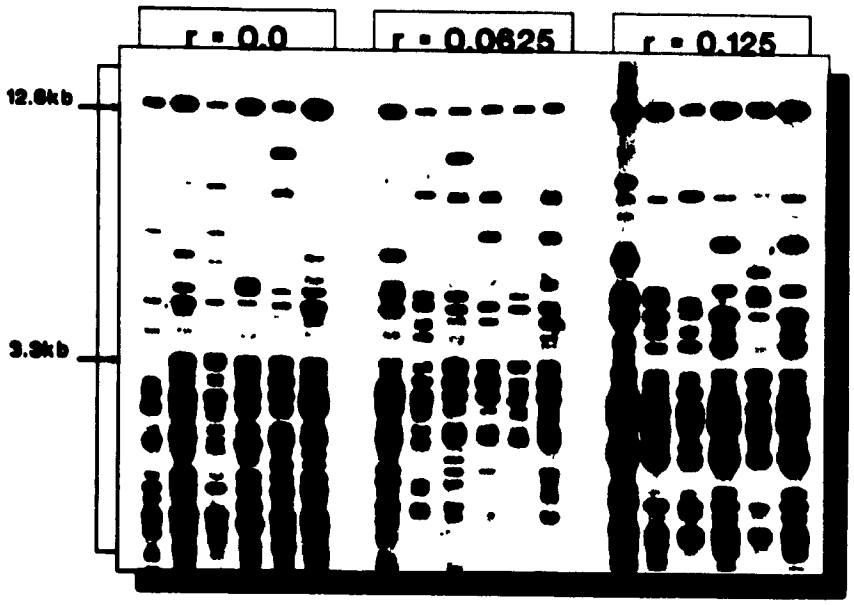

Figure 5.3 DNA fingerprints of 3 groups of animals with relationship coefficients of 0, 0.063 and 0.125, respectively. Each group has 3 pairs of animals. Only bands with apparent molecular weights between 3.3 kb and 12.6 kb (indicated by arrows) were considered in the analysis.

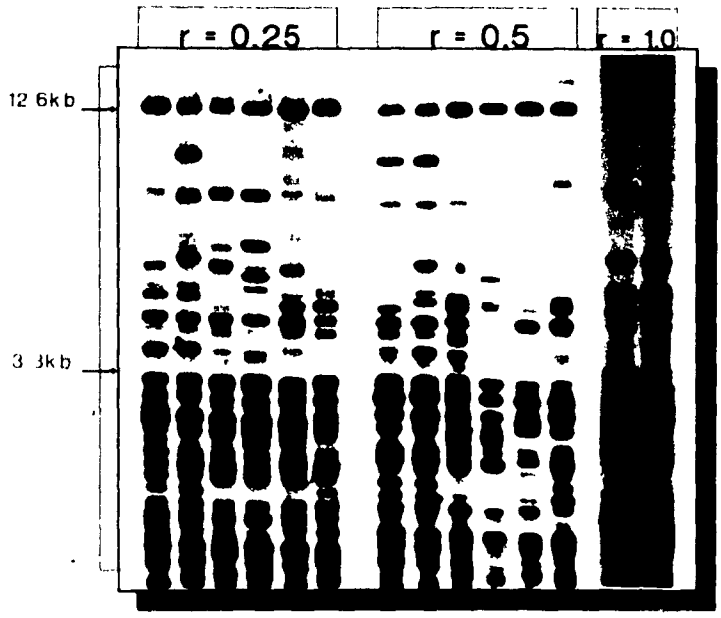

Figure 5.4 DNA fingerprints of 3 groups of animals with relationship coefficients of 0.25, 0.5 and 1 (homozygous twins), respectively. Group I and II have 3 pairs of animals for each. Only bands with apparent molecular weights between 3.3 kb and 12.6 kb (indicated by arrows) were considered in the analysis.

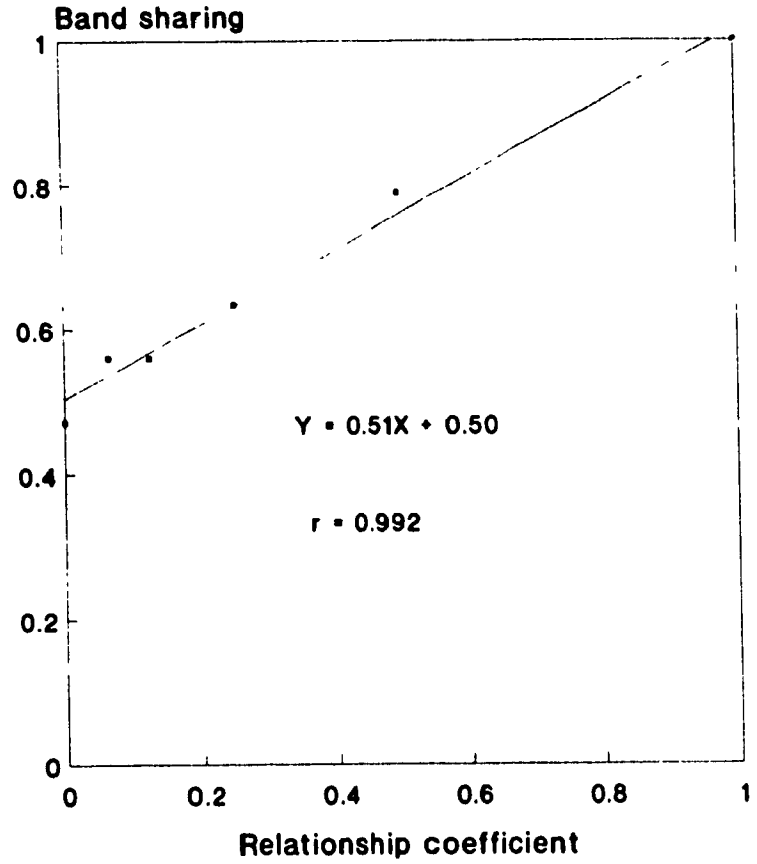

Figure 5.5 Dependence of band variability on relationship coefficient in Holstein dairy cattle. The DNA fingerprints used to derive this relationship were based on the analysis of 6 pairs of dairy cattle per genetic group with microsatellite probe, $(CAC)_5$ following digestion with RsaI. The slope of the linear approximation was 0.51 (± 0.09), and the intercept at no relationship was 0.50 (± 0.04) with correlation coefficient of 0.992.

6. Experiment III: Assessment of Genetic Variability of Holstein Dairy Cattle using DNA Fingerprinting

6.1 Introduction

The detection of the degree of genetic relatedness among individuals is important for many population and behavioral studies (Hamilton, 1964). Methods of calculation of inbreeding and relationship coefficients have been developed by Wright (1921), but the procedure requires an accurate pedigree record. A calibration curve which reflects the linear correlation between band sharing of DNA fingerprints and coefficient of relationship of dairy cattle was previously established (Chapter 5).

The equation in Figure 5.5 can be rearranged to generate an estimate of the degree of relatedness between individuals:

$$X = 1.96Y - 0.98$$

where Y is the band sharing between 2 tested animals and X is the estimated relationship coefficient.

In this chapter, the assessment of relationship coefficients for individual animals will be discussed, and the calibration curve previously obtained will be applied to estimate the genetic variability of Holstein dairy cattle population by comparing the results from DNA fingerprinting and computer assessment.

6.2 Materials and Methods

6.2.1 Genetic Group

Blood samples were obtained from 14 Holstein cows maintained at the Macdonald Campus. These cow were chosen randomly and represent about 17% of the herd. Pedigrees were not analyzed until the average relationship of the population was estimated using DNA fingerprinting.

Pedigrees were subsequently analyzed using computer program which was based on Wright's (1917) methods.

6.2.2 DNA Fingerprinting

DNA fingerprinting were carried out using the methods described previously in Chapter 3 and Chapter 5.2.2.

Band sharing (BS) of DNA fingerprints between 2 individuals were computed according to Chapter 5.3.1.

6.3 Results and Discussion

6.3.1 Assessment of Relationship Coefficients for Individual Animals

Using DNA fingerprinting, the first order of relatives ($r = 0.5$) could be statistically distinguished from individuals with relationship coefficients ≤ 0.063, with respect to the variance of each point as shown in Figure 6.1. Mostly, but not completely, the first order of relatives could be distinguished from animals with relationship coefficients of 0.125 and 0.25. Half sibs ($r = 0.25$) could be distinguished from animals with

relationship coefficients ≤ 0.063. Animals with the relationship coefficients of 0.063 or 0.25, could not be distinguished from those with relationship coefficient of 0.125. Animals with relationship coefficient of 0.063 could not be distinguished from non-related individuals. Homozygous twins could be easily distinguished by their completely identical band pattern of DNA fingerprints.

6.3.2 Assessment of Genetic Variability in the Dairy Herd

Average band sharing between all possible pairs of 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 animals were calculated in sequence (Figure 6.2). As the number of pairwise comparisons increased, the average amount of band sharing was observed to become constant at a value of 0.55. Thus, the inclusion of additional animals beyond a sample size of 6 had no apparent effect on the estimate of average band sharing (Figure 6.2). At a band sharing value of 0.55, according to the equation in chapter 6.1 the degree of relationship in the animals was estimated to 0.089. At a significance level of 0.5, this value is not different from the one by the computer analysis of pedigree 0.051. Therefore, DNA fingerprinting can likely be used to estimate the level of genetic relationship in animals for which pedigree data are not available. Of note (Table 5 and Figure 5.5) in animals with r values of 0.63 and 0.125, band sharing estimates overlap, hence animals in these classes cannot be differentiated with certainty on the basis of DNA analysis. In terms of analysis of individuals, this clearly limits the usefulness of the curve, at least in animals which have relatively low degrees of relationship. The reasons for this lack of discriminatory power at low levels of relationship are not clear, but have

been observed in other species as well (chickens: Kuhnlein et al., 1990; lion: Gilbert et al., 1991). The choice of enzyme and probe combination will influence the slope of the curve and increase the discriminatory power as previously described. It is likely that composite curves (i.e. different enzymes and different probes for describing different parts of the empirical relationship) would be useful, but the requisite studies have not, as yet, been carried out.

6.3.3 Potential Applications of DNA Fingerprinting

Inbreeding is known to decrease genetic variation in the population and thus limit the selection potential. In addition, inbreeding is associated with the unmasking of deleterious genes which may lower the performance of phenotypic traits such as fertility, survivability and size of offspring (Dalton, 1985). Thus, inbreeding is generally avoided in the breeding strategy.

The major tool used by breeders to avoid increasing levels of inbreeding in the population has been the maintenance of pedigree records but never-the-less the average amount of inbreeding has been shown to be increasing in the Holstein population. In 1981 - 1982, inbreeding and inter se relationship of bulls in CIAQ were 0.92% and 1.44%, whereas in 1987 - 1988 they were estimated to be 1.58% and 3.42%, respectively (Su, 1990). The widespread use of artificial insemination and the development of new reproductive strategies such as in vitro maturation and fertilization and embryo transfer is likely to contribute to this problem.

The application of DNA fingerprinting to dairy cattle management is a useful

adjunct to pedigree analysis: Firstly, DNA fingerprints allow an estimate of the degree of allelic variability between the mating pairs, thus providing an estimate of the potential response to selection. Since heterozygosity can be estimated, mating pairs can be selected to maximize the phenotypic benefits associated with heterosis. Secondly, the average inbreeding of a herd can be estimated using DNA fingerprinting. Currently, the main selection pressure is applied to the male, but as new reproductive strategies such as embryo splitting and transfer are more widely used, selection pressure will increasingly be applied to the female. DNA fingerprinting could be used to monitor variability within the herd. This would be especially important where adequate records are not maintained or in herds of cattle where natural mating is still used (e.g. beef cattle which are mainly raised on pasture land) (Ensminger, 1983).

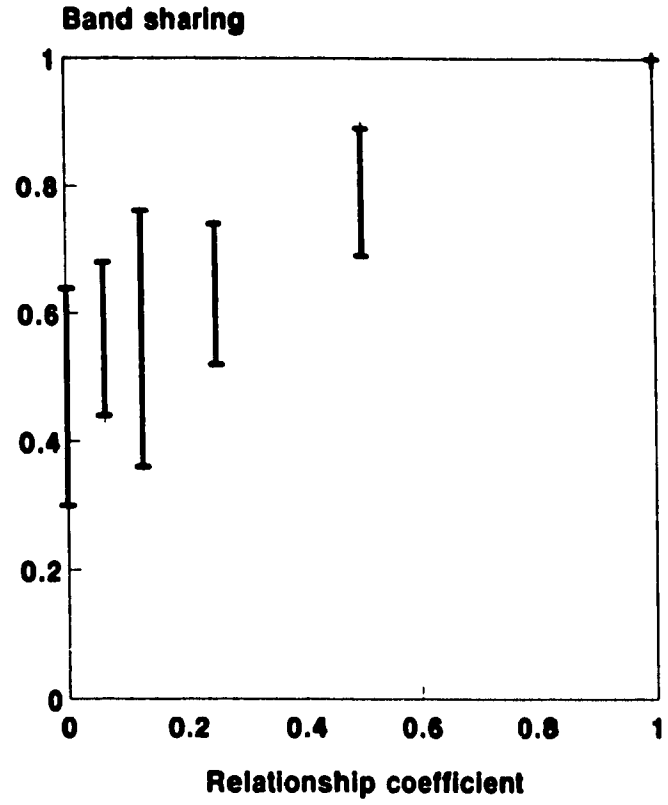

Figure 6.1 Variance at each sample point, indicating the resolution of DNA fingerprinting for the assessment of relationship coefficients of individual dairy cattle.

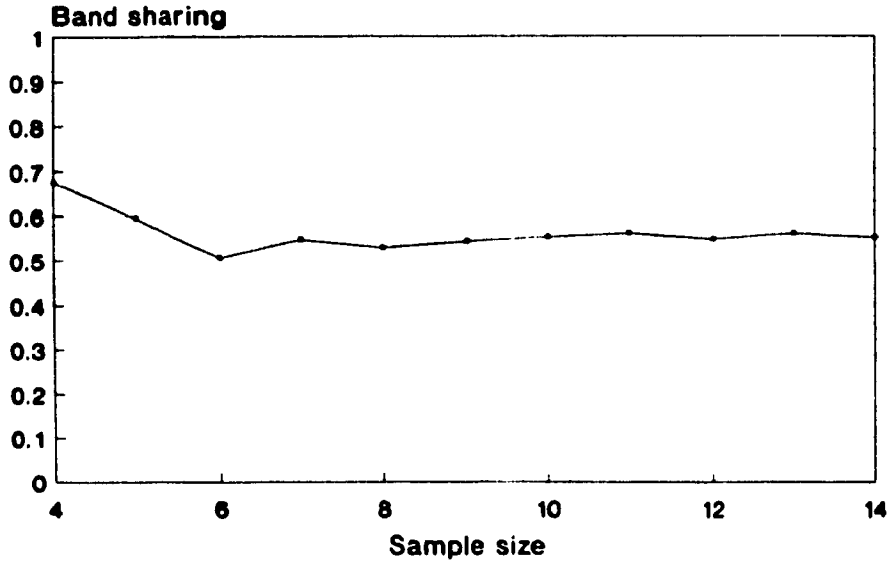

Figure 6.2 Average band sharing of DNA fingerprints of Holstein dairy cattle with different sample size. DNA fingerprints were based on the hybridization with the microsatellite probe, $(CAC)_5$ following digestion with RsaI. When the sample size was increased, the average band sharing became constant which represented the genetic variability of the dairy herd.

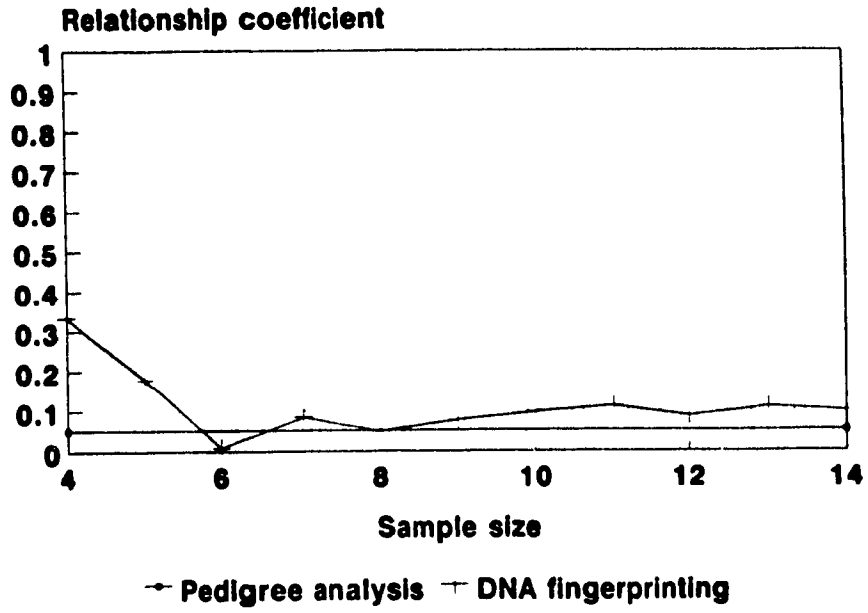

Figure 6.3 Genetic variability in a Holstein dairy cattle population with different sample sizes by comparing pedigree analysis and DNA fingerprinting. DNA fingerprints were based on the hybridization with the microsatellite probe, $(CAC)_5$ following digestion with RsaI.

7. Conclusion

1. Microsatellite DNA can be used as a probe for DNA fingerprinting in inbreeding analysis in poultry. Calibration curves relating band variability and inbreeding are variable with different VNTR probes and restriction enzymes.

2. Microsatellite DNA can be used as a probe for DNA fingerprinting in dairy cattle. Compared to a minisatellite DNA probe, it has the advantage that hybridization can be carried out with dried gels and thus does not require lengthy and labour intensive Southern blotting.

3. A calibration curve for Holstein dairy cattle relating the genetic relationship of animals to the band variability of their DNA fingerprints was established. The ability of this curve to determine relative kinship among individuals would have potential applications in animal breeding, as well as in wildlife biology.

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