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DNA profiling and its applications

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Every individual in the world can be identified at the molecular level on the basis of an extremely high level of polymorphism in the sequence of his or her DNA, which he or she inherits from his or her biological parents and is identical in every cell of the body. DNA fingerprinting, as this technique of identification is called, can confirm with certainty the parentage of an individual. Its applications are, therefore, in the establishment of paternity for personal reasons, in affiliation, wardship or divorce proceedings, in testacy disputes, and in provision to immigration authorities of clear evidence of a family relationship. The test has major applications in the field of forensic science, where samples left at the scene of a crime may be analysed against those from a person suspected of committing that crime; in the veterinary field, where pedigree confirmation may be required; in the medical world, for the diagnosis of genetic diseases; and in agriculture, for identification of seed stocks and germplasm. DNA probes derived from a DNA sequence first isolated from an Indian snake are useful in forensic investigation by DNA fingerprinting.

Crime is as old as human civilization and so is man's ingenuity in crime investigation. The accumulation of special knowledge and inventiveness has been a hallmark of our approach in solving all types of problems. Application of developments in the field of science and medicine in solving legal problems is a good example. It is fascinating how the idea of fingerprints was first introduced in India in 1860 as a proof of identity for the payment of military pensions to illiterate ex-soldiers by a British administrator, William Herschel, to stop widespread swindling. The extensive study and classification of fingerprints, necessary as prelude to their full use in crime investigation, was carried out by the English biologist Francis Galton, whose classic book *Fingerprints* was published in 1892. Based on this, the first murder case was solved in 1892 in Argentina. In Britain, the full adoption of fingerprints had to wait a few more years until the introduction of a new system of classification by Edward Henry, which is even now used all over the world. The estimated probability of fingerprint match in the human population is one in 10^{10}.

It has long been the ambition of the forensic scientist to be able to identify the origin of blood and body-fluid stains found at the scene of a crime with the same degree of certainty as fingerprints. Development of electrophoretic and immunologic techniques to type material in respect of polymorphic proteins and cellular antigens helped in fulfilling this ambition to a certain extent. However, while discrimination using genetic markers is conclusive in exclusion, the chance of inclusion or positive identification using these markers does not exceed 99.7% for a total of 20 polymorphic systems used together (HLA, red cell antigen, serum proteins, red cell enzymes)[1]. Besides, blood evidence from the scene of crime usually enters the laboratory in the form of dried stains, and the drying, ageing and contamination associated with these stains limits the number of markers for which a sample can be typed. There has been, therefore, clearly a need to have another type of marker that not only survives better than proteins but also possesses a larger number of distinguishable alleles. This need was fulfilled by the

recent discovery by Jeffreys *et al.*[2,3] by which individual-specific DNA polymorphism can be detected. This technique is popularly known as DNA fingerprinting or DNA profiling.

Principle

Encoded in human DNA are about 100,000 genes. These represent only about 5% of the total DNA in the chromosomes. The function of the remaining 95% of the genome is not yet understood. One of the components of this 'extra' DNA consists of sets of base sequences repeated numerous times and called minisatellites. The origin and significance of these tandem repeats are a mystery, but the minisatellites show a very high level of allelic variation in the number of repeat units and therefore in the length of the minisatellite. They offer a means of distinguishing one individual from another through DNA typing[3].

Knowledge of this variation is not new, but it was only recently that methods became available for its study at molecular level. Mutational processes that bring about variation in the human genome include transposition, gene conversion and unequal exchange during recombination between duplicated DNA sequences, and polymerase slippage at replication forks. Botstein *et al.*[4] suggested the restriction-fragment-length-polymorphism (RFLP) technique of DNA analysis as an approach to mapping the human genome. Wyman and White[5] discovered the first hypervariable locus (HVR) in the human genome which was shown to be tandemly repeated. Since then many families of HVRs dispersed in the genome have been discovered[6]. Jeffreys *et al.*[2,3] found that myoglobin minisatellite detected other human minisatellites, some of which are highly polymorphic. By hybridizing a cloned probe containing one of the four different repeat units of 33-base-pair-long sequences from the first intron of the myoglobin gene to restriction enzyme-digested genomic DNA, Jeffreys *et al.* demonstrated a highly polymorphic but individual-specific hybridization pattern.

Human DNA fingerprints detected by these polycore probes are highly individual-specific (with the exception of monozygotic twins who cannot be distinguished using these or any other known DNA probes) and are inherited in typical Mendelian fashion; they assort independently from parent to offspring. There is no detectable linkage disequilibrium between any pair of resolved DNA fragments[7], which act as independent genetic markers[8,9]. The chance that a second, unrelated individual would contain all of the first individual's DNA-fingerprint bands detected by probes 33.6 and 33.15 is estimated at 2×10^{-22}.

Technique

The methods used in DNA profiling are conventional techniques of molecular biology: isolation of DNA from the biological sample (such as blood, blood stains, semen stains, vaginal swabs or bone marrow), digestion of the DNA with restriction enzymes, fractionation of the resultant DNA fragments on the basis of size by agarose gel electrophoresis, transfer of the fractionated DNA fragments onto blotting membrane, hybridization of the DNA on the membrane with labelled probe, removal of nonhybridized probe by washing, and detection of hybridized probe.

There are a number of alternative techniques in vogue for each of the above steps and these are evolving rapidly. In my laboratory, DNA is isolated by SDS–proteinase K digestion and phenol–chloroform extraction. After digestion of the DNA with the appropriate restriction enzyme, the samples are run on a 0.8% agarose horizontal slab gel at 3–4 V cm^{-1}. Some workers prefer vertical agarose gels. Field inversion gel electrophoresis (FIGE) has been introduced to increase the resolution of the bands. Samples are transferred to blotting membranes by vacuum blotting[10], Southern blotting[11], or electrotransfer[12]. A variety of blotting membranes are available. These include nitrocellulose, charged or uncharged nylon, and polyvinyldifluoride (PVDF) membranes. We find that the combination of vacuum blotting and nylon membranes results in quick and efficient transfer. Recently, some workers have revived the method of employing dried gel directly for subsequent hybridization, getting reasonable results with small-sized oligo probes. The DNA on the blotted membrane is hybridized with the labelled probe in the appropriate solution and at the right temperature. The probe can be labelled by a variety of methods, which include nick translation, random hexamer-primed labelling, and single-strand M13 labelling using an M13 probe primer[13]. Absence of self-annealing in the latter labelling method makes it advantageous over other methods. A variety of labelled nucleotides are available, including ^{32}P-, biotin- and digoxigenin-labelled nucleotides. A number of protocols are known that allow optimum hybridization. We hybridize the membrane in a solution containing 7% SDS in 0.5 M phosphate buffer (pH 7.5) at 60°C. The hybridized blot is washed with a series of solutions that provides the desired level of stringency of hybridization. The radioactively labelled hybridized probe on the membrane is detected by autoradiography. Nonradioactively labelled probe is detected by appropriate enzymatic or chemiluminescent detection method.

Probes

A stretch of DNA that can detect a target sequence in the genome is called a probe. Almost any cloned segment of a unique sequence of human DNA can detect RFLPs in human DNA, but these variations

result from single-base substitutions or micro-deletions/-insertions that create or destroy restriction endonuclease cleavage sites. The resulting RFLPs are restriction enzyme-specific and dimorphic and therefore not useful for forensic use On the other hand, tandem-repeated minisatellite regions are variable enough for efficient fingerprinting. There are mainly two types of probes that detect such minisatellites.

Multi-locus probes

A subset of human minisatellites share a common 'core' sequence embedded in each repeat unit, which is involved in the generation of hypervariable tandem-repeated loci by serving as polymerase slippage signal[2,3]. These core-sequence probes detect variable-number tandem repeats (VNTR) in the genomes of other vertebrates as well as plants[8,9,14–22]. Some of the multi-locus probes (MLPs) that have been extensively used are 33.6 and 33.15 (ref. 3), M13 (ref. 23), Bkm and Bkm-derived clone 2(8) (refs. 24–26), *Drosophila per* gene related to 6-base-pair tandem repeat[27], tandemly repeated core sequence downstream to α-globin gene[28], and synthetic oligodeoxyribonucleotide probes[29–31]. Probes 33.6 and 33.15 have already been extensively used in the UK. We, in India, are using the Bkm probe in paternity disputes and crime investigations. Singh *et al.*[32] isolated a sex chromosome-associated repetitive DNA from female Indian banded krait *Bungarus fasciatus* as a minor satellite-DNA component and designated it Bkm. Bkm sequences have been shown to be present in many eukaryotes but absent in any appreciable quantity in prokaryotes[33]. These are preferentially concentrated in the sex-determining region of the sex chromosomes of *Drosophila*, snakes, birds, mouse and man[34–36,24], but are also present scattered in other parts of the genome. The conserved components of Bkm are long arrays of repeats of the tetranucleotide GATA (ref. 35). These scattered copies of Bkm can be used to detect RFLP after restricting human DNA with the restriction enzymes *Hin*fI, *Alu*I and *Bst*NI and Southern hybridizing using Bkm probe or Bkm-associated clone 2(8) (ref. 24). The use of this variability, detected by us earlier, for DNA fingerprinting has now been thoroughly tested and its potential use in forensic investigations confirmed. The Bkm probe 2(8) detects qualitatively more polymorphic regions in the human genome than the probe used by Jeffreys. A mean allele frequency of approximately 0.2 and heterozygosity of approximately 90% are found in the population. Because of the association of Bkm sequences with the sex-determining chromosome, by using the Bkm probe we have recovered clones from human and mouse genomic libraries that are Y-chromosome-specific and, therefore, can be used for sexing biological samples. Population analysis of 300 individuals using a combination of Bkm and Bkm-associated probes has revealed a novel amplification of specific DNA fragments that exist as free copies in the cell (our unpublished results). After extensive population studies and calculation of allele frequencies for different populations in India, Bkm is being successfully used in forensic investigation. There are 30 court cases, mainly involving paternity disputes, that have been forwarded to the Centre for Cellular and Molecular Biology for DNA-fingerprint analysis. In one such case from Kerala, the DNA-fingerprint evidence was presented in the court and, for the first time in India, accepted as an infallible evidence by the court of law (see Figure 1)

The banded krait *Bungarus fasciatus*, a poisonous Indian snake, from which a minisatellite DNA, Bkm, was isolated A Bkm-derived probe is being used for DNA fingerprinting in forensic and other investigations

The probability of false association between unrelated individuals in the use of an MLP in combination with one or two enzymes is between 4×10^{-16} and 4×10^{-30}. DNA fingerprints developed by MLPs therefore help in establishing positive associations[37,38]. Mutations altering the number of repeats, and therefore length of minisatellite, detected by MLPs have been observed to occur in DNA fingerprints at a mean frequency of 0.006 in the germline; hence 25% children will show one mutant band, 1% will show two, and 0.05% will show three. However, one to three such non-ascribable bands can be accommodated in statistical analysis, but three or more pose uncertainties[39].

Advantages and disadvantages. A single MLP provides sufficient numbers of variable bands to establish positive identity of an individual beyond reasonable doubt. It therefore constitutes a single powerful test for positive identification of stain or tissue, or of parentage, including paternity The probability of observing identical patterns for two individuals with an MLP is of the order of one in 10^{14} to one in 10^{30}. Considering that the world population is less than 5×10^9, DNA-fingerprint patterns are highly unique.

An MLP cannot, however, be used reliably to type mixed samples, for example in the case of multiple rape.

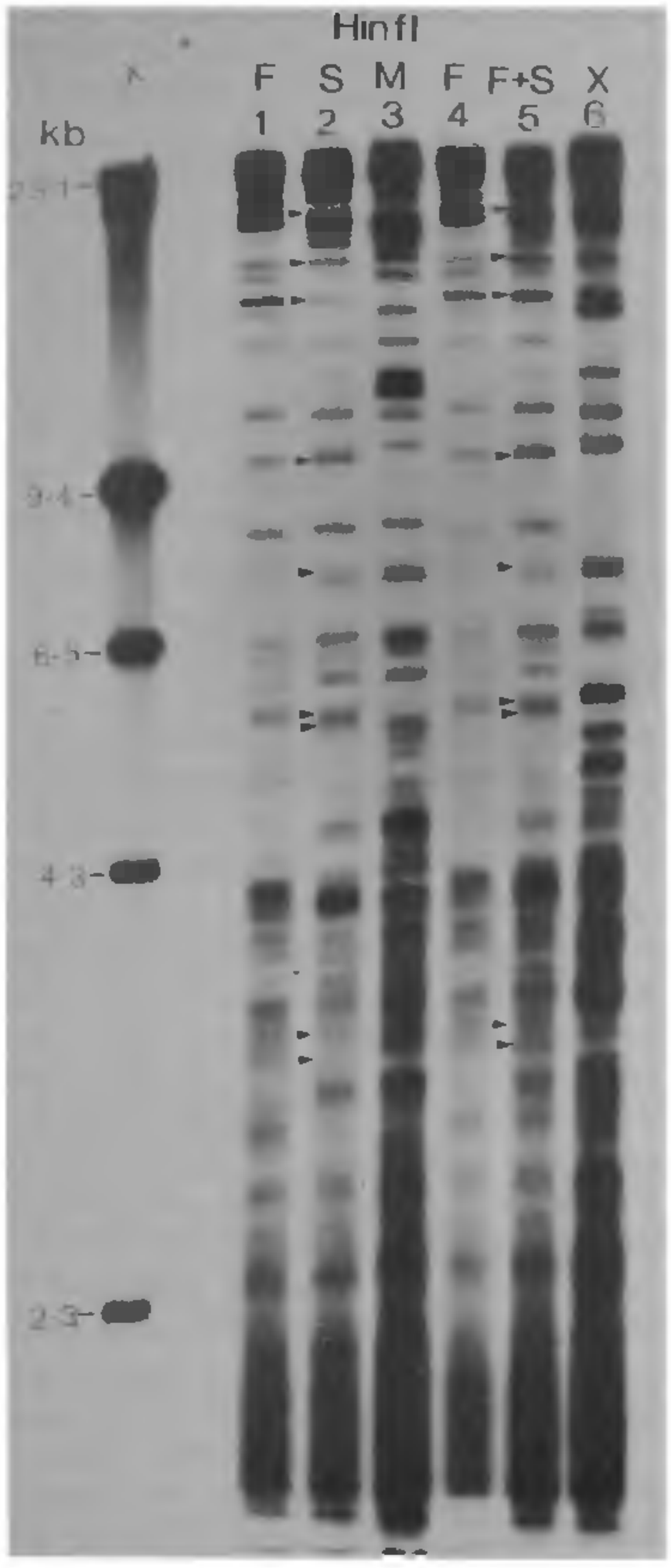

Figure 1. A case relating to the paternity of a child solved by using the Bkm probe DNA fingerprints of track 1, alleged father (F); track 2, the male child (S); track 3, the mother (M), track 4, alleged father (F); track 5, equal amounts of DNA of alleged father and the male child, track 6, an unrelated individual (X).
(i) From a comparison of the DNA profile in track 2 with those in track 1 and track 3, it is obvious that every band in track 2 is derived (inherited) from either the alleged father (track 1) or the mother (track 3). The mother and the alleged father are, therefore, the biological parents.
(ii) In track 2 there are nine bands (marked with arrows) that are not present in track 3 (the mother). These bands, therefore, must be inherited from the biological father. When these bands are compared with the DNA profile in track 1 (the alleged father) all the 9 bands shown by the arrows, without any exception, are detected. To confirm that these bands are exactly the same, equal amounts of DNA of the alleged father and the child were run in track 5 along with the alleged father's DNA in track 4. In track 5, all the nine bands shown by arrows are exactly the same as in track 4. Had these bands been different, additional bands would have appeared adjacent to the bands shown by the arrows. But this is not so.
(iii) Three additional restriction enzymes *Hae*III, *Alu*I and *Bst*NI were used. The DNA profile with these enzymes revealed six additional bands in the child's DNA inherited from the father, which were present in the alleged father (not shown).
(iv) The DNA profile of an unrelated individual in track 6 (X) used as a control shows, as expected, a very different pattern.
Thus all the 15 bands inherited by the child from his biological father are detected in the DNA profile of the alleged father, without an exception. This proves beyond any reasonable doubt that the alleged father is the biological father of the child. The chance that he is unrelated to the child in question but happens to share all 15 fragments is $0.2^{15} = 3.27 \times 10^{-11}$. The figure 0.2 is the mean probability of occurrence of a fragment in two unrelated individuals. It is therefore concluded that the alleged father must be the true father of the child

It requires high-molecular-weight DNA in larger quantities. With MLPs the alleles are not well defined and their specific association with particular chromosomes is difficult to show. The probability of shared bands between individuals increases for smaller fragments. This may be due to comigration of 'unrelated' fragments in such cases. There is also a possibility of low-molecular-weight fragments running off the gel, resulting in an underestimation of the minisatellite alleles. Pooling of loci to determine average frequency of variability may give higher genetic variability than is really present.

Locus-specific probe

A probe that detects a single hypervariable locus is called a locus-specific or a single-locus probe (SLP). By selective cloning of large minisatellites it has been possible to isolate some of the most variable loci in the human genome[40,41]. Fifteen hypervariable loci, with heterozygosities ranging from 60 to 99.4%, with an average value of 95%, have been isolated[42]. Collaborative Research Inc. (Bedford, USA) and Howard Hughes Medical Institute (Salt Lake City, USA) have reported developing hundreds of SLPs. Under high-stringency-hybridization conditions, a large minisatellite-fragment clone acts as SLP. The MS1 probe provides several alleles ranging from 1 to 23 kb in length. The hybridization patterns given by SLPs are very simple and consist of two fragments per individual. Loci with heterozygosities higher than 95% rarely show evidence of common alleles.

Advantages and disadvantages. SLPs are simpler to use compared to MLPs. They require very little genomic DNA, as little as 50 ng, corresponding to 2 μl blood, 1 μl semen or 20 μl saliva, or a few pulled strands of hair with roots. Unlike in the case of MLPs, slightly

degraded DNA can also be used. SLPs are most uniquely used in identifying mixed DNA samples, for example in multiple rape cases. They are also extremely useful in screening of large samples (1000 or more) by pool typing to identify a serial rapist or most potential suspects. Patterns obtained are simple and easy to interpret in courts. Chromosomal locations of these probes are well defined The corresponding probabilities of random identity are of the order of one in 1000 to one in 10,000. One therefore has to use a set of five or six different probes to establish identity. Both MLPs and SLPs are currently being used in forensic casework. Multilocus probes, however, are being used more extensively in cases of questioned paternity.

DNA amplification by polymerase chain reaction

The polymerase chain reaction (PCR) is one of the most ingenious developments in molecular biology in recent years. By this procedure, a single copy of a target DNA sequence, defined by oligonucleotide primers flanking the ends of the sequence, can be amplified to millions of copies in a short period. This is ideally suited for forensic use because it allows DNA profiling of a single strand of human hair or a minute blood stain, which would otherwise be too small to be of value. It also allows the typing of samples too degraded for RFLP analysis. The *HLA-DQ* locus, with six typeable alleles, is the first genetic polymorphism to be analysed by PCR in forensic casework.

Mitochondrial DNA in forensic investigation

Mitochondrial-DNA (mt DNA) fingerprinting is another promising area that, in association with conventional DNA fingerprinting, demands attention. Sequences of mt DNA also are highly polymorphic. Maternal inheritance of mt DNA makes it a unique tool in studies of populations. The use of PCR in association with restriction analysis and sequencing of any given piece of hypervariable mt DNA is ideally suited for forensic investigation, particularly in determining the maternity of any given child[43,44]. Mary Claire-King of the University of California at Berkeley employed mt-DNA analysis to determine the maternal relatives of children who were born in prison in Argentina during the military rule that lasted from 1976 to 1983.

Mapping fine-grained sequence variation in minisatellites

The DNA-fingerprinting technique opens up the prospect of tracing both paternal and maternal lineages during human evolution, because DNA sequences reflect past mutational events. Jeffreys *et al.*[45] have developed a sophisticated method for isolating and then mapping mutation distributions within a single molecule of a given minisatellite-DNA locus derived from blood cells and sperm. Isolation of a single allele of a given array has been achieved with the use of two rounds of DNA amplification by PCR. The mapping procedure is based on an 'end-labelling' technique to locate the precise position of any repeat in an array that contains one *Hae*III restriction site, with reference to a *Hin*fI restriction site that exists in all repeats of the locus in question. By this means an exact map of the distribution of repeats containing a *Hae*III site within an array could be determined. There is sufficient variation in *Hae*III-site-distribution maps for the system to be capable of distinguishing 10^{70} different allelic states and of measuring somatic and germline mutation rates as they occur within a single individual.

Applications

1. In pedigree analysis and establishing paternity: Approximately half of a DNA-fingerprint pattern is derived from the father and half from the mother. DNA fingerprinting can therefore be used to trace the pedigree and to establish paternity.
2. In establishing family relationship for immigration authorities[37,46]
3. In rape cases: A few hair roots, or a small sample of blood, buccal smear, semen spots or skin tissue left behind by the criminal is sufficient to obtain genetic fingerprints. These can be compared with that of the suspect for confirming the rape charge beyond any doubt. Vaginal swabs are particularly suited for DNA analysis because they are generally dried and stored frozen. Blood stains stored under favourable conditions have been successfully analysed up to three years and semen stains up to four years after collection. It is possible to separate the DNA of male origin when semen is mixed with vaginal secretions. A vaginal swab taken up to 20 hours after intercourse can be used to isolate DNA from sperm.
4. In identification of mutilated dead bodies from their tissue remnants with the help of DNA fingerprints of close relatives
5. In social security record identification
6. In solving murder cases by DNA-fingerprint analysis of blood swabs taken from the murder weapon used and found in the possession of the accused, or of blood spots on accused's clothes, and comparing the fingerprints with the DNA fingerprints of the victim; the two should be identical
7. In sexing biological samples by *in situ* hybridization with Y chromosome-specific probes
8. In detecting specific bands in close linkage with disease loci in large pedigrees, and marker loss in tumours, which then could be used for diagnosis purposes; this may help in identifying and cloning the defective gene[47-49]
9. In characterization of cell cultures: Fingerprinting mammalian cell cultures to confirm genetic homogeneity

or detect contamination, verification of cell hybrids and monoclonal cell lines[50,51]

10. In identification of post-transplant cell population[52]

11. In animal-breeding programmes: DNA fingerprinting can be used in providing assurance in livestock breeding[53]

12. In plant-breeding programmes: DNA fingerprinting can be used for authentication of seed stocks and germplasm[54,55]

13. In demographic studies of animal populations[56,57]

Precautions

DNA profiling is technically demanding. There are cases on record in the US where mistakes seem to have been prompted by human errors. There is a need for quality control. Every precaution should be taken to ensure preparation of high-molecular-weight DNA, complete digestion of the samples with appropriate enzymes, and perfect transfer and hybridization of the blot to obtain distinct bands with appropriate controls. If necessary the analysis should be repeated. The DNA-fingerprinting test, if performed properly, is infallible. However, there is need to interpret DNA typing with a thorough understanding of the populations involved[58].

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Application No. 1000 DEL 88 for patenting Bkm and Bkm-derived probes is under consideration.