5 Multilocus DNA Fingerprinting

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1 Introduction

To many workers in the field, the classical multilocus fingerprinting approaches based on the electrophoretic separation of DNA restriction fragments followed by probing with suitable probes is now an obsolete methodology. This is because such procedures are laborious, require much technical skill, depend on high molecular weight DNA to start with and do not easily lend themselves to automation and electronic data storage. Multilocus DNA fingerprinting also requires a different statistical approach than single locus profiling (see the chapter of Krawczak, this volume). For three main reasons, however, this approach should remain in the discussion: (1) It is the method in kinship testing and trace analysis work least likely to unravel unwanted additional information on other personal genetic traits: other than with single locus techniques, the assignment of multilocus DNA fingerprint bands to alleles to loci is not obvious. (2) The shortcomings listed above are not unsurmountable in principle; technological progress may, at some future time, manage to overcome the deficits while retaining the benefits. (3) Some multilocus DNA systems work well beyond the species on which they have been developed, which is due to the fact that the target sequences of many multilocus DNA probes are both conserved and variable throughout the plant and animal kingdoms.

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2 Methods

2.1 Principles

The multilocus DNA approach stood at the very beginning of the DNA fingerprinting era, and in fact the latter term originally was associated exclusively with this technique [1]. The method was developed in the early 1980s by Alec Jeffreys and co-workers, and was soon thereafter complemented by Jörg T. Epplen and his group [2, 3]. In principle, a multilocus DNA fingerprint could be generated by simultaneously applying several DNA probes, each of which is specific for one particular locus, or by the application of a single DNA probe which reacts simultaneously with several loci. Indeed, both approaches have been adopted, but one should be careful not to misinterpret a single probe/multilocus approach as a simple superimposition of single locus patterns.

Multilocus fingerprints make use of the fact that many tandem repeat arrays recur in various parts of the genome and in variable numbers of tandem repeat elements. The repeat arrays themselves are used as a probe, which is applied to a Southern blot of genomic DNA cut with restriction enzymes and fractionated by electrophoresis. The blotting step can be avoided when probes such as oligonucleotides are used that are capable of penetrating dried agarose gels.

Multilocus DNA fingerprint patterns closely resemble the bar codes used in supermarkets for the identification of goods. Both the position and the intensity of the contributing bands differ between individuals (Figs 1–3). Highly informative test systems reveal so much variability that the chance of bands comigrating in unrelated individuals is minimized, and qualitative decision making is hardly ever problematic. An inspection of Figures 1–3 reveals intuitively that the band patterns in Figure 1 are likely to derive from a random sample of individuals, in Figure 2 will be taken from pairs of identical twins and in Figure 3 will derive from pairs of closely related people, such as parent and child or brother and sister. The variability found in multilocus DNA fingerprints is thought to be due to two factors: (1) the average number of repeat elements contained in any one array, and (2) the distance between the repeat array itself and the nearest recognition sites of the enzymes used for digesting the genomic DNA sample. Both these variables determine the proportion of each of the visualized restriction fragments actually occupied by the repeat array. Let us assume that this proportion is large; then, both the band position and its intensity will be roughly proportional to the number of elements of which the repeat array consists. If one assumes, however, that the proportion of the restriction fragment occupied by the repeat itself is very small, then the band position will depend on the sequence characteristics outside this array, and the repeat element number will determine the band intensity only. A complication arises by the fact that it is virtually impossible to decide whether a band shared between two individuals in terms of position, but exhibiting different intensity, reflects variability in the number of repeat elements on allelic stretches or results from comigration of nonallelic fragments polymorphic with respect to their length.

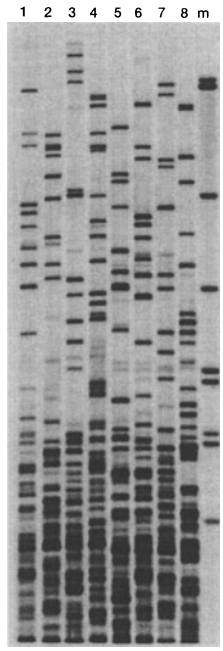

Figure 1 $(CAC)_5$ multilocus DNA fingerprint of *Hinf* I-digested DNA of eight unrelated individuals (m = size marker).

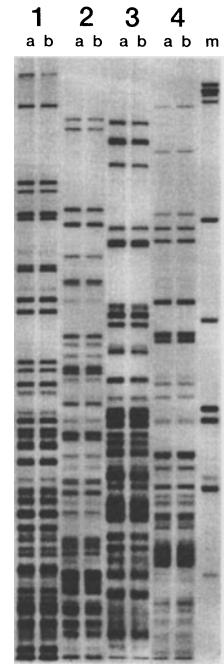

Figure 2 $(CAC)_5$ multilocus DNA fingerprint of *Hinf* I-digested DNA of four pairs of identical twins (m = size marker).

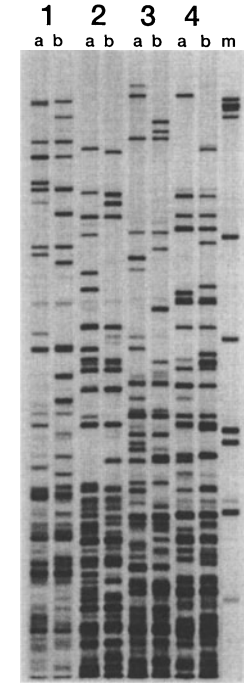

Figure 3 $(CAC)_5$ multilocus DNA fingerprint of *Hinf* I-digested DNA of four mother-infant pairs (m = size marker).

2.2 Strategic considerations

It is obvious that there must be an optimum of loci contributing to a multilocus DNA fingerprint on a finite, predefined gel length. Too few loci would generate little more information than single locus strategies; too many would overcrowd the pattern and ultimately lead to a noninterpretable smear. While gel systems differ with their overall resolving ability, they all share the feature that there are intermediate molecular weight regions for which resolution is optimal; both very large and very small molecules are poorly discernible. Therefore, a size "window" is usually chosen, often between 3 or 4 and 20 kbp, within which the interpretation of the electrophoretic pattern is most accurate and convenient, while the other gel regions are disregarded. This precaution minimizes the aforementioned risk that nonallelic fragments comigrate and thus cannot be distinguished as such.

Many different multilocus DNA systems have been developed, and a few of them will be discussed below in some more detail. A cautionary *note:* Other

than in well-defined single locus systems, where, by definition, each is unique, the multilocus systems that have been put to use have been shown to overlap with respect to their genomic target sequences; therefore, other than in single locus work, where adding new polymorphic systems to an already existing set will always increase informativity, expanding a set of multilocus DNA systems may not always fulfill this expectation. Unless the molecular basis of a multilocus DNA fingerprint is fully understood, it may be dangerous to apply several such systems simultaneously and treat their results as independent evidence.

2.3 Examples

The "classical" multilocus DNA probes, developed in the laboratory of Alec Jeffreys, named 33.6 and 33.15, are derived from a human minisatellite [2]. In the fragment size window usually chosen for these systems, an average of 17 variable DNA bands per individual is produced. The repeat arrays in genomic DNA detected by these probes have a core sequence of GGGCAGGANG (where N stands for any of the four bases). The number of core elements per array varies between 3 and 40. Furthermore, there is considerable interlocus variation with respect to the extent of sequence heterogeneity among the elements that constitute a repeat array. This observation dates back to the early 1980s [4], but was not exploited for practical use before 1991, when Jeffreys and coworkers invented the technique of "minisatellite variant repeat mapping" by PCR, also called "digital fingerprint" [5]. This approach lends itself optimally to single locus application and is therefore not discussed here in detail. One observation, however, is of interest in terms of understanding the basic principles of variability at minisatellite loci: Probes derived from repeat arrays with high element sequence homogeneity identify a particularly high degree of repeat number polymorphism in the population; conversely, probes derived from repeat arrays with a high degree of sequence heterogeneity identify a relatively lower degree of repeat number polymorphism. One would expect such a result, when, as hypothesized, the minisatellite repeat copy number polymorphism is generated mainly by crossing-over events during meiosis [6].

One further consequence for correctly interpreting multilocus DNA fingerprint patterns follows from the described observation of sequence heterogeneity within minisatellite repeat arrays: hybrid molecules formed between a probe and a not perfectly matched target sequence are less stable than perfectly matched DNA duplexes. Depending on the experimental conditions, i.e. the stringency of molecular hybridisation as determined by temperature and ionic strength of the reaction assay and molecule length, band intensity may vary, thus forming a further variable besides repeat element number variation and fortuitous comigration of nonallelic fragments.

Several further minisatellite probe systems used for the generation of multilocus DNA fingerprints include sequences from bacteriophage M13 [7], the 3′

hypervariable region of α-globin [8], the major eggshell protein of *Schistosoma mansonii* [9] and the mouse homolog of the *Drosophila* "Per" gene [10].

Jörg Epplen and co-workers have developed an approach to multilocus DNA fingerprinting that differs from the ones so far discussed in many theoretical and technical properties. The procedure is based on an earlier observation that the occurrence of very simple repeat sequences are a common feature of nearly all eukaryotic genomes [3]. Their initial finding was that the Bkm"satellite" DNA sequence isolated from the genome of a snake, which cross-reacts with many other species' DNA and generates interindividual band variation on Southern blots, contains stretches of the tetranucleotides GATA or GACA [11, 12]. This finding was then systematically explored in that further oligonucletide probes consisting of multimers of bi-, tri- and tetranucleotide cores were synthesized and tested for their ability to generate fingerprints. These include $(CA)_n$, $(CT)_n$, $(GATA)_n$, $(GACA)_n$, $(GAA)_n$, $(GGAT)_n$ and $(TCC)_n$. In humans, the highest degree of genetic individualisation was achieved, however, using $(CAC)_5$ or its complement, $(GTG)_5$ [13]. This system has been popular in paternity testing (Fig. 4) for many years, especially in German laboratories [14], but has received attention from elsewhere as well [15]. The full laboratory procedure is given below. Since there is considerable inconsistency regarding repeat DNA nomenclature, it should be pointed out here that the Epplen methodology uses *micro*satellite probes for generating *multi*locus DNA fingerprints resembling *mini*satellite patterns (I follow the definitions given in [6], according to which minisatellites have a degree of repetition per locus of 10 to 10^3 and repeat unit lengths of 9 to 100, while the corresponding figures for microsatellites are 10 to 10^2 and 1 to 6).

It has never been clarified how polymorphisms detected by $(CAC)_5$ actually come about. It may be speculated that the minisatellite appearance of the bands in this system are due to perfectly and imperfectly matching genomic counterparts located side by side.

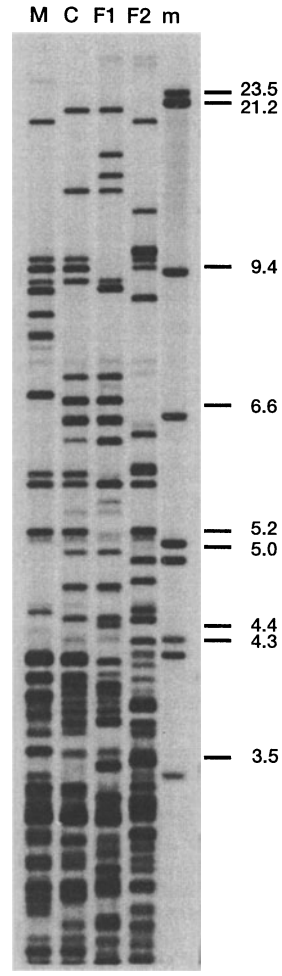

Figure 4 $(CAC)_5$ multilocus DNA fingerprint of *Hinf* I-digested DNA of a mother (M), her child (C) and two putative fathers (F1, F2) (m = size marker).

All nonmaternal bands seen in the child are contained in the pattern of F1, but not F2. The qualitative interpretation is that F1 is likely to be the true father of C, while F2 can be excluded. The quantitative approach to decision making is outlined in the chapter of Krawczak in this volume.

Armour and co-workers have contributed an interesting extension of the oligonucleotide fingerprint methodology by polymerizing random oligonucleotides to form synthetic tandem repeat probes; they were able to show that such probes hybridize to loci which are detected also by probes derived from naturally occurring minisatellite sequences [16].

Another further development of the basic methodology of multilocus DNA fingerprinting is 2D DNA fingerprinting, which allows simultaneous analysis of both size and sequence of multilocus DNA probe targets. This approach is discussed in detail in the chapter of Marczinek et al. in this volume.

Protocol 1 Multilocus DNA fingerprinting in man using $(CAC)_5$ as DNA probe

1. **Extraction of high molecular weight DNA**: This can be done using conventional procedures, such as phenol-chloroform extraction or proteinase K salting out methods (see also the chapter of Lubjuhn and Sauer, this volume). The final concentration of genomic DNA should be 0.5 µg/ml.
2. **Restriction enzyme digestion**: Digest 4.5 µg of genomic DNA with 8 units of restriction enzyme (*Hinf* I or *Hae* III) per µg of DNA for at least 6 hours in the presence of 1 mM spermidine in a final volume of 32 µl. Use 0.5-µg aliquots to check for completeness of digestion by running on agarose gels (alongside aliquots of undigested DNA – see *Troubleshooting*), and document these gels for later inspection in the case of suspected "hidden partials".
3. **Gel electrophoresis**: Two to four micrograms of DNA are run on a 0.95% horizontal agarose gel, 25 cm long and 0.45 cm thick; gel slots should be 1 mm long by 5 mm wide and at most 2 mm apart. Loading more than 2 µg requires prior sample concentration. The electrophoresis buffer is made up from 40 mM Tris-HCl, pH 8.3, 12 mM Na-acetate and 2mM EDTA. Add bromophenol blue when loading the samples. Run the gel at 4 °C for approximately 60 hours in order to bring the bromophenol blue front to a theoretical distance of about 36 cm from the start. For monitoring the run, one can reload bromophenol blue into one slot every 24 hours. Replace the electrophoresis buffer every 24 hours.
4. **Blotting**: Whereas radioactively labelled probes can be used for in-gel hybridisation, digoxigenin (DIG)-labelled probes work much better on blots, which also yield sharper bands. Wet one layer of Whatman paper 3MM and one Hybond-N (Amersham-Pharmacia) membrane in 2 × SSC, and put the Whatman paper, the Hybond-N membrane and the gel onto a vacuum blotter. Start the vacuum pump, and add and remove by gently decanting the following solutions: 0.2 M HCl for 10 min; 0.5 M NaOH, 0.15 M NaCl for 15 min; 1 M Tris, 2 M NaCl, pH 5.5 for 15 min, and finally 20 × SSC for 40 min. Make sure that the gel never dries during the entire process. After transfer, wash the Hybond-N membrane twice in 2 × SSC for 5 min, dry it between two layers of Whatman paper 3MM, and bake it for 2 hours at 80 °C. Alternatively, the DNA can be cross-linked to the membrane by ultraviolet (UV) treatment.

5. **Hybridisation**: Soak the membrane in 2 × SSC, transfer it into a glass tube with silicone stoppers, add 25 ml of (pre)hybridisation solution consisting of 7% SDS, 0.25 M $NaPO_4$ (pH 7.2), 1% Blocking reagent (Roche-Boehringer), brought into solution at 55 °C, and prehybridise in a rotating incubator for 2 hours at 45 °C. Dissolve the oligonucleotide probe in distilled water (1 pmol/µl), and add 35 µl to 10 ml of hybridisation solution. Decant the prehybridisation solution and replace it by the hybridisation solution containing the probe. Hybridise at 45 °C for 4 hours. Both prehybridisation and probe-containing hybridisation solutions can be stored at –20 °C and reused a few times.
6. **Washing**: Wash the membrane in 200 ml 6 × SSC: three times for 30 min at room temperature, once for 90 sec at 45 °C, and once including 0.25% Blocking reagent (Roche-Boehringer) at room temperature overnight. (Blocking reagent will dissolve in 6 × SSC by heating up to 70 °C.) Use glass trays throughout the washing procedure and make sure that the membrane always remains wet.
7. **Blocking and detection by chemoluminescence**: Strictly follow the manufacturer's recommendations (Roche-Boehringer).

3 Results and Discussion

3.1 Technical considerations

The multilocus DNA fingerprint, as the first DNA-based methodology of individualisation of biological samples, revolutionised forensic medicine. It surpassed traditional hematogenetic techniques in almost every respect, especially in terms of informativity and efficiency [17]. Immediately after its invention, DNA fingerprinting was officially accepted as legal proof, and it is noteworthy that it was a deficiency case of kinship testing where it first demonstrated its power [18]. Kinship testing is still the primary domain of this methodology, but it has also proved useful in trace analysis [19]. Many other fields of medicine and biology have immensely profited from its introduction, including oncology and transplantation medicine [20], monitoring of cell culture stability [21, 22], animal and plant breeding [23, 24], sociobiology and ethology [25; see also the chapter of Lubjuhn and Sauer, this volume].

In order to be useful, any one system applied to genetic individualisation of biological samples must be analytically and biologically stable. Synthetic oligonucleotides are advantageous over probe systems generated biologically in that a constant quality of the former can be guaranteed with no need to worry over possible sequence alterations by recombination. Target sequences must be somatically stable, and the germline mutation rate must be tolerable. Nürn-

berg and co workers [26] have shown that $(CAC)_5/(GTG)_5$ oligonucleotides do indeed fulfill these requirements. From their data, a mutation rate of approximately 0.0005 per gamete per band position can be derived [27, 28].

A disadvantage of multilocus DNA fingerprinting may be seen in the fact that the banding pattern is not entirely objectifiable as such. In a cooperative study comprising 256 paternity cases contributed by seven laboratories [14], it was shown that all parameters characteristic of multilocus DNA fingerprints differ significantly between the contributing centers, including the number of analysed gel positions, the number of bands scored per individual, the probability of occurrence of a band at a particular position, and the band sharing probabilities between the mother and both child and putative father. In spite of these differences, paternity cases could be divided clearly into two distinct subgroups on the basis of (1) offspring bands that could not be assigned to either the mother or the putative father, and (2) the extent of band sharing between child and putative father. There is no doubt whatsoever that these two subgroups correspond to true and false paternity. Thus, multilocus paternity testing yields reliable results in spite of technical inconsistencies between laboratories.

Even under highly controlled conditions, e.g. within one and the same laboratory, the banding patterns obtained from a particular sample are not totally reproducible. Reliable conclusions regarding banding match or mismatch can only be drawn when all samples to be compared are run side by side on one and the same gel. In a recent study, Nürnberg and co-workers [29] addressed a number of complex settings where up to 55 rhesus monkey infants born to up to 25 females had to be assigned to their true fathers from groups of up to 166 potential sires. They first explored whether or not multilocus DNA fingerprints could be digitalised and standardised in order to enable intergel comparisons. This was clearly not feasible in that the computer-assisted image analysis did not appear to be capable of properly adjusting banding patterns for their intergel variability. However, multilocus DNA fingerprinting proved to be of great value in this study when, after the complex task had been largely solved by a single locus approach, several of the still unsolved cases could be clarified by multilocus tests. Thus, multilocus DNA fingerprinting is impractical in other than simple constellations where direct comparisons are possible.

3.2 Ethical and legal considerations

The one and only aim of sample individualisation is precisely that: to assign it to a particular person. Any further information about this individual is unnecessary and unwanted, because it may interfere with personal privacy. The genetic polymorphisms currently in use for kinship testing and trace analysis are more or less "neutral" in that they do not allow any direct inferences about any phenotypic characters of its carrier. However, in many systems allelic associa-

tions with certain diseases have been established, and therefore these alleles are – usually weak – predictors of future disease. It is well known, for example, that carriers of blood group A suffer from gastrointestinal tumors more frequently than others, whereas carriers of blood group B have a disposition to gastrointestinal ulcers. While the relative risk in these cases is between 1.2 and 2, it is much more pronounced – some 90-fold – in, for example, carriers of the HLA allele B27 with its strong association with ankylosing spondylitis; HLA-B27 is nevertheless still a weak predictor, because the prior risk for this disease is low.

The problem of surplus information is becoming more obvious when more common diseases are at stake. The APOE-E4 variant confers a significantly increased risk of Alzheimer's disease at some stage of life [30], and with this knowledge, one would clearly avoid using such a disease predictor in kinship testing. Prior to the research that led to finding out about this association, however, the APOE polymorphism would have appeared to be a useful test system. It follows that one should beware of one's own ignorance, because an association between a polymorphic variant and a disease or character trait not known today may be revealed tomorrow. The use of DNA polymorphism at individually defined noncoding loci does not totally circumvent the issue, because such polymorphisms may stand in linkage disequilibrium with a neighbouring locus with direct relevance for the phenotype. Forensic DNA databases themselves could become a source of such knowledge: "This allele, at this locus about which I know nothing, tends to come up in rapists" [31]. Multilocus DNA fingerprinting is the method least likely to generate such knowledge, because – although possible in principle [32] – the assignment of bands to alleles to loci is hardly feasible. Thus, what in one way appears as a drawback of the method (see the chapter of Krawczak, this volume), is an "inbuilt protective" against genetic data misuse. Without access to the DNA itself, even a computer-stored multilocus DNA fingerprint image cannot yield data on phenotypic associations. Thus, multilocus DNA fingerprinting remains the method of choice in most cases of paternity testing, where the disadvantages of the method do not usually play any significant role.

4 Troubleshooting

1. The most important problem that may arise in multilocus DNA fingerprinting is the possible occurrence of artifactual bands, the "hidden partials". Depending on the quality of the DNA (purity) and its methylation status, some restriction sites are obstinately resistant to enzymatic action. Remedies include a high concentration of restriction enzyme, the inclusion of spermidine [1 mM] in the digestion assay, and the preferential use of methylation-insensitive enzymes.

2. In any comparative analysis, the presence or absence of weak bands may simply be due to different amounts of DNA having been loaded onto the gel. Great care should therefore be exerciced to accurately measure the DNA concentrations actually applied. It is usually advisable to perform a photometric analysis of the DNA stock solution, and to inspect the relative strengths of the ethidium bromide signals on the restriction control gels.
3. Always strive to start out with high molecular weight DNA. Degradation may lead to the artifactual weakening or loss of bands preferentially in the highest molecular weight region of the gel, and it will contribute significantly to lane background with the consequence that bands of low intensity might be overlooked.

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