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Review Article

Revisiting Restriction Fragment Length Polymorphism (RFLP) for Human DNA Profiling: A Practical Evaluation Using *MspI* Digestion and Agarose Electrophoresis

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Abstract

Restriction Fragment Length Polymorphism (RFLP) was the first widely accepted DNA-based method for forensic human identification. Although it has been largely replaced by PCR-based techniques such as short tandem repeat (STR) analysis, RFLP remains historically significant and educationally valuable. This study evaluates the practical utility of a classical, whole-genome RFLP approach for human DNA profiling under routine laboratory conditions. High-molecular-weight genomic DNA was isolated from human peripheral blood using the phenol-chloroform-isoamyl alcohol (PCI) method, digested with the restriction endonuclease *MspI*, and analyzed by agarose gel electrophoresis. The PCI extraction yielded intact, high-quality DNA with A260/A280 ratios between 1.7 and 1.9, suitable for enzymatic digestion. *MspI* efficiently cleaved the DNA at its CCGG recognition sites, as evidenced by the disappearance of high-molecular-weight bands and the appearance of fragmented DNA across the gel lanes. However, the resulting restriction patterns were predominantly smear-like, with only a few weakly resolved bands, making individual differentiation impossible under the conditions tested. These findings illustrate the fundamental principle of RFLP, detecting length variations in restriction fragments, while also highlighting its major practical limitations: reliance on high-quality, high-quantity DNA, susceptibility to incomplete digestion, and poor resolution of complex fragment mixtures without Southern blotting and probe hybridization. The study concludes that while whole-genome RFLP with *MspI* successfully demonstrates enzymatic fragmentation, it does not produce interpretable, individual-specific profiles for forensic casework. This reinforces the need for modern forensic laboratories to transition to more sensitive, rapid, and standardized PCR-based methods.

Keywords

Restriction Fragment Length Polymorphism; RFLP; *Mspi*; Genomic DNA; PCI Extraction; Agarose Gel Electrophoresis; Human Identification; Forensic DNA Profiling

1. Introduction

The advent of DNA fingerprinting by Alec Jeffreys in 1985 revolutionized forensic science by enabling the identification of individuals from biological samples with high scientific rigor [1]. Every human individual, except monozygotic twins, possesses a unique combination of genetic markers that can be exploited for applications ranging from criminal investigation and paternity testing to

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disaster victim identification and anthropological research [2,3]. DNA's chemical stability and its presence in diverse biological materials such as blood, saliva, semen, hair roots, and bone make it an exceptionally valuable tool when other identification methods are inadequate.

The first widely implemented laboratory technique for exploiting DNA variation was Restriction Fragment Length Polymorphism (RFLP) [4]. This method involves isolating high-molecular-weight genomic DNA, digesting it with restriction endonucleases (e.g., *HaeIII*, *HinfI*) that recognize specific short nucleotide sequences, and separating the resulting fragments by gel electrophoresis [5]. Sequence differences between individuals, such as single-nucleotide polymorphisms (SNPs) or variable number tandem repeats (VNTRs), can create or abolish restriction sites or alter the distances between them, generating fragment length polymorphisms that serve as individual-specific genetic fingerprints [6].

Despite its historical success and high discriminatory power, RFLP has significant technical limitations. It requires relatively large amounts (micrograms) of high-quality, undegraded genomic DNA, which is seldom available from crime scene samples exposed to environmental insults [7]. The procedure is labor-intensive, time-consuming (often requiring several days), and traditionally involves Southern blotting and radioactive or chemiluminescent probe hybridization to visualize specific loci [8]. The development of the Polymerase Chain Reaction (PCR) by Mullis and Faloona [9] transformed forensic genetics by enabling the amplification of specific DNA regions from picogram quantities of even partially degraded template DNA. This led to the widespread adoption of PCR-based STR profiling, mitochondrial DNA sequencing, and SNP typing, which are now the gold standards in forensic casework [10,11].

Nevertheless, RFLP retains educational and historical importance. It introduced the core concept of detecting inherited DNA polymorphisms and laid the groundwork for modern molecular biology techniques [12]. Many principles underlying RFLP, restriction enzyme usage, fragment separation, and pattern interpretationremain relevant. Comparative studies between RFLP and contemporary methods help clarify the strengths, weaknesses, and technical demands of each approach [7,13].

In this study, we revisited a classical RFLP strategy using a simplified, practical design. Human genomic DNA was isolated from peripheral blood via the PCI method, digested with the restriction endonuclease *MspI* (recognition sequence CCGG), and analyzed on agarose gels. The aim was not to develop a forensic casework protocol but rather to evaluate, under routine laboratory conditions, whether a basic whole-genome RFLP approach can generate discernible individual-specific patterns. By doing so, we sought to understand more concretely why such methods have been superseded by targeted PCR-based profiling.

2. Materials and Methods

2.1. Sample Collection and Ethical Compliance

Peripheral blood samples (2–3 mL) were collected from five healthy adult volunteers (three males, two females, aged 22–30 years) by venipuncture following written informed consent. The study protocol was approved by the Institutional Ethics Committee of SAM Global University (approval no. SAM/EC/2022-03). Blood was drawn under aseptic conditions into sterile EDTA-coated vacutainer tubes, labeled uniquely, transported on ice, and stored at 4°C until processing within 48 hours.

2.2. Genomic DNA Extraction by the PCI Method

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Genomic DNA was extracted using a phenol-chloroform-isoamyl alcohol (PCI) protocol adapted from Sambrook and Russell [14]. Red blood cells were lysed by adding 3 volumes of RBC lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM NaCl) to 500 µL whole blood, incubating on ice for 10 minutes, and centrifuging at 2,500 × g for 10 minutes at 4°C. The leukocyte pellet was washed twice with lysis buffer. The final pellet was resuspended in 500 µL Solution B (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 2% SDS) and treated with 20 µL Proteinase K (20 mg/mL) at 55°C overnight with gentle shaking.

After complete lysis, an equal volume (500 µL) of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0) was added, mixed by inversion for 5 minutes, and centrifuged at 12,000 × g for 10 minutes at room temperature. The upper aqueous phase was carefully transferred to a fresh tube, avoiding the protein interphase. A second extraction was performed using chloroform:isoamyl alcohol (24:1). DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ice-cold absolute ethanol, followed by incubation at −20°C for 1 hour. The DNA pellet was collected by centrifugation at 12,000 × g for 15 minutes at 4°C, washed twice with 70% ethanol, air-dried for 10 minutes, and dissolved in 50–100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was stored at −20°C.

2.3. DNA Quality and Quantity Assessment

DNA integrity was assessed by electrophoresis of 2 µL DNA on a 0.8% agarose gel prepared in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA) containing 0.5 µg/mL ethidium bromide. Electrophoresis was performed at 80 V for 45 minutes, and gels were visualized under UV light (GelDoc system, Bio-Rad). DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Only samples with A260/A280 ratios between 1.7 and 1.9 and exhibiting high-molecular-weight bands with minimal smearing were used for downstream analysis.

2.4. Restriction Digestion with *MspI*

For RFLP analysis, 1 µg of genomic DNA was digested with *MspI* (New England Biolabs) in a 20 µL reaction volume containing: 1 µg DNA (volume adjusted to 10 µL with nuclease-free water), 2 µL 10× CutSmart® buffer, 1 µL *MspI* (10 U/µL), and nuclease-free water to 20 µL. Reactions were set up on ice, with the enzyme added last. After gentle pipetting and brief centrifugation, tubes were incubated at 37°C for 3 hours. The reaction was terminated by heating at 65°C for 10 minutes. Undigested DNA controls (without enzyme) were processed identically. Digested samples were stored at −20°C until electrophoresis.

2.5. Agarose Gel Electrophoresis of Restriction Fragments

Digested DNA samples (20 µL each) were mixed with 4 µL 6× gel loading dye (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) and loaded onto a 3% agarose gel prepared in 1× TAE buffer with 0.5 µg/mL ethidium bromide. A 100 bp DNA ladder (Thermo Fisher) was included as a size marker. Electrophoresis was carried out at 100 V for 1.5 hours. Gels were visualized under UV light and documented using a gel documentation system (Bio-Rad ChemiDoc). Restriction patterns were compared visually between individuals.

2.6. Data Analysis

No statistical analysis was performed as the study was qualitative and observational. Banding patterns were visually assessed for the presence of discrete bands versus smearing, and for inter-individual differences in fragment distribution.

3. Results

3.1. Genomic DNA Quality and Quantity

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The PCI extraction method yielded visible white thread-like DNA pellets from all five blood samples. Agarose gel electrophoresis (0.8%) showed intense, high-molecular-weight bands near the well with minimal smearing, indicating intact genomic DNA (Figure 1A). NanoDrop spectrophotometry gave A260/A280 ratios ranging from 1.72 to 1.88, confirming low protein contamination. DNA concentrations ranged from 80 to 150 ng/µL, yielding total DNA amounts of 4–7.5 µg per 500 µL blood. These results indicate that the extracted DNA was of sufficient quality and quantity for restriction digestion.

Figure 1A (suggested placement):

0.8% agarose gel showing high-molecular-weight genomic DNA from five individuals (lanes 1–5). Lane L: 1 kb DNA ladder. No smearing or RNA contamination is observed.

3.2. *MspI* Digestion Efficiency

Undigested control samples (without *MspI*) showed a single high-molecular-weight band near the top of the gel (Figure 1B, lanes U1–U3). In contrast, *MspI*-digested samples displayed a complete loss of the high-molecular-weight band and the appearance of DNA fragments distributed over a broad size range (approximately 100–2,000 bp), confirming that *MspI* efficiently cleaved the genomic DNA at its CCGG recognition sites. The fragment pattern was consistent with frequent cutting, as expected given the estimated occurrence of CCGG sites every 200–300 bp in the human genome.

Figure 1B (suggested placement):

0.8% agarose gel comparing undigested (U) and *MspI*-digested (D) genomic DNA from three representative individuals. L: 1 kb ladder.

3.3. Restriction Fragment Patterns on 3% Agarose Gels

When *MspI*-digested DNA was resolved on 3% agarose gels to improve the separation of small fragments, the predominant feature was a continuous smear extending from approximately 100 bp to 1,500 bp (Figure 2). No discrete, well-resolved bands were observed in any of the five individual samples. In two samples, a few faint, broad bands (approximately 500–700 bp) were visible against the smear, but these were too poorly resolved to be considered reproducible or individual-specific markers. No consistent inter-individual differences in band patterns could be discerned; all lanes appeared essentially similar in their smear distribution.

Figure 2 (suggested placement):

3% agarose gel of *MspI*-digested genomic DNA from five individuals (lanes 1–5). Lane L: 100 bp ladder. Note the continuous smearing and absence of discrete, interpretable bands.

These results demonstrate that whole-genome digestion with a frequent-cutting restriction enzyme (*MspI*), followed by direct agarose gel electrophoresis without Southern blotting or probe hybridization, generate complex fragment mixtures that cannot be resolved into individual-specific fingerprints under standard laboratory conditions.

4. Discussion

This study successfully isolated high-quality genomic DNA from human blood using the classical PCI method, which remains a reliable technique for obtaining intact, high-molecular-weight DNA suitable for restriction analysis [14,15]. The A260/A280 ratios obtained (1.72–1.88) indicated minimal protein contamination, and the high-molecular-weight bands on agarose gels confirmed the absence of significant degradation. These results align with previous reports that phenol-chloroform extraction, when carefully performed, yields DNA of sufficient purity for enzymatic manipulations [16].

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Restriction digestion with *MspI* was clearly effective, as evidenced by the complete disappearance of the high-molecular-weight band and the appearance of fragmented DNA across the gel. *MspI* recognizes the palindromic tetranucleotide CCGG, which occurs with high frequency in the human genome (estimated once every 200–300 bp on average due to 5-methylcytosine depletion in CpG islands, but effectively frequent in non-methylated regions) [17]. Consequently, digestion of whole human genomic DNA with *MspI* generates tens of thousands of fragments of varying lengths, ranging from very short (<100 bp) to several kilobases.

The key finding of this study is that when such a highly complex mixture of fragments is resolved by agarose gel electrophoresis, even at a higher concentration (3%) intended to improve resolution, the resulting pattern is a continuous smear rather than discrete, interpretable bands. This outcome is entirely consistent with the principles of RFLP analysis. Successful forensic RFLP, as historically practiced, did not rely on visualizing all restriction fragments simultaneously. Instead, after restriction digestion and gel separation, DNA fragments were transferred to a membrane (Southern blotting) and hybridized with labeled probes specific to hypervariable minisatellite or VNTR loci [1,6,8]. This detection step reduces the complex smear to a small number of highly polymorphic bands (typically 10–30), enabling individual differentiation.

Without such locus-specific detection, as in our simplified protocol, the inherent complexity of the restriction digest overwhelms the resolving power of agarose gels. Even high-percentage agarose gels cannot separate the thousands of fragments that differ in length by only a few base pairs. The result is the observed smear, which masks any underlying length polymorphisms. The few faint, broad bands seen in some samples likely represent highly repetitive sequences (e.g., satellite DNA) that are present in many copies and thus appear as dominant features, but they are not sufficiently polymorphic or reproducible for individual identification.

These findings directly explain why whole-genome RFLP with general DNA staining is unsuitable for forensic casework. The method requires: (1) micrograms of high-molecular-weight DNA, which is rarely obtainable from degraded forensic samples; (2) complete restriction digestion, which is challenging with partially damaged DNA; (3) high-resolution gel electrophoresis, often using pulsed-field or long-run conditions; (4) Southern transfer; and (5) hybridization with radioactive or chemiluminescent probes [7,10]. This multi-step process is time-consuming (days), labor-intensive, and prone to technical variability.

In contrast, PCR-based STR profiling overcomes virtually all these limitations. PCR amplifies specific short tandem repeat loci (typically 100–500 bp) from picogram quantities of even partially degraded DNA, generates clean electrophoretic patterns with single-base resolution using capillary electrophoresis, and produces results in a few hours with high sensitivity and standardization [11,18]. The discriminatory power of 15–20 STR loci exceeds that of RFLP, and the ability to multiplex amplifies dozens of loci simultaneously [19]. These advantages have rendered RFLP obsolete for routine forensic casework.

Nevertheless, this study has educational value. It demonstrates the core principle of RFLP: restriction enzymes cut DNA at specific sequences, and sequence variation between individuals can theoretically alter fragment lengths. It also highlights the practical challenges that motivated the transition to PCR-based methods. For teaching laboratories, performing a simplified RFLP experiment as described here provides students with hands-on understanding of

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restriction digestion, gel electrophoresis, and the concept of DNA polymorphism, while also illustrating why modern forensic genetics has moved toward targeted amplification.

Limitations of the study:

This work used a single restriction enzyme (*MspI*), a small sample size (n=5), and did not employ Southern blotting or probe hybridization. The agarose gel system, even at 3%, has limited resolution compared to polyacrylamide or capillary electrophoresis. Therefore, the absence of interpretable individual-specific profiles should not be interpreted as evidence that RFLP cannot work; rather, it shows that a simplified, whole-genome visualization approach is insufficient. Future studies could explore the use of multiple restriction enzymes, improved gel systems (e.g., pulse-field gel electrophoresis), or the incorporation of locus-specific probes to demonstrate full RFLP capacity.

5. Conclusion

This study evaluated a classical whole-genome RFLP approach using *MspI* digestion of human genomic DNA extracted via the PCI method. While the extraction protocol yielded intact, high-quality DNA and *MspI* demonstrated efficient enzymatic cleavage, electrophoretic analysis on 3% agarose gels revealed predominantly smeared patterns with no clear, individual-specific restriction fragment profiles. These results confirm that whole-genome RFLP with frequent-cutting enzymes, visualized only by general DNA staining, lacks the resolution and selectivity required for forensic human identification. The findings underscore why RFLP has been replaced by PCR-based STR profiling in modern forensic laboratories. Nonetheless, the principles demonstrated here remain fundamental to molecular biology and provide a valuable educational framework for understanding DNA polymorphisms and the evolution of forensic genetic technologies.

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Conflict of Interest

The authors declare no conflicts of interest. No external funding was received for this research.

Author Contributions

Priyanka Dhurvey:
Conceptualization, methodology, investigation, writing – original draft.
Varun Jain:
Sample collection, DNA extraction, data curation.
Vaishali Vardiya:
Restriction digestion, electrophoresis, visualization.
Lakshya Bhardwaj:
Data analysis, validation.
Nikita Tomar:
Formal analysis, writing – review & editing.
Deepak Bharti:
Supervision, project administration, funding acquisition (institutional support), final approval.

Ethical Approval

This followed the ethical standards of the Declaration of Helsinki and its later amendments.

Informed Consent

Written informed consent was obtained from all individual participants included in the study.

Data Availability

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

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References

[1] Jeffreys, A. J., Wilson, V., & Thein, S. L. (1985). Individual-specific fingerprints of human DNA. *Nature*, 316(6023), 76–79.

[2] Jobling, M. A., & Gill, P. (2004). Encoded evidence: DNA in forensic analysis. *Nature Reviews Genetics*, 5(10), 739–751.

[3] Butler, J. M. (2015). *Forensic DNA typing: Biology, technology, and genetics of STR markers* (2nd ed.). Academic Press.

[4] Botstein, D., White, R. L., Skolnick, M., & Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics*, 32(3), 314–331.

[5] Gill, P., Jeffreys, A. J., & Werrett, D. J. (1985). Forensic application of DNA 'fingerprints'. *Nature*, 318(6046), 577–579.

[6] Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., et al. (1987). Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science*, 235(4796), 1616–1622.

[7] Goodwin, W., Linacre, A., & Hadi, S. (2011). *An introduction to forensic genetics* (2nd ed.). Wiley-Blackwell.

[8] Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*, 98(3), 503–517.

[9] Mullis, K. B., & Faloona, F. A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods in Enzymology*, 155, 335–350.

[10] Butler, J. M. (2012). *Advanced topics in forensic DNA typing: Methodology*. Academic Press.

[11] Parson, W., & Bandelt, H. J. (2007). Extended guidelines for mtDNA typing in forensic science. *Forensic Science International: Genetics*, 1(1), 13–19.

[12] Kayser, M., & de Knijff, P. (2011). Improving human forensics through advances in genetics and genomics. *Nature Reviews Genetics*, 12(3), 179–192.

[13] Alaeddini, R., Walsh, S. J., & Abbas, A. (2021). Forensic implications of DNA extraction methods and their applications in human identification. *Forensic Science International: Genetics Supplement Series*, 8(1), 12–18.

[14] Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). Cold Spring Harbor Laboratory Press.

[15] Green, M. R., & Sambrook, J. (2012). *Molecular cloning: A laboratory manual* (4th ed.). Cold Spring Harbor Laboratory Press.

[16] Phillips, K., & McCallum, N. (2019). Comparison of DNA extraction methods for forensic applications. *Forensic Science International: Genetics*, 38, 204–211.

[17] Roberts, R. J., Vincze, T., Posfai, J., & Macelis, D. (2003). REBASE, restriction enzymes and methylases database. *Nucleic Acids Research*, 31(1), 418–420.

[18] Gill, P., Fereday, L., Morling, N., & Schneider, P. M. (2006). The evolution of DNA databases, Recommendations for new European STR loci. *Forensic Science International*, 156(2-3), 242–244.

[19] Butler, J. M., & Hill, C. R. (2012). Biology and genetics of new autosomal STR loci useful for forensic DNA analysis. *Forensic Science Review*, 24(1), 15–26.

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