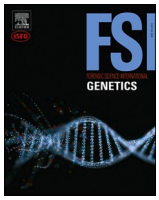
Forensic Science International: Genetics 81 (2026) 103368



Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsigen



Nanopore sequencing and haplotyping of mitochondrial DNA hypervariable regions and its application on mixed stain

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ARTICLE INFO

Keywords:
Mitochondrial DNA
Hypervariable regions
Nanopore sequencing
Forensic DNA analysis
Mixed biological samples

ABSTRACT

Objective: To explore the feasibility of mitochondrial DNA (mtDNA) haplotype detection by nanopore sequencing and its potential value in the identification of mixed biological samples in forensic DNA analysis.
Methods: Firstly, validation of the method for long fragments detection of human mtDNA hypervariable regions (HVRs) was carried out on the G-seq500 nanopore sequencing platform. Then, the amplified products of two DNA standards were mixed at different ratios to be sequenced and analyzed. Finally, the method established in this study was used to separate haplotypes of the mixed biological samples from simulated and real crime scenes.
Results: The nanopore sequencing protocol of mtDNA HVRs established in this study demonstrated human-specificity, accuracy comparable to Sanger sequencing and Next Generation Sequencing (NGS), high sensitivity, repeatability and broad applicability. Using Sanger sequencing results as the standard, the accuracy of DNA standards sequencing was 100 %. The sequencing results for random individual blood cards were consistent with that of NGS. The detection limit of DNA template could be as low as 0.0625 ng. The method was also applicable to various sample types, including blood, oral swab, hair with follicles, different tissues and exfoliated cells, and can effectively interpret the Poly-C region. The mixtures of amplified products at ratios of 1:1, 1:9, 1:19, 1:49 and 1:99 from two DNA standards, as well as amplified products of mixed blood samples from two unrelated individuals at ratios of 3:2, 17:3, and 24:1, were accurately sequenced and haplotype-separated using the G-seq500 nanopore sequencing platform. Using this technical scheme to detect the mixture of exfoliated cells in a rape case can accurately separate the haplotypes to identify the suspect.
Conclusion: The long-read nanopore sequencing of human mtDNA HVRs based on the G-seq500 platform meets forensic DNA methodological requirements and is operationally feasible. It holds great potential and application value in the identification of mixed biological samples in forensic DNA analysis.

1. Introduction

Human mitochondrial DNA (mtDNA), located within the cytoplasm, constitutes the primary extranuclear genetic material. It holds significant value in forensic research and application. The standard mtDNA sequence known as Anderson sequence revised in 1999 is 16,569 base pairs in length composed of 13 genes, 22 tRNAs, and 2 rRNAs [1,2]. Traditional studies highlight mtDNA is of particular value in forensic science for analyzing complex kinship relationships and identifying trace or degraded samples [3–6]. Furthermore, the genetic polymorphism within its hypervariable regions (HVRs), coupled with its haploid nature, has rendered mtDNA a valuable tool in recent years for

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https://doi.org/10.1016/j.fsigen.2025.103368
Received 7 March 2025; Received in revised form 22 September 2025; Accepted 23 September 2025
Available online 24 September 2025
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identifying contributors to mixed biological samples [7–12]. At present, the primary methods for mtDNA sequence detection are Sanger sequencing and Next Generation Sequencing (NGS). However, due to the disadvantages of quantitative ability (Sanger sequencing) and short-read length (NGS), these methods cannot effectively perform phasing of mtDNA haplotypes with long length from mixed samples [13–16], which has limited the forensic research and application of mtDNA to a certain extent.

Nanopore sequencing has evolved significantly over the past decade. With substantial improvement in sequencing accuracy in recent years, this technology presents a promising solution to the aforementioned challenges. In 2014, Oxford Nanopore Technologies (ONT) launched the world's first commercially available nanopore sequencer, the MinION [17], followed by numerous technology companies introducing nanopore sequencers based on the same concept of current change while single-stranded DNA passing through a nanopore. The key features of nanopore sequencer include single-molecule sequencing and long-read length. In a single sequencing run, the recorded read length could reach up to 2.3 Mb [18–20], and the accuracy of single-base sequencing has been improved to ~ 99 % [21–25].

In 2023, Geneus Technologies unveiled the G-seq500 nanopore sequencer, which is predicated on the concept of DNA nanopore sequencing by synthesis (NSS), using a nanopore to identify nucleotide bases during DNA synthesis. G-seq500 builds upon similar strategy of the prototype platform, which was introduced by Fuller et al. and Stranges et al. [26,27], but shows significantly enhanced performance with extensive optimization of the biochemical system. The G-seq500 Sequencer corresponds to a chip with 512K sequencing channels, which theoretically can sequence up to 512K DNA molecules simultaneously. Each effective sequencing unit corresponds to a nanopore, which covalently couples polymerase and binds to the DNA molecule to be sequenced. During polymerase-mediated replication of the target nucleic acid, the labeled nucleotides being incorporated interact with the nanopore, thereby producing a highly distinctive blockade currents. Real-time monitoring of these signals enables highly accurate sequencing of the target nucleic acid. It is important to note that in a single sequencing cycle, each unit only sequences the same DNA molecule that is pre-bound to the polymerase. Consequently, when the read length of the polymerase exceeds the length of the DNA molecule, it is possible to sequence a cyclized DNA molecule for multiple times and results in higher accuracy [28–30]. The read length of G-seq500 is at the range of tens of kilobase pairs and can provide high consensus accuracy exceeding Q50 at the coverage of 50X, making it a powerful sequencing platform in many applications, such as genome assembly, structural variation detection and long-read amplicon identification. Recently, Zhou et.al used the full-length *mtlA* gene for *V. parahaemolyticus* strain typing and epidemiological surveillance, confirming high sequencing accuracy of G-seq500 [25].

This study employed the G-seq500 platform to sequence the human mtDNA HVRs (HVR-I, HVR-II and HVR-III) with a total of ~ 1.4 kb length, which are of significant value in forensic DNA identification. Initially, we performed a comprehensive evaluation of the approach based on parameters such as species specificity, accuracy, sensitivity, repeatability and adaptability. Subsequently, an assessment was conducted on the nanopore sequencing results of amplicon mixtures, simulated mixtures of blood samples and mixtures of exfoliated cell samples from a rape case. Furthermore, we performed the separation of haplotypes to evaluate its practical value in forensic DNA analysis of mixed biological samples.

2. Material and methods

2.1. Samples

This study conforms to the Declaration of Helsinki and was approved by the Shanghai Ethics Committee for Clinical Research (Approval Number: SECCR/2023-118-01). Human tissues used in this study were procured in line with WHO Guiding Principles on Human Cell, Tissue and Organ Transplantation. All procedures were performed in compliance with relevant laws and institutional guidelines. Written informed consents were obtained from all volunteers.

Human genomic DNA standards 9947A and 9948 were purchased from Peoplespot (Beijing) Co., Ltd. Various human samples, including blood stain, oral swab, hair with follicles, tissues (miscarriage tissue obtained with the written informed consent of the donor in a rape case, muscle, and costal cartilage), exfoliated cells (cigarette butt, toothbrush, and chewing gum) and vaginal swabs from a rape case, were routine case samples preserved by Sichuan Genegle Forensic Center. Non-human DNA samples were extracted from muscle tissues of various species, including cat, fish, chicken, sheep, duck, pig, mouse, rabbit, horse, dog and cattle. *E. coli* DNA was extracted from DH5α competent cells (TransGen Biotech, China).

2.2. DNA extraction, quantification and amplification

Genomic DNA was extracted using the QIAamp® DNA Investigator kit (QIAGEN, Germany) with the final elution volume of 50 μL, and quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, USA). PCR amplification of mtDNA HVRs was performed using Veriti 96-Well Gene Amplifier (Thermo Fisher Scientific, USA) in a total volume of 25 μL, containing 12.5 μL of 2 × GoTaq® Green Master Mix (Promega, USA), 0.0625–2 ng of DNA template (2 ng of 9947A DNA or each species' DNA was input in species specificity study; 2 ng of 9947A DNA or 9948 DNA was input in accuracy study; 0.0625 ng, 0.125 ng, 0.25 ng, 0.5 ng, 1 ng or 2 ng of 9947A DNA with three replicates per group was input in sensitivity and reproducibility study; 0.25–2 ng of each human sample's DNA was input in adaptability study; 2 ng of human DNA extracted from each dried blood card of three unrelated individuals was input in comparison of NGS and Nanopore Sequencing study; 2 ng of 9947A DNA or 9948 DNA was input in amplicon mixtures study; 2 ng of mixed DNA extracted from each cotton swab of mixed two unrelated individuals' blood at volume ratios of 3:2, 17:3 and 24:1 was input in simulated mixtures study; 2 ng of human DNA extracted from vaginal swab was input in case study), 0.5 μL of forward/reversed primer (forward, 5'-tactcaaatgggcctgtcct-3'; reverse: 5'-tgttccttttgatcgtggtg-3') and nuclear-free water. Amplification was performed as following condition: pre-denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 90 s, and extension at 72 °C for 60 s; final extension at 72 °C for 10 min.

2.3. Sanger sequencing and data analysis

PCR amplification products were confirmed by electrophoresis of 1 % agarose gel (condition: 3 μL of loaded volume, 120 V of voltage for 30 min and 100–2000 bp sizes of DNA marker used) and sent to Sangon Biotech (Shanghai) Co., Ltd. for Sanger sequencing. Briefly, BigDye™ Terminator v3.1 (Thermo Fisher Scientific, USA) and ABI 3730 (Thermo Fisher Scientific, USA) were employed, and 100–200 ng of DNA template from PCR produce of each sample was used. Sequencing data was analyzed using SnapGene (v5.1.5) software.

2.4. NGS and data analysis

Following the protocols of the MGIEasy Individual Identification Library Preparation Kit (MGI, China) and DNBSEQ-G99RS High Throughput Sequencing Kit (MGI, China), samples were subjected to library preparation, nanoball preparation and sequencing on the DNBSEQ-G99ARS Sequencer (MGI, China). Briefly, dried blood card with a diameter of 1.2 millimeter from each unrelated individual, 1 ng of 9947A DNA as positive control or DNA free water as negative control was input, and a total of 5 samples were sequenced in a single run. Sequencing data were processed using FIS software (MGI, China) for

quality control and analysis of mtDNA HVRs sequences.

2.5. Nanopore sequencing and data analysis

DNA libraries were prepared using the Universal Sample Preparation Kit (Geneus Technologies (Chengdu), China), and nanopore sequencing was performed on the G-seq500 Sequencer (Geneus Technologies (Chengdu), China) following the protocols of Universal Sequencing Reagent Kit (Geneus Technologies (Chengdu), China) and Sequencing Chip (Geneus Technologies (Chengdu), China). Briefly, 200–800 ng of purified amplicon DNA (200 ng amplicon of per sample was input in species specificity study, accuracy study, sensitivity and reproducibility study respectively; 250 ng amplicon of per sample was input in adaptability study; 800 ng amplicon of per sample was input in comparison of NGS and Nanopore Sequencing study; in amplicon mixtures study, the amplified fragments of HVRs from 9947A or 9948 were purified and quantified to 200 ng/μL, which were then mixed at volume ratios of 1:1, 1:9, 1:19, 1:49 and 1:99, and 200 ng amplicon of per sample was input; 800 ng amplicon of per sample was input in simulated mixtures study and case study respectively), which was modified to create blunt ends suitable for ligation with sequencing adapters and cyclization, was then subjected to end repair at 20 °C for 30 min. After end repair, the DNA fragments are ligated to the sequencing adapters at 20 °C for 15 min, followed by digestion at 37°C for 30 min, the addition of sequencing primers and annealing at 25 °C for 20 min. Finally, sequencing complexes were obtained after protein complexes were combined with the library at 25 °C for 10 min, followed by magnetic bead purification. Sequencing was performed on the G-seq500 sequencer using one 512 K chip.

In this study, in order to improve the sequencing accuracy at the single-molecule level, we performed cyclization treatment on the tested DNA, thereby enabling the polymerase to replicate sequencing of the tested DNA multiple times during the Rolling circle amplication (RCA) extension process. Based on the extension speed of polymerase and the setting of sequencing time, the mitochondrial amplicon sequencing of a single molecule was usually repeated 7–12 times. Subsequent to processing with built-in algorithms, each DNA molecule obtains a single sequence (read) based on 7–12 times repeated sequencing (Fig. 1).

Quality control was performed by filtering reads based on quality scores, which are based on the overall quality of the entire reads instead of single bases. A Q score of 20 means the entire read sequence most likely shows an identity of ~ 99 % relative to the template sequence. In this study only high-quality reads above Q20 will be selected for typing analysis. The pivotal threshold for typing is the mutation rate, which refers to the percentage of bases other than the highest frequency base at each site in nanopore sequencing results by G-seq500 platform. Although quality threshold (Q20) was employed in this study, possible errors in PCR amplification and sequencing processes were still unavoidable, resulting in random background mutations. We sequenced the amplicons from single source mitochondrial samples, and then calculated the level of background mutations in the sequencing results. The results showed that for these samples, the level of random background mutations at all loci was below 0.9 %. Therefore, when performing sequence typing, all point heteroplasmy (PHP) sites exceeding the mutation rate threshold of 0.9 % were used as seed sites for analysis. Since observations of two and even three points heteroplasmic sites in a single sample have been reported [31–33], among these seed sites, three differences in PHP are used as a threshold to determine whether they are a mitochondrial typing type, in order to distinguish between different individual sources, and mutation sites except C-stretch regions with fewer than or equal to 3 mismatches were merged into a single type in this study. Finally, the consensus sequences of all reads of the same type were obtained and aligned with the Anderson sequence (GenBank accession number: NC_012920.1) using BLASR (v5.3.5), and characteristic sites were analyzed. The T-test was employed to analyze the significance of the inter group data in Fig. 5, Fig. 6 and Fig. 8, and a p-value greater than or equal to 0.05 was considered significant.

3. Results

3.1. Species specificity

PCR amplification was performed using human mtDNA HVRs-specific primers with DNA from samples of *E. coli*, cat, fish, chicken,

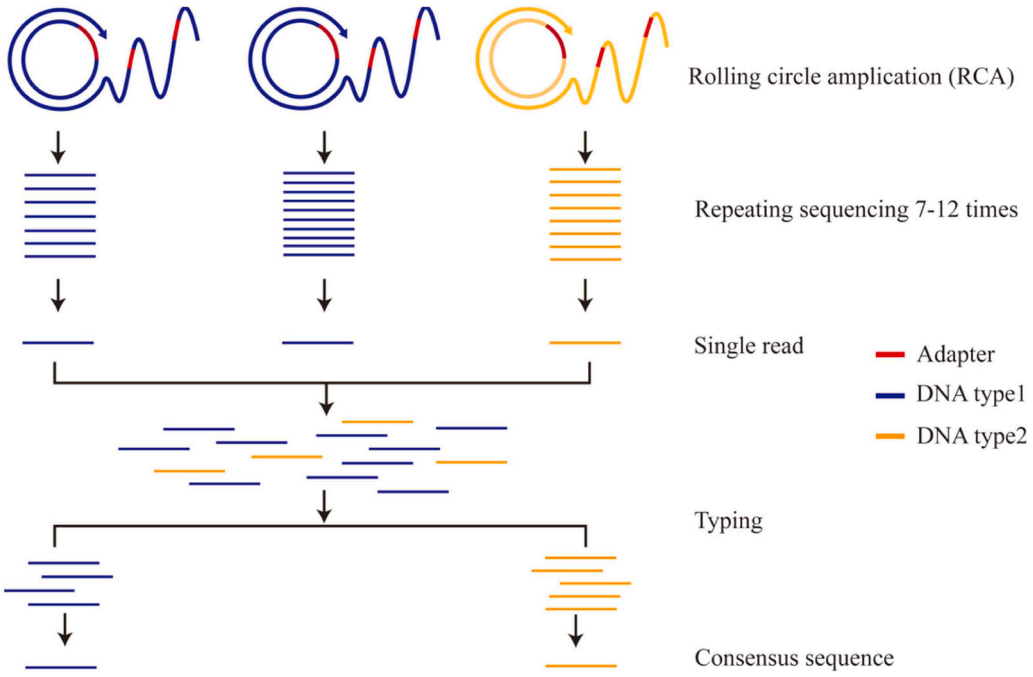

Fig. 1. Illustration of general workflow of G-seq500 sequencer. Firstly, target DNA undergoes cyclization and is sequenced 7–12 times during the Rolling circle amplification (RCA) extension process in a single nanopore. Then, each DNA molecule obtains a single read after processing with built-in algorithms. Finally, the consensus sequence was obtained through consistency analysis of multiple single reads in a sequencing run.

sheep, duck, pig, mouse, rabbit, horse, dog, cattle and human. The results of agarose gel electrophoresis demonstrated that only human DNA amplified a ~1.4Kb product with a single and distinct band of the expected size (Fig. 2). Certainly, it cannot be excluded that sequencing of other products might reveal low levels of non-human DNA.

3.2. Accuracy

DNA standard 9947A or 9948 was utilised as PCR template for the amplification of mtDNA HVRs and nanopore sequencing by G-seq500. A total of 95,936 and 111,223 reads were obtained for 9947A and 9948, respectively, and the read length and Q-value distributions of all single reads were analyzed. The results showed that for both 9947A and 9948, the read length was concentrated between 1400 bp and 1500 bp, and the accuracy of over 99 % of single reads reached Q10, over 95 % reached Q20, and over 50 % reached Q30 (Fig. 3, Table 1 and Fig. 4).

In the single sequencing results of 9947A or 9948 using the G-seq500 system, random sampling of 4–30 reads was performed. Each sampling result generated a consensus sequence, which was compared to the Sanger sequencing results to confirm a threshold of number of reads required to generate a high accuracy. The results showed that for both 9947A and 9948, when the number of reads reached 20 or more, the haplotype obtained by nanopore sequencing was completely consistent with the Sanger sequencing results, with the accuracy of 100 % (Fig. 5, Table 2 and Table 3). We conservatively set the threshold for the number of reads required to generate a high accuracy consensus sequence to 50 instead of 20 in this study. The comparison with Anderson reference sequence revealed 9 bases differences for 9947A (Table 2), which is consistent with previous report [34,35], and 14 bases differences for 9948 (Table 3), respectively.

3.3. Sensitivity and reproducibility

DNA standard 9947A was divided into six groups with DNA amounts of 0.0625 ng, 0.125 ng, 0.25 ng, 0.5 ng, 1 ng and 2 ng, with three replicates per group. The samples were subjected to amplification, library preparation and sequencing on the G-seq500 platform to assess the sensitivity and reproducibility. Through the *t*-test analysis between groups, there was no significant difference in number of reads between other groups except the group of 0.0625 ng, and there was a significant difference in number of reads between the group of 0.0625 ng and other groups ($p < 0.05$). It was noteworthy that while the group of 0.0625 ng had fewer reads compared to the others, all sample groups met the detection threshold established in this study ($\geq$ 50 reads) (Fig. 6). Consensus sequence from each group was compared with the Anderson reference sequence, and the identity values for all groups reached 100 % (data not shown).

3.4. Adaptability

Various types of forensic samples, including blood stain, oral swab, hair with follicles, tissues (miscarriage tissue, muscle, and costal cartilage) and exfoliated cells (cigarette butt, toothbrush, and chewing gum), were subjected to DNA extraction, mtDNA HVRs amplification, and nanopore sequencing using the G-seq500 system. The results showed that all types of samples successfully amplified ~ 1.4 kb fragments and obtained single-source haplotypes (Table 4). The samples of oral swab and miscarriage tissue exhibited length heterogeneity in the C-stretch area of HVR-I, causing a shift in Sanger sequencing that hindered accurate interpretation (e.g., the miscarriage tissue, as shown in Fig. 7). By contrast, nanopore sequencing could accurately perform separation of haplotypes for these samples (Table 5).

3.5. Comparison of NGS and nanopore sequencing results

In order to establish a basis for comparison with the current mainstream high-throughput sequencing (NGS), three unrelated individuals' blood card samples were randomly selected to perform HVRs detecting by both NGS using the BGI system and nanopore sequencing using the G-seq500 system. For NGS, the sequences were obtained from seven amplicons in HVR-I, HVR-II and HVR-III [36]. Conversely, nanopore sequencing furnished a continuous sequence of ~ 1.4 kb covering HVR-I, HVR-II and HVR-III without assembly. For the common fragments detected by both NGS and nanopore sequencing, the genotyping results of the three samples on the two platforms were consistent, with 11, 10, and 8 bases differences compared to Anderson reference sequence, respectively (data not shown).

3.6. Nanopore sequencing of amplicon mixtures

To further evaluate the potential of the G-seq500 nanopore sequencing platform for mixed stain identification, amplicon DNA of 9947A and 9948 were mixed at volume ratios of 1:1, 1:9, 1:19, 1:49 and 1:99 for the purpose of sequencing. The results showed that in all mixed samples, both haplotypes type 1 and type 2, corresponding to the HVRs of 9947A and 9948, were detected. The ratios of number of reads corresponding to the two haplotypes generally reflected the mixing proportions of 9947A and 9948 (Table 6).

Random sampling of 4–30 reads of major or minor component generated from theses mixtures was performed to confirm the threshold of number of reads required to generate consensus sequences with high-accuracy. The results showed that as the mixing ratio decreased, the requirements for number of reads increased. When the number of reads reached 10 or more, whether for the major or minor component of mixtures, the consensus sequences with accuracy of over 99.9 % were



Fig. 2. The results of agarose gel electrophoresis for human mitochondrial DNA HVRs amplification products with DNA from various species as templates. Lanes 1–13: *E. coli*, cat, fish, chicken, sheep, duck, pig, mouse, rabbit, horse, dog, cattle and human, respectively (DNA input: 2 ng). Lane N: Negative control. Lane M: DNA molecular weight marker (from top to bottom: 2000, 1000, 750, 500, 250, and 100 bp, respectively).

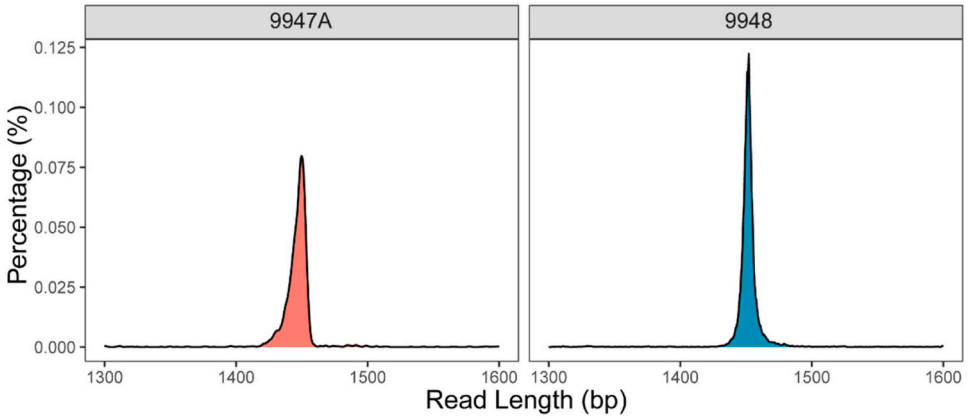

Fig. 3. Read length distribution of all single reads for DNA standards by nanopore sequencing using the G-seq500 system. The read length was concentrated between 1400 bp and 1500 bp for both 9947A and 9948.

Table 1
Q-value distribution of all single reads for DNA standards by nanopore sequencing using the G-seq500 system. A total of 95,936 and 111,223 reads were obtained for 9947A and 9948, respectively.

Sample	Q10	Q20	Q30
9947A	99.70 %	95.39 %	55.99 %
9948	99.67 %	95.94 %	53.60 %

obtained (Fig. 8).

3.7. Nanopore sequencing of simulated and real mixed samples

Blood from two unrelated individuals was mixed at volume ratios of 3:2, 17:3 and 24:1. Cotton swabs were used to collect the simulated mixtures, followed by DNA extraction, amplification of the HVRs and nanopore sequencing using the G-seq500 system. The results showed that all three mixed samples were comprised of two haplotypes (Individual 1 and Individual 2), and the ratios of number of reads were generally consistent with the actual mixing proportions (Table 7). Further Sanger sequencing confirmed that the two haplotypes matched the donors. There were 12 bases differences between the two haplotypes (Table 8), sufficient for effective individual identification according to the guideline of the International Society for Forensic Genetics [33].

Finally, a vaginal swab from a rape case was processed using the method established in this study. Two haplotypes were identified, the dominant haplotype of which matched the victim while the minor haplotype matched the suspect (Table 9). This result ultimately assisted the police in case investigation.

4. Discussion

The identification and examination of mixed biological stains has long been a challenging in forensic science. The research of this field focuses on three directions: development of genetic markers, theories for mixed stain genotyping and sequencing analysis [37–45]. Currently, substantial researches have been dedicated to the development of genetic markers (e.g., microhaplotypes) and mixed stain genotyping theories (e.g., probabilistic genotyping) based on capillary electrophoresis and NGS technologies. However, to our knowledge, there are few studies and application on nanopore sequencing and mtDNA simultaneously for forensic mixture deconvolution, which was mainly based on ONT platform [14,46]. mtDNA, being maternally inherited in a haploid manner, has a relatively short genome length suitable for long-read sequencing. Theoretically, mtDNA possesses inherent advantages and potential in the detection and analysis of mixed samples. Although Sanger sequencing is still widely used in numerous laboratories as the classic method with high accuracy for single source mtDNA HVRs sequencing, and NGS has been increasingly used in many laboratories as the second generation sequencing for both single and mixed mtDNA HVRs or whole genome, they could not perform the proper phasing of mtDNA HVRs haplotype with long length for mixture deconvolution [13–16]. This study integrated the advantages of nanopore sequencing technology (single-molecule sequencing with long-read length) with the haploid characteristics of mtDNA. The focus of the study was on the mtDNA HVRs, which have a length of ~1.4Kb and concentrate with genetic

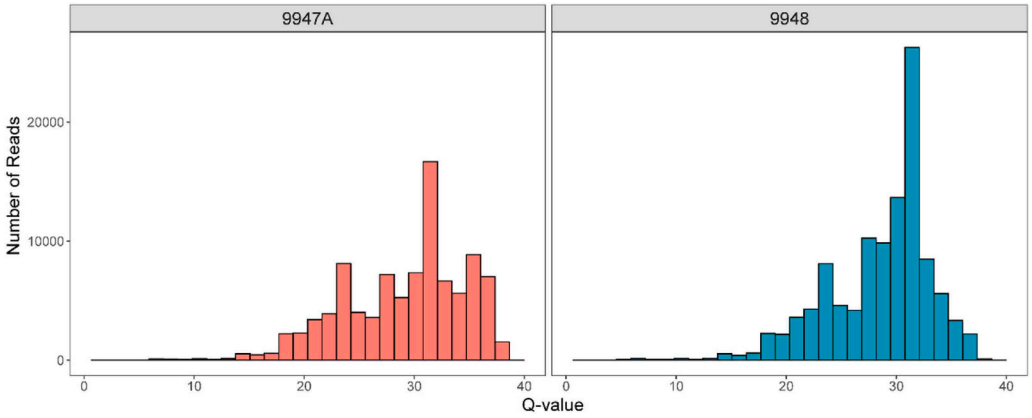

Fig. 4. Q-value distribution of all single reads for DNA standards by nanopore sequencing using the G-seq500 system. A total of 95,936 and 111,223 reads were obtained for 9947A and 9948, respectively.

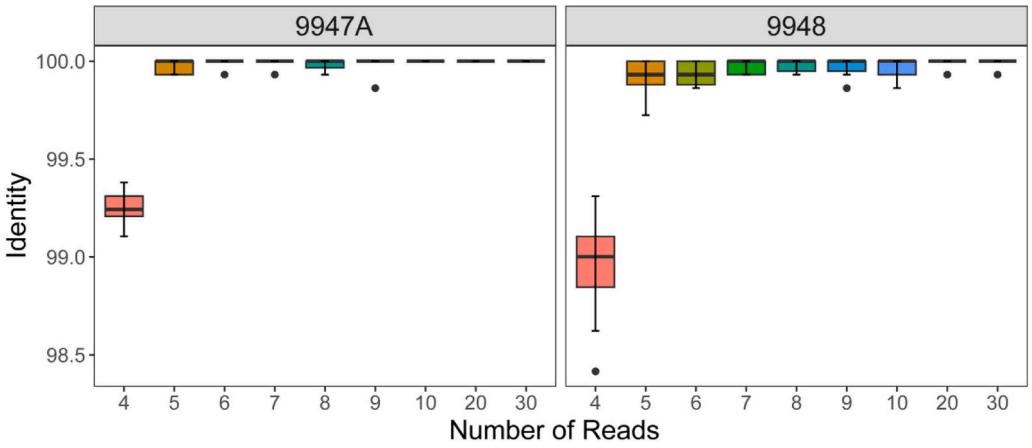

Fig. 5. Average accuracy of consensus sequences by the G-seq500 system for mtDNA HVRs amplicon of DNA standards at different data volumes. For both 9947A and 9948, when the number of reads reached 20 or more, the haplotypes obtained by nanopore sequencing were identical to that of the Sanger sequencing.

Table 2
Genotyping results of 9947A mtDNA HVRs from the Sanger sequencing and nanopore sequencing using the G-seq500 system when the number of reads reached 20 or more and comparison with Anderson sequence.

Mitochondrial SNP sites	Anderson sequence	Sanger sequencing	Nanopore sequencing
93	A	G	G
195	T	C	C
214	A	G	G
263	A	G	G
303	-	CC	CC
311	-	C	C
16,311	T	C	C
16,519	T	C	C

Table 3
Genotyping results of 9948 mtDNA HVRs from the Sanger sequencing and nanopore sequencing using the G-seq500 system when the number of reads reached 20 or more and comparison with Anderson sequence.

Mitochondrial SNP sites	Anderson sequence	Sanger sequencing	Nanopore sequencing
73	A	G	G
152	T	C	C
195	T	C	C
263	A	G	G
303	-	C	C
311	-	C	C
709	G	A	A
15,928	G	A	A
16,126	T	C	C
16,163	A	G	G
16,186	C	T	T
16,189	T	C	C
16,294	C	T	T
16,519	T	C	C

polymorphisms and easily amplifiable via PCR in forensic applications. Utilising the G-seq500 nanopore sequencing platform developed by Geneus Technologies, this study preliminarily evaluated the potential value of effective long-fragment detection of mtDNA HVRs in the forensic identification of mixed biological stains.

Firstly, the results of preliminary performance evaluation indicated that the technical approach established in this study met the general application requirements. Regarding accuracy, the sequencing results obtained by the G-seq500 platform exhibited concordance with those of Sanger sequencing and NGS for both DNA standards and biological samples. This platform exhibited substantial advantages in read length and accuracy of special loci (C-stretch area). Unlike ONT and other domestic nanopore sequencing technologies, this platform employs the concept of DNA sequencing by synthesis. Consequently, in this study, the accuracy of consensus sequence for single-source DNA standards achieved 100 % when the number of reads reached 20. Sanger sequencing is characterized by its low throughput and an effective read length generally limited up to 850 bp. Moreover, heterogeneity of C-stretch area in some samples led to frame shifts and overlapping peaks in Sanger sequencing results. Although NGS provides high throughput, its shorter read length requires assembly of long fragments. In this study, although the sequencing results of the mtDNA HVRs obtained from NGS and nanopore sequencing were consistent, NGS required separate sequencing of seven amplicons, which is unsuitable for mtDNA haplotype analysis in mixed samples. In contrast, the G-seq500 platform enabled high-throughput sequencing of long fragments (~ 1.4 kb) without assembly, while maintaining high accuracy even in homopolymer regions, in term of which other nanopore sequencing platforms have been found to be deficient [46–48]. In general, from the performances of specificity, sensitivity, reproducibility and adaptability, the technical approach in this study based on G-seq500 nanopore sequencing platform met forensic DNA identification requirements for both routine and complex biological samples.

Secondly, the performance of the established technical approach in mixture detection was highly satisfactory. Jiang et al. reported that high accuracy of 99.9 % could be achieved with a 5 % heteroplasmy (no haplotype separation) of mtDNA HVRs by nanopore sequencing on QNome platform [11]. Nakanishi et al. estimated individual mtDNA haplotypes in mixed DNA samples with a minor contribution of 5 % by combining MinION and MiSeq [46]. In our study, the nanopore sequencing results of PCR amplicon mixtures with varying proportions as low as 1 % maintained high accuracy of > 99.9 % for haplotype identity when ≥ 10 reads were obtained for each component. For minor components constituting ≥ 2 % of the mixture, ≥ 30 reads were sufficient to achieve consensus sequence with accuracy of 100 %. Certainly, when the minor component proportion decreased to 1 %, achieving the same accuracy required a larger number of reads. Across all proportions of mixed amplicons, only two haplotypes were detected, and the ratios of number of reads were generally consistent with theoretical mixing ratios. This indicated that the detection system established in this study reliably reflected the relative proportions of each component in mixtures and exhibited high sensitivity for minor components of low-abundance. However, for simulated mixed blood samples, the proportion of reads in 24:1 group deviated significantly from theoretical mixing ratio, likely due to differences in DNA content between components or amplification bias. This also underscored the complexity of mixture detection in practice. Finally, the successful application of this technical approach in

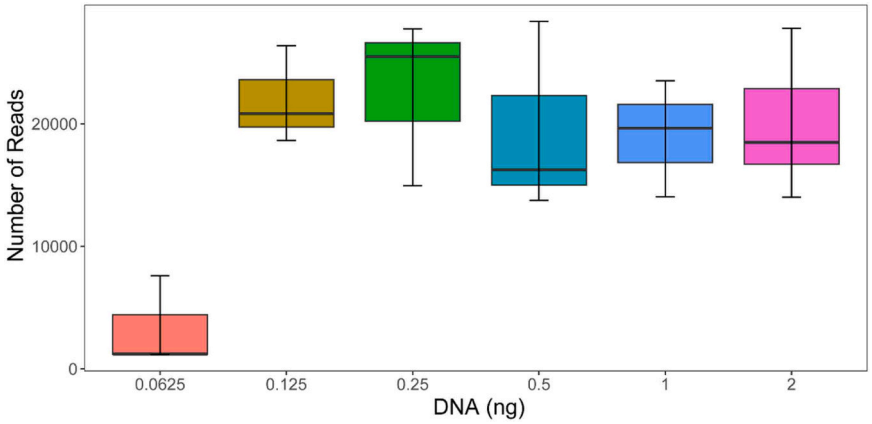

Fig. 6. Numbers of reads obtained from different amounts of 9947A as templates. 0.0625–2 ng of DNA standard 9947A were input for PCR amplification of mtDNA HVRs and nanopore sequencing using the G-seq500 system. The average numbers of reads were 3325, 21,952, 22,729, 19,459, 19,070 and 18,513, respectively.

Table 4
Results of nanopore sequencing using the G-seq500 system for different sample types and comparison with Sanger sequencing. The numbers of reads ranged from 31,856 to 49,267, and each sample obtained single-source haplotype.

Sample	Number of reads	Haplotype	Identity to Sanger sequencing
Blood stain	47,236	Single-source	100 %
Oral swab	49,267	Single-source	Heterogeneity in C-stretch region
Hair with follicles	37,215	Single-source	Undetectable by Sanger sequencing.
Miscarriage tissue	41,454	Single-source	Heterogeneity in C-stretch region
Muscle	32,362	Single-source	100 %
Costal cartilage	40,526	Single-source	100 %
Cigarette butt	34,466	Single-source	100 %
Toothbrush	31,856	Single-source	100 %
Chewing gum	46,005	Single-source	100 %

a rape case demonstrated its feasibility and potential for mixture identification in practice.

5. Conclusion

In summary, this study establishes a preliminary workflow for mtDNA HVRs sequencing and haplotyping based on the nanopore sequencing with the concept of DNA sequencing by synthesis. It

Table 5
Composition of haplotypes in C-stretch regions of HVR-I by nanopore sequencing using the G-seq500 system for the samples of oral swab and miscarriage tissue. Three and four types of haplotypes (reads) in C-stretch regions were detected respectively, and 11 continuous C was dominant for both of the samples.

Sample	Genotype of C-stretch	Proportion of reads
Oral swab	CCCCCCCCCCCC	21.89 %
	CCCCCCCCCC	57.49 %
	CCCCCCCCC	20.53 %
Miscarriage tissue	CCCCCCCCCCCCC	3.78 %
	CCCCCCCCCCC	35.25 %
	CCCCCCCCCC	41.94 %
	CCCCCCCC	19.03 %

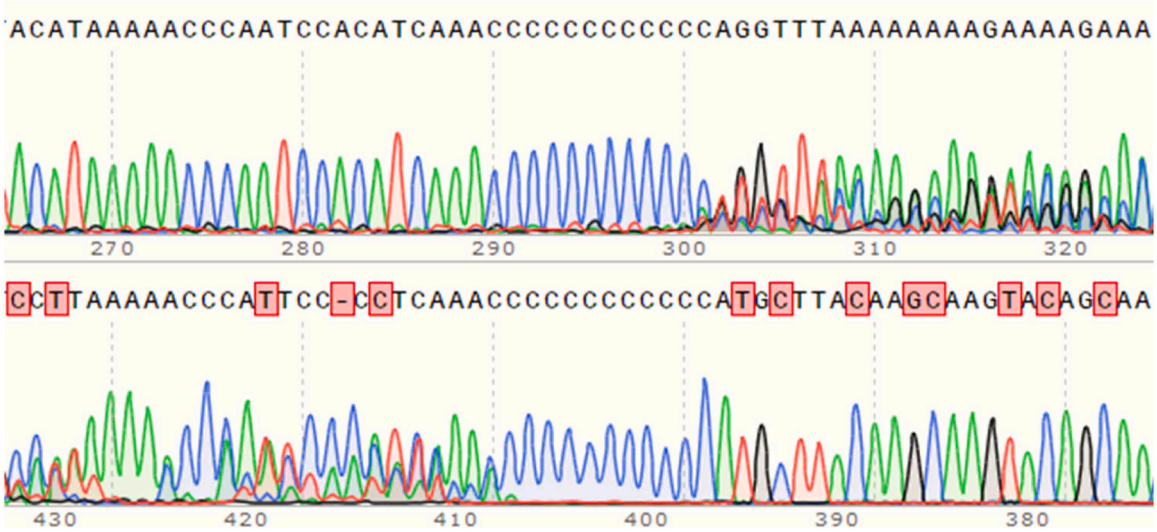

Fig. 7. Results of Sanger sequencing for the C-stretch area in mtDNA HVR-I from the miscarriage tissue. Shifts were observed on both flanks of the C-stretch area of HVR-I. Upper: forward sequencing result. Lower: reverse sequencing result.

Table 6
The deconvolution of results of nanopore sequencing using the G-seq500 system for amplicon mixtures of DNA standards. The mtDNA HVRs amplicon mixtures of 9947A and 9948 were mixed at different volume ratios as below to be sequenced. The haplotypes matched the donors and generally reflected the actual mixing proportions.

Mixing ratio (9947A: 9948)	Genotyping results		
	Haplotype	Number of reads	Proportion of reads
1:1	9947A	4581	57.17 %
	9948	3432	42.83 %
1:9	9947A	917	14.02 %
	9948	5625	85.98 %
1:19	9947A	288	4.17 %
	9948	6616	95.83 %
1:49	9947A	145	1.89 %
	9948	7528	98.11 %
1:99	9947A	110	0.77 %
	9948	14,160	99.23 %

demonstrates long read length and high accuracy consistent with Sanger sequencing and NGS for single-source DNA including DNA standards and biological samples. More importantly, it performs good in mixtures deconvolution with minor contributor as low as 1 %, and indicates significant potential for forensic DNA analysis of mixed biological samples in practice. More work will be involved to further test its effect through more cases validation and whole-genome sequencing of mitochondria using G-seq500 would be tried since the actual total read length have reached tens of kilobase pairs, thereby maximizing the utility of this genetic marker in mixed biological sample analysis.

Table 7
The deconvolution of results of nanopore sequencing using the G-seq500 system for amplified fragments of simulated mixtures of two blood samples. The blood of two unrelated individuals were mixed at different volume ratios as below to perform mtDNA HVRs amplification and be sequenced. The haplotypes matched the donors and generally reflected the actual mixing proportions.

Mixing ratio (Individual 1: Individual 2)	Genotyping results		
	Haplotype	Number of reads	Proportion of reads
3:2	Individual 1	833	54.09 %
	Individual 2	707	45.91 %
17:3	Individual 1	1502	86.37 %
	Individual 2	237	13.63 %
24:1	Individual 1	4586	98.71 %
	Individual 2	60	1.29 %

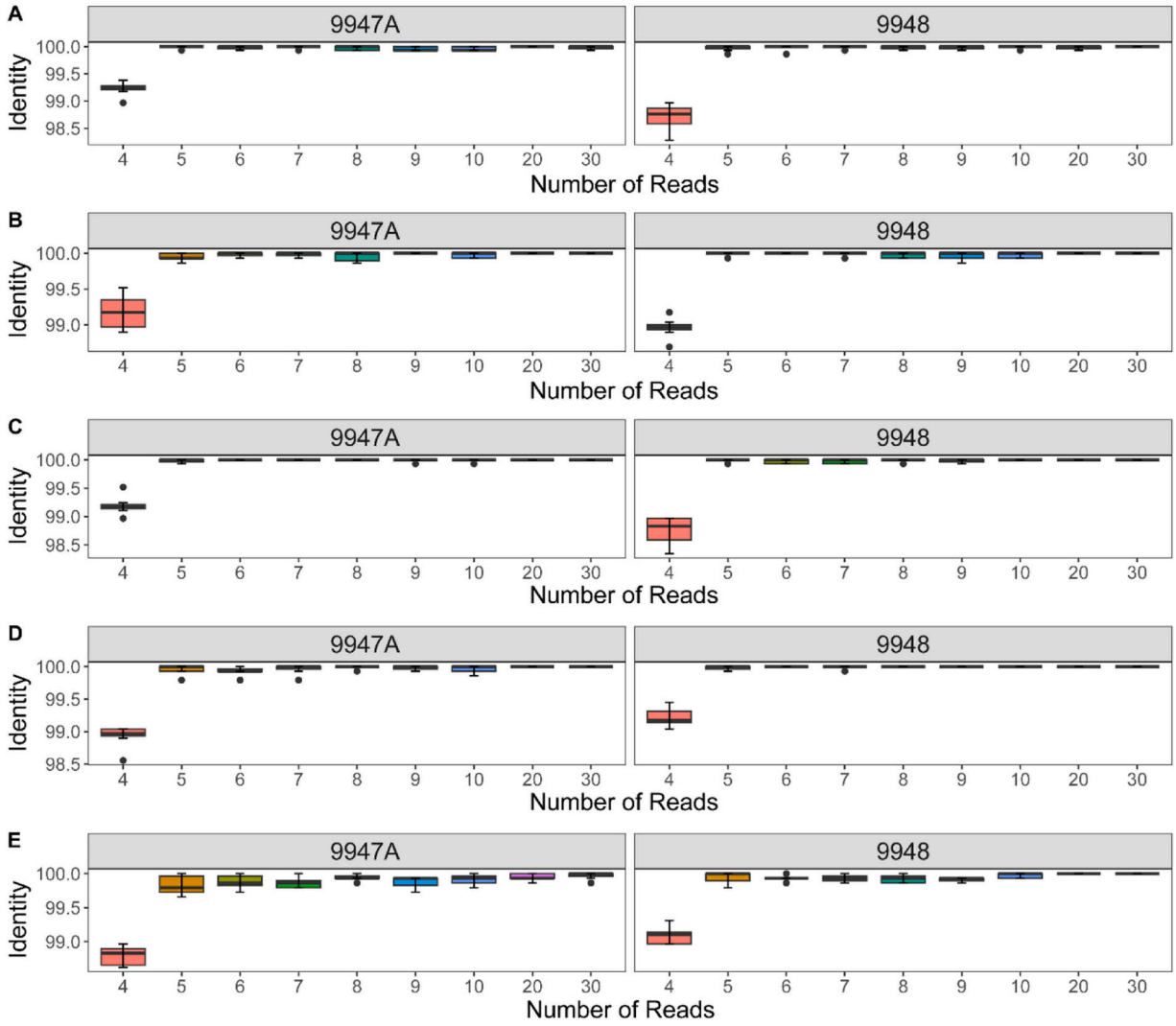

Fig. 8. Average accuracy of consensus sequences by the G-seq500 system for mtDNA HVRs amplicon mixtures of DNA standards at different data volumes. For all mixtures, When the number of reads reached 10 or more, the consensus sequences with accuracy of over 99.9 % were obtained. The ratios of 9947A to 9948: 1:1 (A), 1:9 (B), 1:19 (C), 1:49 (D) and 1:99 (E).

Table 8
SNP differences in the mtDNA HVRs between the two haplotypes from the blood mixtures by nanopore sequencing using the G-seq500 system compared with Sanger sequencing. There were 12 bases differences between the two haplotypes and each one was consistent with the Sanger sequencing.

Mitochondrial SNP sites	Sanger sequencing of Individual 1	Nanopore sequencing of Individual 1	Sanger sequencing of Individual 2	Nanopore sequencing of Individual 2
248	-	-	G	G
489	C	C	T	T
514	C	C	-	-
515	A	A	-	-
593	T	T	C	C
709	G	G	A	A
16,093	C	C	T	T
16,140	T	T	C	C
16,182	-	-	C	C
16,183	A	A	C	C
16,189	T	T	C	C
16,223	T	T	C	C

Table 9
The deconvolution of results of nanopore sequencing using the G-seq500 system for amplified fragments of the mixture from a rape case. The mtDNA HVRs amplification and sequencing were performed. The haplotypes matched the victim and suspect respectively.

Sample	Genotyping results		
	Haplotype	Number of reads	Proportion of reads
Vaginal swab	victim	37,202	98.54 %
	suspect	552	1.46 %
Victim's blood	victim	100,868	100 %
Suspect's blood	suspect	88,785	100 %

CRediT authorship contribution statement

Yanan Liu: Writing – review & editing, Funding acquisition, Conceptualization. **Huan Hu:** Writing – review & editing, Resources, Project administration, Formal analysis, Conceptualization. **Chunying Jiang:** Methodology. **Yaozhong Zou:** Resources, Formal analysis. **Wenjie Chai:** Project administration, Formal analysis. **Junrui Shui:** Validation, Software, Resources, Methodology, Data curation. **Yong Liao:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by grant from Scientific Research Projects of Science and Technology Commission of Shanghai Municipality (24JG0500500). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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