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SANGER SEQUENCING FOR MOLECULAR KINSHIP DETERMINATION IN IRAQI FAMILIES WITH MISSING PATERNAL MEMBERS

Israa Abdulsattar Ali[1], Dhuha Salim Namaa [2], Faridah Mohd Nor[3], Nur Najmi Mohamad Anuar[4], Siti Fathiah Masre[5]

[1] Faculty of Health Sciences, University Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 5300, Kuala Lumpur, Malaysia
[2] Department of Forensic Biology, Higher Institute of Forensic Sciences, Al-Nahrain University, Jadriya, Baghdad, Iraq
[3] Faculty of Medicine, Universiti Kebangsaan Malaysia, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia
[4,5] Centre for Toxicology and Health Risk Studies, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 5300, Kuala Lumpur, Malaysia

ABSTRACT

Background: Kinship testing is crucial during forensic investigations into missing persons, and Sanger sequencing has proven to be an effective method for establishing biological connections. The present work aimed to establish the worth of Sanger sequencing to resolve paternity claims among Iraqi families whose fathers have gone missing.

Materials and Methods: Genomic DNA was extracted from 300µl of whole blood, collected from six cases (n=120 samples) at the Medical Legal Directorate in Baghdad, Iraq. Each case comprised three relatives of the missing father, the mother, and the putative child. Polymerase Chain Reaction (PCR) procedures were performed using specific primers for the D18S, TPOX, CSF, and FGA genes. Sanger sequencing was utilised to compare the sequences from the relatives with those of the child.

Results: The findings of the present study revealed the sequence of outcomes for six Iraqi cases of missing fathers. The range of DNA concentration was (75 to 110 ng/µL). The findings revealed a high degree of sequence similarity between the grandson and his grandparents, as well as among the siblings, aligning with the expected patterns of inheritance. The sequencing data for six families revealed the matching alleles used in the study for each family member, which supported the pedigree hypothesis and confirmed the biological connections between the grandparents, siblings, and the grandson.

Conclusion: In conclusion, Sanger sequencing is widely utilised in kinship analysis due to its accuracy, reliability, and specific application scope. In certain situations, such as cases involving a missing father where traditional methods fall short, it is considered a crucial method for confirming paternity alongside other techniques like Short Tandem Repeats**.**

KEYWORDS: Sanger Sequence, Kinship analysis, missing father cases, Forensic application.

INTRODUCTION

Forensic genetics encompasses kinship analysis. Identifying kinship between relatives based on blood markers may be involved. The techniques used in the forensic Laboratory are utilised in paternity analysis and cases involving missing individuals, as well as in court for inheritance or immigration matters. Since the start of this millennium, there has been a sudden rise in interest in the field known as "kinship" due to its increasing popularity in forensic and anthropological applications. Regardless, DNA-based techniques have enhanced the accuracy and effectiveness of kinship analyses compared to serological-type solutions (Alketbi, 2023). Establishing parentage is a significant issue that affects families on multiple levels, including legal, social, and emotional. If a father is not present, like in violence after 2003 or displacement, there is significant uncertainty about inheritance and right of descent. In Iraq, years of turmoil and gun violence have unfortunately resulted in numerous families in which the paternity of children is not known, as fathers have gone missing or have been killed (Human Rights Watch, 2020).

These instances must be addressed by having sound and precise methods for establishing biological connections. Sanger sequencing has long been considered the "gold standard" among numerous DNA-based technologies due to its reliability and precision in identifying DNA sequences (Goodwin et al., 2016). This method enables the precise analysis of single genetic markers, including short tandem repeats (STRs) and single-nucleotide polymorphisms (SNPs), which exhibit high polymorphism rates within the human population and are consequently highly informative for establishing kinship (Butler, 2012) . Sanger sequencing is helpful when an intensive analysis of precise loci is required, and a large amount of genomic data is not necessary. However, more recent high-throughput sequencing technologies are now available. (Schmid et al., 2022)

The mechanism behind DNA chain termination is the Dideoxy Method of DNA sequencing/Sanger sequencing, which was pioneered in the late 1970s (Nicouleau, 2020). Sanger sequencing is used in forensic applications to produce high-quality sequence data of targeted regions, as anything less than that would be unacceptable in forensic applications. In kinship testing, sequencing mitochondrial DNA, Y-chromosome markers, or selected autosomal loci by Sanger sequencing provides the peak of evidence in proving genetic relationships. With its precision and reliability, it is used to confirm results achieved by another genotyping method, especially in cases where STR-based analyses are inconclusive. With the advent of next-generation sequencing (NGS), one would assume that it would replace Sanger sequencing. However, Sanger sequencing remains the most widely used sequencing methodology in forensics because it is cost-effective and has established protocols in forensic laboratories (Butler, 2022). Due to its high precision, reliability, and established standards globally, Sanger sequencing remains a valuable technique for kinship identification in forensic genetics. The process is based on chain-termination chemistry, which enables accurate reading of nucleotide sequences in specific areas of DNA. (Melkova et al., 2023)

When it comes to accuracy, Sanger sequencing delivers exceptionally dependable results for short DNA fragments. On the other hand, next-generation sequencing (NGS) can sometimes introduce errors due to the specific context of the sequences or biases related to the platform used. This means that careful validation is crucial before applying NGS in routine rhetorical contexts. As a result, Sanger sequencing remains the preferred method for targeted kinship testing, particularly when factors such as cost, simplicity, and accuracy take precedence over throughput. (Bruijns et al., 2018)

The research aims to determine the important role of Sanger sequencing in resolving controversial paternity disputes among families and to obtain conclusive findings regarding biological parentage, thereby addressing crucial legal and social needs within the families. The findings of this study will not only shed light on the affected families but also contribute to the general knowledge about the application of Sanger sequencing in complex kinship analysis from an Iraqi perspective.

MATERIALS AND METHODS

Sample collection

A total of 120 samples were collected from six cases involving fathers who were absent from their children's lives. Each case comprised three relatives of the missing father (grandparents, uncle, and aunts), in addition to the mother and the child concerned, [Table 1]. The Sanger sequencing method was employed to obtain data and compare the genetic sequences of the relatives with that of the child in instances where the father was absent. The inclusion criteria focused on kinship analysis for Iraqi families with missing fathers between 2014 and 2016 from three cities: Baghdad, Mosul, and Kirkuk. The exclusion criteria encompassed paternity analysis cases involving trios, incest, kidnapping, and neonatal misidentification.

Table 1 : The type of kinship in each case for thirty samples

Case No.	Grand mother	Grand father	uncle	Aunt	Child	Mother
Case 1		√	√	√	√	√
Case 2			√	2√	√	√
Case 3	√			2√	√	√
Case 4	√	√	√	√	√	√
Case 5			√	√	2√	√
Case 6			√	2√	√	√

Genomic DNA extraction

Genomic DNA extraction. Genomic DNA was extracted from the collected whole blood samples (300 µL) utilising two commercial kits: the Promega kit (Ambion Inc., USA) and the Qiagen kit (QIAamp DNA Investigator kit, 50 samples, Germany). The extraction process followed a protocol consisting of the following steps.

DNA Concentration and Purity Assessment

The concentration of DNA samples exhibiting satisfactory integrity was assessed using a Nanodrop spectrophotometer 2000c (Thermo Fisher Scientific), which was operated with basic computerised software for control and data recording. This process was preceded by using the TE buffer as a white solution. Two microliters of the isolated DNA were introduced into the Nanodrop to determine the concentration in nanograms per microliter (ng/µL), which was found to range from 75 to 110 ng/µL. Regarding DNA purity, the Nanodrop spectrophotometer 2000c was also employed, with sample absorbance measured at two wavelengths (260 and 280 nm). The A260/A280 ratio was determined to be within the range of 1.7 to 1.9, indicating that the DNA sample was of high purity.

Agarose Gel Electrophoresis

The isolated DNA and amplified PCR fragments were visualised through electrophoresis on an agarose gel and afterwards observed under UV light following ethidium bromide staining. Specifically, agarose was prepared at a

concentration of 0.8% (w/v) to verify the total DNA. One gram of agarose was dissolved in 100 mL of 0.5X TBE buffer (pH 8) and melted by heating and stirring. The agarose was allowed to cool to 60°C, after which ethidium bromide was added to achieve a final concentration of 0.5 µg/ml. The agarose solution was then poured into a tray, and a comb was positioned unreal on one edge of the gel. The gel was left to solidify until it became opaque, at which point the comb was cautiously removed. The gel tray was placed horizontally in the electrophoresis tank, and 0.5X TBE buffer was added to the tank until the gel was submerged by 3-5 mm. The isolated DNA or PCR product (10 µl) was loaded into each well, and five µl of DNA ladder (100 bp) was loaded into a single well, serving as a marker during the electrophoresis process. The electrophoresis was run at 5 V/cm² for 45 minutes, and after that, the Ethidium bromide-stained bands in the gel were visualised using a gel imaging system.

DNA Loading and Electrophoresis

The wells of the gel were filled with a mixture consisting of 3 µl of loading dye and 7 µl of extracted genomic DNA. Once all the wells were loaded, the electrical current was activated for 60 minutes at a voltage of 100 volts (5V/cm²). This process caused the negatively charged DNA to move from the cathode (-) towards the anode (+).

Agarose Gel staining and UV Visualization

Following the staining of the electrophoresis gels with ethidium bromide, the gel was immersed in the solution for 20-30 minutes. Subsequently, the gel was transferred to a gel documentation system for visualisation of the DNA bands.

Polymerase Chain Reaction (PCR)

PCR primers design

The primers used in this study were designed based on their reference sequences available in the National Centre for Biotechnology Information (NCBI) database, GenBank (http://www.ncbi.nlm.nih.gov/), and synthesised by Macrogen Ltd (Korea). They were stored in a lyophilised state at -20°C. The primers are detailed in [Table 2]. The PCR procedures were conducted using specific primers targeting the D18S, TPOX, CSF, and FGA genes.

Conventional PCR Reaction

To initiate the PCR, the reaction was optimised by testing four annealing temperatures: 56 °C, 54 °C, 52 °C, and 50 °C. These annealing temperatures were found to be optimal for producing clear and sharp bands in agarose gels, and therefore, they were used in the current study. This protocol employs 2xEasyTaq® PCR SuperMix. All PCR reactions were performed in a 25 µL final volume, following the manufacturer's instructions. SuperMix.

Detection of PCR Products

Products of PCR and the DNA ladder were separated using a 1.5% agarose gel (1.5g agarose/100 ml 1X TBE buffer) and run at 75 V for 90 min of electrophoresis. A DNA ladder (5 µl) was loaded onto a 2% agarose gel, which was used to estimate the molecular size of the PCR product bands. Additionally, five µL of the PCR product was loaded. The gel was stained with a (2 µl of 10 mg/ml) red safe solution. Bands were visualised on a UV transilluminator and then photographed using a photo documentation system.

Table 2: the mrepeats sequence primer size for the four primers used in the study

Primers	SequenceDirection	Primer Size	Annelling Temp.
D18S51	FGTCTCAGCTACTTGCAGG RGGAGATGTCTTACAATAACAGTTG	833pb	56C°
TPOX	FCTTAGGGAACCCTCACTG RGCAGCGTTTATTTGCCCAA	1209pb	54C°
CSF1PO	FACTGCCTTCATAGATCTTCCT RGCCCTGTTCTAAGTAACCA	804pb	54C°
FGA	FCTCACAGATTAAACTGTAACCA RTTGTCTGTAATTGCCAGC	828pb	52C°

Sanger Sequencing technique

The procedure for submitting a DNA template for sequence analysis encompasses several critical steps, beginning with identification and isolation, which were conducted at the Medical Legal Directorate in Baghdad, Iraq. Subsequent purification and quantification of the target DNA template, as well as sequencing, were performed at Macrogen in Korea. Typically, the DNA submitted for Sanger sequencing is an amplicon derived from an accepted assay employed for rhetorical purposes. The Sanger sequencing workflow comprises six direct steps from sample preparation to data acquisition [Figure 1]. When initiating the process with a small quantity of DNA, the initial step involves PCR amplification of the target to ensure adequate template availability for the sequencing reaction. This is followed by a cleanup step to eliminate superfluous primers. The PCR products are then utilised in a cycle sequencing reaction to produce chain-terminated fragments. A subsequent cleanup step is necessary to remove unincorporated ddNTPs, which could impede the transmissible analyser's capacity to detect the dyes. The

fragments are afterwards separated via capillary electrophoresis (CE), and the sequence is understood using data analysis software.

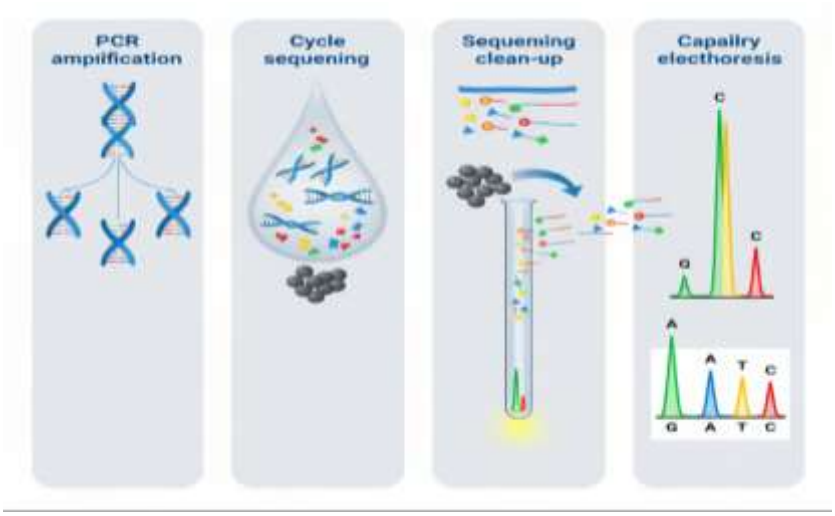

Figure 1: Sanger Sequence Steps

Data Analysis

The Genius software program was used to analyse the results of the Sanger sequence for each member of the family. Geneious software is a powerful bioinformatics platform designed to help researchers manage, analyse, and visualise DNA sequence data, including data generated by Sanger sequencing. Geneious streamlines the process by providing user-friendly tools for assessing chromatogram quality, base calling, aligning sequences to a reference, and detecting variants. Researchers can import Sanger chromatograms (trace files), visualise peaks, and identify SNPs or mismatches that may impact paternity determinations. Interactive visualisation features enable the straightforward interpretation of ambiguous or mixed peaks, a common challenge in forensic DNA analysis. Moreover, Geneious helps organise large datasets, annotate sequence regions, and generate publication-ready figures for reporting results (Kearse et al., 2012). In paternity analysis, using Geneious can reduce manual errors and speed up the investigation, providing standardised documentation and facilitating communication among forensic teams. This enhances confidence in the final conclusions on biological relationships.

RESULTS

The integrity of the DNA samples was evaluated using a Nanodrop Spectrophotometer 2000c from Thermo Fisher Scientific, USA. Absorbance readings were taken at 260 nm and 280 nm to calculate the A260/A280 ratio, which was found to be between 1.7 and 1.9. This indicates that the DNA is of high purity (Walter et al., 2016). The sequence results for samples received from Macrogen were analysed using Geneious (https://geneious.com) software [figure 2]. The repeat motifs of each primer utilised in the comparison among grandparents, siblings, and the grandson in this study, along with their chromosomal locations, are presented in [Table 3]. The comparison between the sequences of the relatives of the missing father and the child in question shows a match [Table 4]. Sanger sequencing results for all family members yielded DNA fragment sequences of sufficient quality and length for comparative analysis.

Sequence alignments indicate a high proportion of homologous regions among the samples, with minor nucleotide variations distinguishing unrelated or more distantly related individuals. The highest degree of sequence identity was observed between samples attributed to close kin relationships (i.e., between siblings and their direct descendants), confirming the expected Mendelian inheritance patterns. A lower concordance was observed between the grandson and the more distantly related grandparent; yet the shared allelic features remain consistent with a biological relationship. In this study, Sanger sequencing was employed to analyse specific genetic loci in DNA samples collected from grandparents, siblings, and grandsons to determine their kinship relationships. The sequencing chromatograms were successfully generated for all samples, showing clear peak patterns corresponding to the four DNA bases (A, T, C, G). The DNA sequences were aligned and compared to identify genetic similarities and variations among the family members. The results revealed high sequence similarity between the grandson and both grandparents, as well as among the siblings, consistent with expected inheritance patterns. Analysis of specific short tandem repeat (STR) markers verified familial connections by identifying shared alleles. The sequencing data corroborated the hypothesis that grandparents are biologically linked to both siblings and grandchildren. Table 1 summarises the sequence of identity percentages and key polymorphic loci comparisons among individuals in the family trio. The grandson's sequence showed approximately 99.5% homology with the grandparents' sequences, while siblings shared about 98–99% identity, illustrating close

genetic relatedness.

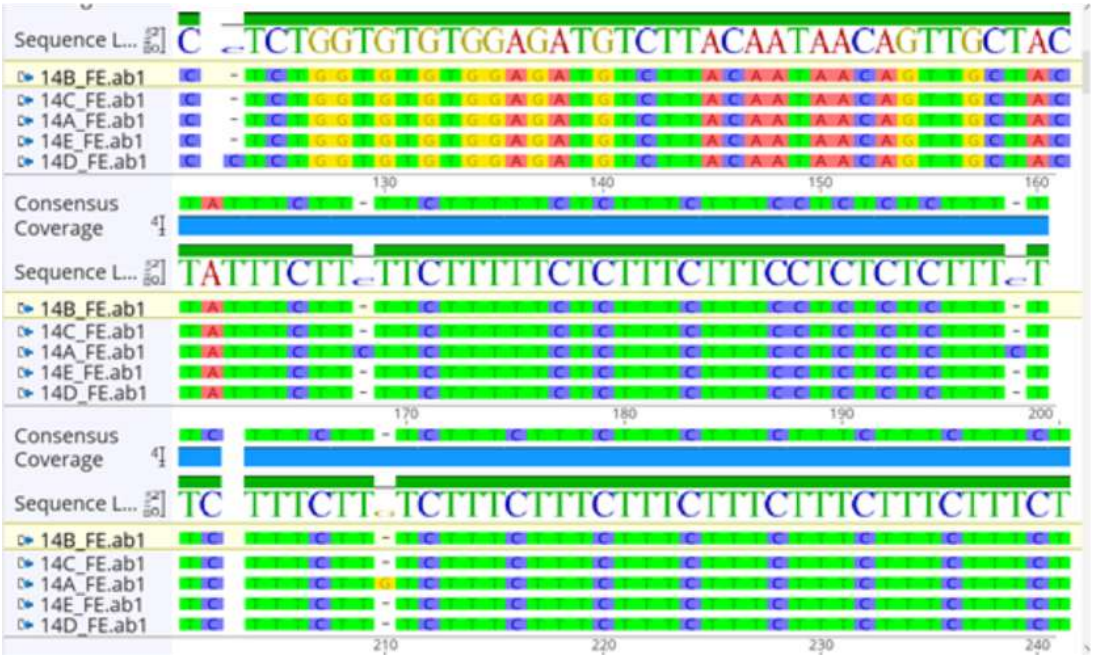

Figure 2: Alignment and comparative visualisation of multiple Sanger sequencing chromatogram files using Geneious software. The figure shows sequence reads from different family members labelled as 14B_FE.ab1 to 14E_FE.ab1, aligned against a consensus sequence. Colored base peaks highlight nucleotide variations and sequence quality, with coverage bars indicating the depth of read support for the consensus at each position.

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location in planning primers. However, in known polymorphic sites, family-based kinship studies, Sanger sequencing is a straightforward and cost-effective method. Overall, the research demonstrates that Sanger sequencing is successful in determining kinship relationships through careful analysis of DNA sequence compatibility between kin. Sanger sequencing determines the biological relationship between grandmothers, siblings, and grandsons, providing a solid genetic foundation for kinship verification in forensic, genealogical, or clinical testing. This study reminds us of the continuous usefulness of Sanger sequencing in kinship testing, where precise sequence data is critical, complemented by larger-scale, high-throughput sequencing techniques.

Table 3: the modif repeats for the four primers Used in the study and their location on chromosome

Primers	Repeat Modif	Chromosomal loction	Allele Range
D18S51	AGAA	18q21.33	7-27
TPOX	AATG	2p25.3	6-13
CSF1PO	AGAT	5q33.1	6-15
FGA	CTTT	4q28	18-51

Table 4: Sanger sequence results for the six families for the four primers

Kind of relatives	FGA	D18S	TPOX	CSF
Case 1 Mother	16	9	13	24
Child	16	8	13	23
Grandfather	16	9	13	24
Uncle	16	9	12	24
Aunt	16	9	12	23
Case2				
Mother	12	9	10	22
Child	12	11	10	22
Grandfather	12	11	9	22
Uncle	12	9	10	22
Aunt	12	11	10	22
Case 3				
Mother	16	9	10	21
Child	16	11	10	21
Grandfather	16	11	10	21
Uncle	16	11	10	21
Aunt	16	9	10	21
Case 4				
Mother	12	12	10	22
Child	12	11	10	22
Sister	12	11	10	22
Grandfather	12	11	10	22
Grandmother	11	11	12	22
Case 5				
Mother	15	8	10	23
Aunt	14	8	10	23
Uncle	14	8	10	23
Sister	14	8	10	23
Child	14	8	10	23
Case 6				
Mother	12	8	10	21
Uncle	12	8	10	21
Aunt	12	8	10	21
Aunt	12	8	10	21
Child	12	8	10	21

DISCUSSION

This study suggests that Sanger sequencing gives a foundation for DNA analysis with high reliability and accuracy, particularly for small-scale sequencing experiments or for targeting specific areas. Loci D18S51, TPOX, CSF1PO, and FGA were selected based on their high polymorphism, good amplifiability, and general use as standard markers in forensic DNA typing, which renders them highly informative for kinship and paternity testing. Major forensic organisations endorse these markers and routinely include them in validated commercial STR kits, ensuring compatibility with international databases and robust discrimination across diverse populations. (Butler, 2020 ; Schindler et al. 2020)

Current Sanger sequencing automation generally supports the generation of DNA sequences up to 800–1,000bp (Gupta et al., 2020). Typically, the most significant limitations of the approach are the presence of low-quality sequences within the first 15–40 bp due to primer binding, and an inability to distinguish single-base pair differences in longer segments (e.g., >900 bp) (Brodin et al., 2013). For optimal sequencing results, the target submitted for Sanger sequencing should exhibit a single product, or band, as confirmed by capillary electrophoresis or gel electrophoresis procedures. If one "band" is present, indicating a homogeneous product, the amplicon can be purified and used for sequencing. (Butler , 2012 ; Sambrook, 2001).

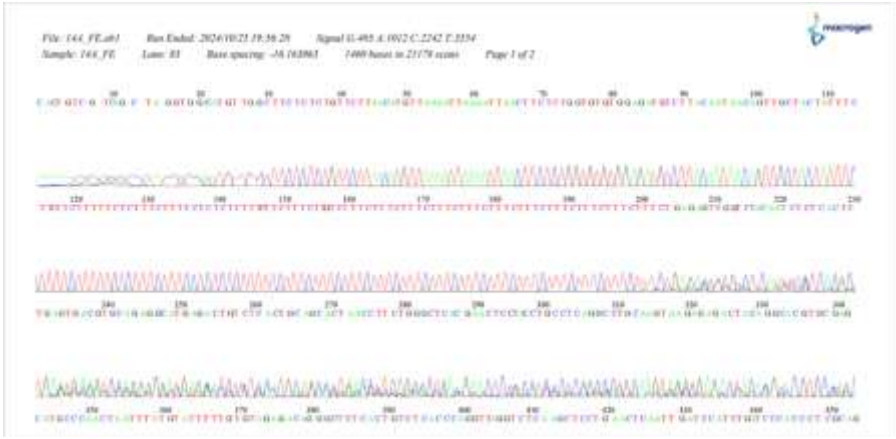
The primary methodological technique evident in these studies involves utilising chain-terminating dideoxynucleosides to yield random-length DNA fragments that are still being resolved and analysed for sequence content purposes (Chen, 2014). This approach is fundamental in instances where resolution is crucial, and mutation detection and verification of NGS findings are necessary, as well as in the analysis of mitochondrial DNA for both forensic and clinical purposes (Santani et al., 2017). The technique provides very high discriminatory power when examining single-nucleotide changes or small indels and is therefore still considered the gold standard for verifying sequences (Al-Shuhaib et al., 2023). The results showed a very close sequence of Identity between the grandson and both grandparents, as well as between siblings, consistent with the expected pattern of inheritance. However, partial homology and individual-specific alleles, as well as single-nucleotide polymorphisms (SNPs), enabled the distinction of individuals from one another within the pedigree (Melkova et al., 2023). It is clear from the data that full siblings and their offspring share a large number of alleles. As one progresses through generations (e.g., from grandparent to grandson), the agreement is substantially less. However, it is still significant, indicating a biological relationship(Borecki et al., 2008). These findings support Sanger sequencing as a valid alternative to STR profiling in stringent kinship cases where fragment size, sequencing detail, and base-calling accuracy are of paramount importance (Al-Shuhaib et al., 2023). The study demonstrates the ongoing applicability of the Sanger sequencing method for targeted kinship analysis and paternity testing. High sequence homology and expected Mendelian inheritance patterns substantiate the use of this method for confirming biological relationships in forensic casework (Browne et al., 2024). While increasing availability of NGS provides greater throughput and marker panel diversity, Sanger sequencing accuracy and availability still provide value in resource-limited forensic situations. (Bruijns et al., 2018)

A limitation of this methodology is its focus on predefined loci, which may restrict resolution in highly admixed or complex pedigrees. However, for direct kinship and straightforward paternity testing, it provides reliable results. Expanding the panel of analysed loci or transitioning to SNP-based NGS panels could further increase discrimination power for challenging cases (Colucci et al., 2025). Due to its precision and reliability, Sanger sequencing has relevance in both forensic and non-forensic fields. Forensic uses include confirming the Identity of persons in question, mitochondrial DNA evidence, and confirming DNA evidence in a criminal investigation. In contrast, non-forensic applications are clinical diagnostics, genetic tests, and validation of synthetic or therapeutic DNA constructs (Dahal et al., 2023). In large part, the greatest strength of Sanger sequencing is its high accuracy, long read lengths, and capacity to target specific sequences. Essentially, it is still the only method for confirming variants identified by NGS, or in cases where high error rates cannot be tolerated. However, it offers lower throughput than NGS, is less cost-effective per base, and is not scalable to large-scale or genome-wide projects (Ferreira et al., 2025). Another identified research gap is the lack of studies to provide evidence of the use of Sanger sequencing to detect distant kinship. While it is well-established for close family relationships and direct identification, there is a scarcity of research providing evidence for distant genetic relationships (more than first-degree), thus constraining its use in complex forensic cases and in population genetic studies where long pedigree analysis should be conducted (Schmid et al., 2022). The ability and routine developments in automation for analysing Sanger data are significant for improving turnaround time and reducing manual and human error. (Mostafa, 2023)

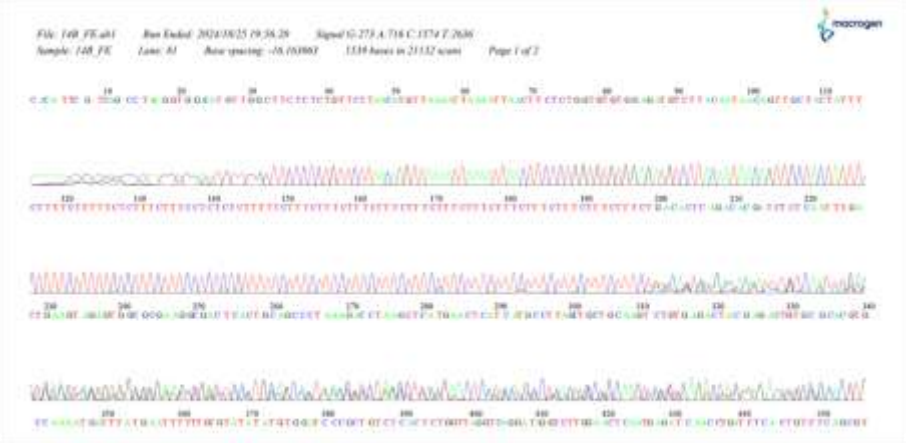
CONCLUSION

In conclusion, Sanger sequencing is widely utilised in kinship analysis due to its accuracy, reliability, and specific application scope. It is regarded as the gold standard for sequence verification and variant confirmation when precision is essential, serving as a confirmation method for other paternity techniques, such as Short Tandem Repeats. In certain situations, such as cases involving a missing father where traditional methods fall short, it is

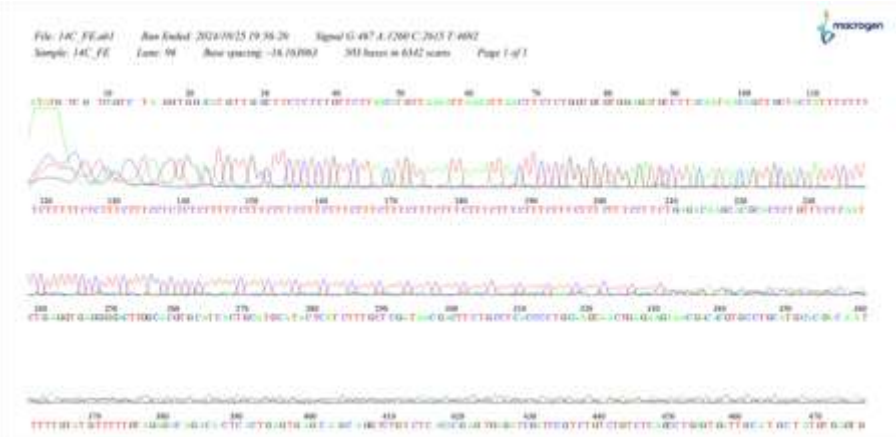
considered a crucial method for confirming paternity alongside other techniques like Short Tandem Repeats Continuous advancements in automation and the integration of Sanger sequencing with high-throughput platforms, along with efforts to improve population coverage, will ensure the sustained relevance and significance of both forensic and non-forensic genetic studies over time.



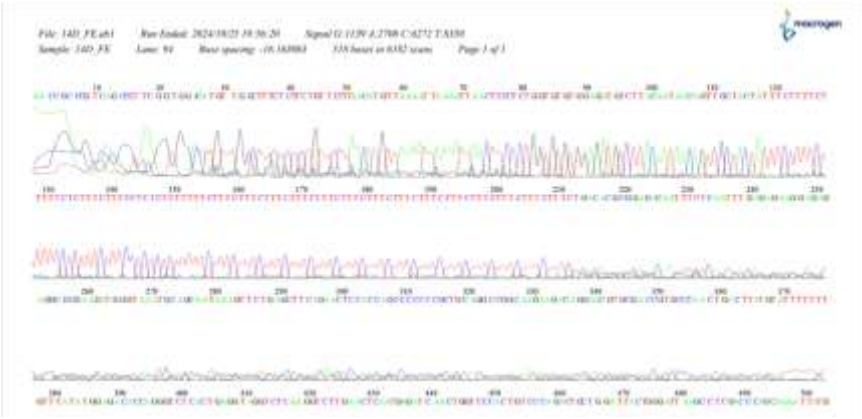
A- Grandfather



B – Child



C-Uncle



D- Aunt

Figure 3: Sanger sequencing results for a case involving the grandfather, uncle, aunt, and child. The results were obtained from Mcrogen Lab in Korea. Ethics approval and consent to participate

This study was approved by the Medical Legal Directorate in Baghdad/Iraq, with number 17656 on September 16, 2024.

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Conflicts of interest: There are no conflicts of interest**.**

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