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Targeted Sanger Sequencing as a Confirmatory Adjunct to Short Tandem Repeat-Based Paternity Analysis in Six Iraqi Families with Missing Fathers

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Abstract: Paternity testing in families with missing fathers depends on indirect genetic comparison because direct paternal DNA is unavailable. Such cases create legal and familial uncertainty and require methods that can strengthen kinship interpretation when conventional comparison becomes more difficult. The study aims to evaluate whether targeted Sanger sequencing could support short tandem repeat (STR) profiling in six Iraqi families with missing fathers. Inferred paternal profiles were reconstructed from the child, the mother, and available paternal-side relatives and were assessed descriptively against the routine short tandem repeat interpretation. Thirty individuals were analyzed by routine STR profiling across 20 autosomal loci, and targeted Sanger sequencing was performed for D18S51, TPOX, CSF1PO, and FGA. STR profiling generated the primary kinship dataset for all six families and revealed several microvariant alleles across the examined loci. Sequence analysis showed complete child-to-inferred-paternal agreement across the four sequenced loci in five cases, whereas one case showed agreement at two loci and disagreement at two loci. Comparison between sequence-based calls and fragment-length STR designations showed broad family-level concordance while also clarifying locus-specific differences. Routine short tandem repeat profiling remained the principal method for missing-father paternity assessment, whereas targeted Sanger sequencing served as a focused confirmatory approach for selected loci. Given the small case series and the four-locus sequencing design, the findings should be interpreted as exploratory.

Key Words: Forensic Genetics, Parentage Analysis, Deficiency Cases, Allele Inheritance, Sequence Confirmation, STR (Short Tandem Repeats)

INTRODUCTION

Establishing parentage is a central concern within the legal, social, and emotional dimensions of family life. Uncertainty surrounding parentage, particularly in cases involving a missing father, can lead to large-scale complications related to inheritance, legal rights, and lineage, especially in societies affected by violence or displacement [1]. In Iraq, for example, years of conflict and wars have led to many families in which the father of a child is unknown because men have gone missing or died. Therefore, reliable methods for confirming biological relationships carry clear legal and social value.

The STRs, also known as microsatellites, are highly polymorphic DNA sequences with repetitive units usually 2 to 7 base pairs in length. The high variability in repeat numbers among individuals makes STRs particularly valuable markers for human identification and kinship analysis [2]. In forensic paternity analysis, the core principle is that a child receives one allele from each parent at every STR locus. Such an inheritance pattern provides allele differentiation between maternal and paternal alleles establishing the possibility for direct or indirect paternity testing without genetic material of a biological father [3]. By analysing a standardised panel of parental STR loci and comparing the genetic profiles of the child, putative father, and ideally the mother, forensic examiners can evaluate familial relationships through comprehensive allele profiling

[4]. When the putative father's STR profile matches the paternal alleles identified in the child at all analysed loci, the results provide strong statistical evidence consistent with paternity [5]. Conversely, the presence of multiple allele mismatches between the child and the putative father at paternally inherited loci leads to the exclusion of that individual as the biological father [6].

Multiplexed STR testing uses modern technologies that permit the simultaneous analysis of many STR loci in a single reaction, which strengthens paternity testing through greater discriminatory power. The approach yields high probabilistic support for biological fathers and strong exclusionary power for non-fathers, which improves the reliability of forensic conclusions. Its value becomes especially clear in cases involving limited or degraded DNA, where robust amplification remains essential for forensic analysis [7]. Despite that strength, routine STR analysis may not fully resolve all deficiency paternity cases, particularly when the alleged father is missing and interpretation must rely on indirect comparison with relatives [8,9]. Under such conditions, length-based STR profiles may leave uncertainty in paternal allele assignment or fail to clarify locus-level discrepancies.

Sanger sequencing has long been regarded as a highly accurate method for determining nucleotide sequences, and that feature becomes particularly relevant when additional clarification is required at selected loci [10]. Such value extends to STRs and single-nucleotide polymorphisms (SNPs) as both marker types show substantial polymorphism across human populations and thus support kinship determination and relationship testing [11]. Therefore, Sanger sequencing may serve as a targeted STR-confirmatory approach when sequence-level evaluation of specific loci is needed and comprehensive genomic information is not required [12].

The present study aims to evaluate the practical feasibility of applying Sanger sequencing in paternity disputes involving six Iraqi families in which the fathers were missing. The analytical framework relied on DNA obtained from the child together with available relatives, including mothers, grandparents, and siblings, in order to strengthen the assessment of biological parentage when direct paternal DNA was unavailable. The primary endpoint of the study was descriptive concordance between targeted sequence-based calls and the kinship interpretation derived from routine short tandem repeat profiling. Secondary aims included clarification of locus-level discrepancies and assessment of whether sequence-level data strengthened interpretation in missing-father cases.

METHODS

Study Design and Ethical Approval

A case-series design was used to investigate paternity disputes involving six Iraqi families in which the alleged fathers were missing. The study was conducted at the Paternity Laboratory of the Medical-Legal Directorate, Baghdad, Iraq, from September 2024 to September 2025. Six missing-father paternity cases were included. The cases were selected through a random process, and each case included the mother, the child, and three available paternal-side relatives. Ethical approval for sample collection and laboratory analysis was obtained from the Medical Legal Directorate, Baghdad, Iraq, under approval number 17656 dated 16 September 2024. Routine STR profiling was performed for all 30 individuals, and targeted sequencing of four loci generated 120 locus-specific sequence observations. Detailed demographic characteristics were not reported because the study involved a small number of sensitive forensic family cases, and broader demographic disclosure could increase identifiability.

Sample Collection and Family Structure

Fresh blood specimens were collected on Flinders Technology Associates (FTA) cards from six families with missing fathers through the paternity laboratory of the Medical-Legal Directorate in Baghdad, Iraq. The cases originated from Baghdad, Kirkuk, and Mosul. The FTA cards were stored overnight at room temperature and were labelled clearly with the case number and sample identity. Each case included the child and the mother together with available paternal relatives, such as grandparents, uncles, aunts, or siblings, depending on family availability. Cases with incomplete family structure or insufficient analyzable material were not included. The distribution of kinship categories across the six cases is presented in Table 1.

DNA Preparation from FTA Cards

DNA for STR analysis was prepared directly from the blood spots on the FTA cards. A 1.2 mm punch was used to remove a small disc from each card, and the disc was transferred directly into a PCR tube for downstream amplification.

STR Amplification

Routine autosomal STR profiling was performed with the PowerPlex® Fusion System. The system was used for co-amplification and fluorescent detection of 20 STR loci

Table 1: A Summary of the Kinship Composition of the Six Investigated Families

Case No.	No. of samples	Grandfather	Grandmother	Uncle	Aunt	Sister	Child	Mother
1	5	✓	-	✓	✓	-	✓	✓
2	5	-	-	✓	✓✓	-	✓	✓
3	5	-	✓	-	✓✓	-	✓	✓
4	5	✓	✓	-	-	✓	✓	✓
5	5	-	-	✓	✓	✓	✓	✓
6	5	-	-	✓	✓✓	-	✓	✓

Routine STR analysis

loci together with amelogenin. The analyzed loci were D3S1358, D1S1656, D6S1043, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, and FGA. The panel included CODIS core loci and loci from the European Standard Set. Amplification was carried out in a final reaction volume of 25 µL according to the manufacturer's instructions. The master mix was vortexed for 3 seconds, centrifuged briefly, and dispensed into the reaction wells. A positive amplification control was included by adding 1 µL of control DNA at 10 ng/µL to a reaction well containing 25 µL of amplification mixture.

Capillary Electrophoresis and Allele Designation

Following PCR amplification, the STR products were analyzed by capillary electrophoresis on a 3130 Genetic Analyzer (Applied Biosystems, USA). Sample preparation for capillary electrophoresis included deionized formamide, a 500 bp LIZ internal size standard, and an allelic ladder from Promega, USA. Electrophoretic data were processed with GeneMapper® ID-X software (Thermo Fisher Scientific, USA). Panel, bin, and stutter files specific to the PowerPlex® Fusion system were used for allele designation. The resulting STR profiles were used for kinship comparison across the six families. Electrophoretic data were reviewed for peak clarity and allele assignment. Many samples were analyzed more than once on the ABI 3130 platform to verify profile clarity and reproducibility. Borderline or unclear profiles were reviewed manually before final interpretation.

Targeted Sanger Sequencing Analysis

Dna Extraction from Whole Blood: Genomic DNA for targeted sequencing was extracted from 300 µL of whole blood with two commercial extraction approaches. One approach used a Promega kit from the United States, whereas the second used the QIAamp DNA Investigator Kit from QIAGEN, Germany. DNA extraction was performed according to the manufacturers' protocols.

DNA Concentration and Purity Assessment

DNA concentration and purity were assessed with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). Absorbance was measured at 260 and 280 nm, and DNA purity was evaluated through the A260/A280 ratio. The recorded values ranged from 1.7 to 1.9. The sequencing section also reported DNA concentrations ranging from 14 to 20 ng and purity values between 1.7 and 1.8.

Agarose Gel Electrophoresis

The integrity of isolated genomic DNA and amplified PCR products was assessed by agarose gel electrophoresis. Genomic DNA was examined on 1.0% agarose gel, whereas amplified DNA was examined on 1.5% agarose gel. DNA samples of 7 µL were loaded into the wells, and a 100 bp DNA ladder was loaded as the molecular size marker. Electrophoresis was performed at 5 V/cm² for 45 minutes. The bands were stained with ethidium bromide and visualized under ultraviolet illumination. For detection of PCR products selected for sequencing, electrophoresis was also carried out on 1.5% agarose gel at 75 V for 90 minutes, followed by visualization and photographic documentation.

Target Loci and Primer Design

Four loci were selected for targeted sequencing, namely D18S51, CSF1PO, TPOX, and FGA. The four loci are well-established is forensic studies, suitable for locus-specific primer design, and can provide sequence-level targets for focused comparison with routine short tandem repeat profiling in the present case series. Primer design was based on reference sequences obtained from the National Center for Biotechnology Information database. D18S51 is located on 18q21.33 and carries the repeat motif (AGAA) [13,14]. CSF1PO is located on 5q33.1 and carries the repeat motif (AGAT) [15]. TPOX is located on 2p25.3 and carries the repeat motif (AATG) [16]. FGA is located on 4q28 and carries the repeat motif $(TTTC)_3$ $(TTTTTTCT[CTTT)_n$ $(CTCCTTCC)_2$ [13]. Primer sequences, expected amplicon sizes, and annealing temperatures are presented in Table 2. The reported amplicon sizes were 833 bp for D18S, 804 bp for CSF, 828 bp for FGA, and 1209 bp for TPOX, whereas annealing temperatures ranged from 52°C to 56°C.

PCR Amplification for Sequencing

PCR amplification for sequencing was carried out with 2× EasyTaq® PCR SuperMix (Transgen Biotech, China). Each reaction contained 12.5 µL of 2× EasyTaq® PCR SuperMix, 4 µL of template DNA, 1 µL of forward primer, 1 µL of reverse primer, and 6.5 µL of nuclease-free water, giving a final reaction volume of 25 µL. Amplification was performed on an ABI 9700 thermal cycler (Applied Biosystems, USA) configured for 96-well operation. The PCR program began with an initial denaturation step at 94°C for 5 minutes, followed by the cycling conditions specified for the assay.

Table 2: A List of the Primer Sequences, Amplicon Sizes, and Annealing Temperatures used for Targeted Sanger Sequencing

Primer	Sequence (5′–3′)	Amplicon size (bp)	Annealing temp. (°C)
D18S	F: GTC TCA GCT ACT TGC AGG R: GGA GAT GTC TTA CAA TAA CAG TTG	833	56°C
CSF	F: ACT GCC TTC ATA GAT AGA AGA R: GCC CTG TTC TAA GTA CTT CCT	804	54°C
FGA	F: CTC ACA GAT TAA ACT GTA ACC A R: TTG TCT GTA ATT GCC AGC	828	52°C
TPOX	F: CTT AGG GAA CCC TCA CTG R: GCA GCG TTT ATT TGC CCA A	1209	54°C

Sanger Sequencing and Sequence Analysis

Sanger sequencing was performed at Macrogen, Korea, using an ABI3730XL automated DNA sequencer. The resulting sequences were reviewed after alignment to reference sequences, and only interpretable chromatograms were retained for comparison with the corresponding short tandem repeat profiles. Sequence-based findings at D18S51, CSF1PO, TPOX, and FGA were then compared with the corresponding length-based STR profiles. Core repeat regions and flanking sequences were examined during sequence review.

Comparison Strategy

Inferred paternal profiles were reconstructed from the child, the mother, and the available paternal-side relatives in each case and were compared descriptively with the inheritance pattern derived from routine short tandem repeat profiling. Routine STR profiles obtained from the child, mother, and available paternal relatives were used as the primary basis for kinship assessment. Targeted Sanger sequencing results obtained for D18S51, CSF1PO, TPOX, and FGA were compared with the corresponding STR designations in order to assess concordance between sequence-based and length-based findings in the six families. Concordance was defined descriptively as agreement between the child and the inferred paternal profile at the sequenced loci. Complete concordance (%) referred to agreement at all four loci, whereas partial concordance referred to agreement at fewer than four loci.

RESULTS

The study examined whether targeted Sanger sequencing at four loci could support routine STR-based kinship assessment in six Iraqi families in which the fathers were missing. Routine STR profiling generated the primary forensic dataset across 20 autosomal loci, whereas Sanger sequencing provided sequence-level data for D18S51, TPOX, CSF1PO, and FGA.

Composition of the Study Cohort and Overall Genetic Dataset

The six families contributed 30 tested individuals, with five individuals examined in each case. Each family included the mother and child together with three additional paternal-side relatives. Across the full dataset, the cohort comprised 6 mothers, 6 children, 2 grandfathers, 2 grandmothers, 4 uncles, 8 aunts, and 2 sisters. Grandparental references were available in 3 of the 6 families, avuncular references were available in 5 families, and sibling references were available in 2 families. Such family structure provided several indirect kinship configurations for evaluation in the absence of direct paternal DNA. DNA prepared for targeted sequencing showed concentrations ranging from 14 to 20 ng, with purity values between 1.7 and 1.8.

STR Profile Findings Across the Six Families

Table 3 summarizes the STR profiles obtained across 20 autosomal loci. The STR dataset covered D3S1358, D1S1656, D6S1043, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, and FGA for all 30 individuals, which yielded 600 locus-specific STR observations. Case-wise comparison of the child with the mother and available paternal relatives allowed indirect evaluation of paternal allele transmission in all six families. Several microvariant alleles were present across the dataset, including 15.3, 16.3, 17.3, 9.3, 13.2, 14.2, 15.2, 16.2, 31.2, 32.2, and 33.2. A decimal microvariant pattern appeared in all six families, particularly at D1S1656, TH01, D19S433, D21S11, and FGA.

Table 3: STR Profile Data for the Six Families Across 20 loci

A: D3S1358 to Penta D											
	Relative	D3S1358	D1S1656	D6S1043	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF	Penta D
Case 1	Mother	15.16	15.15	11.13	8.12	10.10	9.11	13.14	17.20	10.13	9.14
	Child	15.15	15.16	11.13	8.11	7.10	9.11	13.15	18.20	12.13	14.14
	Grandfather	16.18	14.16	11.20	11.11	5.10	10.12	16.17	17.23	12.13	10.14
	Uncle	18.18	14.16	18.20	9.11	5.7	12.12	15.17	18.23	12.12	14.14
	Aunt	17.18	13.15	14.19	12.12	9.12	12.13	13.14	17.18	12.12	10.12
	Inferred paternal profile	18.16 17	13.14 15.16	19.20 11.14	11.9 11.12	5.10 9.12 5.7	10.12 12.13	13.14 15.17 16.17	17.23 18.23	12.13	10.12 10.14
Case 2	Mother	16.17	15.17	11.19	11.12	14.14	11.11	12.12	21.23	10.12	13.14
	Child	15.16	15.3.17	11.11	11.11	12.14	10.11	12.17	20.23	10.12	9.13
	Uncle	15.15	12.16	11.11	11.13	12.12	11.11	12.15	17.24	10.12	9.14
	Aunt 1	15.15	15.3.16	11.11	11.13	12.15	11.11	12.15	19.20	10.10	9.11
	Aunt 2	15.15	12.16	11.11	11.13	15.15	10.11	12.17	19.20	10.11	9.14
	Inferred paternal profile	15.15	15.3.16	11.11	11.13	12.15	10.11	15.17	19.20	10.11	9.14
Case 3	Mother	16.17	13.16	12.14	9.9	7.18	9.12	16.17	21.21	10.10	9.12
	Child	15.17	13.16	11.14	9.9	7.10	11.12	16.16	19.21	10.12	9.14
	Grandmother	17.18	11.15	10.11	9.12	9.14	11.12	11.16	17.19	10.12	10.12
	Aunt 1	15.18	11.15	11.12	11.12	10.14	9.11	11.14	17.18	10.12	2.2.12
	Aunt 2	15.17	15.15	10.15	9.11	9.10	9.11	14.16	17.18	10.12	12.14
	Inferred paternal profile	15.18	11.15	11.12	9.11	9.10	9.11	14.16	18.19	10.12	12.14
Case 4	Mother	15.18	13.16	14.15	12.12	16.17	9.13	12.17	27.27	10.10	11.12
	Child	16.18	15.16	14.14	8.12	12.17	13.13	12.17	17.27	10.10	11.13
	Sister	18.18	16.16.3	14.14	12.12	14.16	9.12	12.12	17.27	10.12	12.13
	Grandfather	15.18	16.3.17.3	11.13	8.9	12.12	11.13	12.13	22.24	10.10	13.14
	Grandmother	16.16	15.17.3	14.14	11.12	12.14	12.13	11.12	17.18	12.12	10.10

Table 3: Continued

A: D3S1358 to Penta D											
	Relative	D3S1358	D1S1656	D6S1043	D13S317	Penta E	D16S539	D18S51	D2S1338	CSF	Penta D
Case 4	Inferred paternal profile	15.16	15.16.3	13.14	8.11	12.14	12.13	11.12	17.22	10.12	10.13
Case 5	Mother	16.16	12.17	10.11	11.11	13.13	11.12	14.16	19.20	10.10	11.13
	Child	15.16	11.17	10.11	8.10	13.19	11.12	14.14	19.25	10.10	11.15
	Sister	16.17	17.17.3	11.14	11.12	13.13	12.12	14.16	20.25	10.10	11.15
	Uncle	14.17	11.17.3	11.3.14	12.13	13.19	12.13	14.16	18.20	10.11	9.12
	Aunt	15.17	11.17.3	10.11.3	8.12	13.18	11.12	15.16	25.25	11.11	12.15
	Inferred paternal profile	15.17	11.17.3	11.11.3	8.12	18.19	12.13	14.15	18.25	10.11	12.15
Case 6	Mother	17.18	15.17	11.11	8.12	15.17	9.12	13.17	14.17	10.11	10.12
	Child	17.17	14.17	10.11	8.13	11.15	11.12	12.17	17.18	11.11	10.10
	Uncle	16.17	15.17	10.11	12.13	11.16	11.12	12.15	18.23	11.12	10.13
	Aunt 1	16.17	15.17	10.11	11.12	7.13	12.12	12.15	18.23	10.12	10.13
	Aunt 2	16.17	15.17	10.11	11.12	13.16	11.12	13.15	17.18	10.10	10.10
	Inferred paternal profile	16.17	15.17	10.11	12.13	11.13	11.12	12.15	18.23	11.12	10.13
B: TH01 to FGA											
	Relative	TH01	VWA	D21S 11	D7S 820	D5S 818	TPOX	D8S1 179	D12S 391	D19S 433	FGA
Case 1	Mother	8.9	17.18	28.33.2	8.10	11.13	8.10	10.15	19.22	13.14	19.24
	Child	8.8	15.17	29.33.2	8.10	10.11	8.11	14.15	19.19	13.17	19.23
	Grandfather	6.8	14.16	29.29	9.11	11.12	9.9	10.13	20.21	13.17	21.21
	Uncle	6.8	14.16	29.30	9.10	10.12	9.11	13.14	19.21	13.13	21.23
	Aunt	6.7	16.17	29.29	10.10	11.12	11.11	16.16	18.19	14.2.15	22.24
	Inferred paternal profile	6.8.7	14.16 17	29.30	9.10 11	10.11 12	9.11	10.13 14.16	18.20 19.21	13.15 14.2.17	21.23 22.24
Case 2	Mother	6.6	15.16	29.32.2	8.10	13.13	8.11	15.15	19.21	13.16	22.24
	Child	6.7	15.15	29.32.2	8.10	11.13	11.11	13.15	19.23	13.17	22.23
	Uncle	7.9.3	14.17	28.30	8.12	11.11	11.11	12.14	21.22	13.2.15.2	24.28
	Aunt 1	6.7	16.17	28.29	11.11	11.14	11.11	12.14	19.21	13.17	23.24
	Aunt 2	6.7	16.17	28.29	11.11	10.13	8.10	12.13	19.23	13.2.17	24.28
	Inferred paternal profile	6.7	16.17	28.29	8.11	10.11	10.11	13.14	21.23	13.17	23.28
Case 3	Mother	7.9.3	17.17	28.31.2	10.11	12.13	9.11	9.14	18.20	13.15	21.21
	Child	7.7	16.17	28.28	10.11	11.13	11.11	11.14	20.24	13.15	21.21
	Grandmother	7.7	16.18	28.29	10.10	11.12	11.12	11.13	20.24	13.15	21.21
	Aunt 1	7.9	16.16	29.32.2	10.12	11.11	11.11	13.14	24.24	13.14	22.24
	Aunt 2	7.9	16.18	28.32.2	10.11	11.12	9.12	11.14	20.24	13.15	21.24
	Inferred paternal profile	7.9	16.18	28.32.2	10.11	11.12	9.11	11.14	20.24	13.14	21.22
Case 4	Mother	7.7	14.17	32.2.32.2	10.11	12.12	12.12	14.15	22.23	16.2.16.2	22.23
	Child	7.9	16.17	28.32.2	10.10	12.12	11.12	13.13	22.23	15.16.2	22.23
	Sister	7.9.3	14.16	28.32.2	11.11	9.12	11.12	13.14	22.22	15.16.2	20.23
	Grandfather	7.9	17.18	28.31.2	10.11	11.12	11.11	13.15	17.18	14.15	20.24
	Grandmother	6.9.3	16.19	28.28	10.11	9.11	8.11	12.13	17.22	14.2.15	22.24
	Inferred paternal profile	9.9.3	16.18	28.31.2	10.11	9.12	8.11	13.15	17.22	14.15	20.22
Case 5	Mother	8.9	18.18	29.30	7.8	10.11	8.8	13.14	18.24	13.13	22.22
	Child	6.9	18.18	29.30	8.10	10.13	8.8	14.15	18.23	13.14.2	22.25
	Sister	9.10	18.18	29.30	8.10	11.11	8.8	14.15	23.24	13.14.2	22.25
	Uncle	6.9	17.17	29.30	7.11	11.12	8.8	12.15	20.24	14.14.2	20.25
	Aunt	9.10	17.18	29.30	7.10	12.12	8.8	14.15	22.23	12.13	20.21
	Inferred paternal profile	6.10	17.18	29.30	10.11	11.12	8.8	12.15	20.23	13.14.2	20.25
Case 6	Mother	7.8	16.17	30.31	10.11	12.13	8.8	14.14	19.19	13.2.14	23.24
	Child	8.9	16.18	28.30	10.14	12.12	8.10	14.14	19.21	14.14	23.25
	Uncle	6.9	17.18	29.29	11.12	9.11	8.10	14.14	19.21	12.13.2	20.25
	Aunt 1	6.9	17.18	29.29	12.13	8.11	8.9	14.14	20.21	14.14	20.21
	Aunt 2	7.9	16.17	29.29	11.14	9.11	8.9	14.15	20.23	14.14	20.25
	Inferred paternal profile	6.9	17.18	29.29	12.14	9.11	9.10	14.15	20.21	12.14	20.25

Alleles are presented as genotype pairs separated by commas. Decimal values indicate microvariant alleles (e.g., 15.3, 16.3, and 14.2)

Sequence-Level Findings at D18S51, TPOX, CSF1PO, and FGA

Table 4 presents the sequence results for D18S51, TPOX, CSF1PO, and FGA across the six families, whereas Figure 1 shows representative sequence data from Case 1. Child-to-inferred-paternal comparison across the four sequenced loci yielded complete agreement in Cases 2, 3, 4, 5, and 6. Case 1 showed agreement at 2 of the 4 loci, namely D18S51 and FGA, whereas TPOX and CSF1PO differed between the child and the inferred paternal profile. Across the full set of 24 child-versus-inferred-paternal locus comparisons, 22 comparisons were identical and 2 comparisons differed, corresponding to an overall concordance of 91.7% at the targeted loci.

Table 4: Case-wise Sanger sequencing results for D18S, TPOX, CSF, and FGA.

Kind of relative	D18S	TPOX	CSF	FGA
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
Inferred paternal profile	16	9	12	23
Case 2				
Mother	12	9	10	22
Child	12	11	10	22
Uncle	12	11	9	22
Aunt 1	12	9	10	22
Aunt 2	12	11	10	22
Inferred paternal profile	12	11	10	22
Case 3				
Mother	16	9	10	21
Child	16	11	10	21
Grandmother	16	11	10	21
Aunt 1	16	11	10	21
Aunt 2	16	9	10	21
Inferred paternal profile	16	11	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
Inferred paternal profile	12	11	10	22
Case 5				
Mother	15	8	10	23
Child	14	8	10	23
Sister	14	8	10	23
Uncle	14	8	10	23
Aunt	14	8	10	23
Inferred paternal profile	14	8	10	23
Case 6				
Mother	12	8	10	21
Child	12	8	10	21
Uncle	12	8	10	21
Aunt 1	12	8	10	21
Aunt 2	12	8	10	21
Inferred paternal profile	12	8	10	21

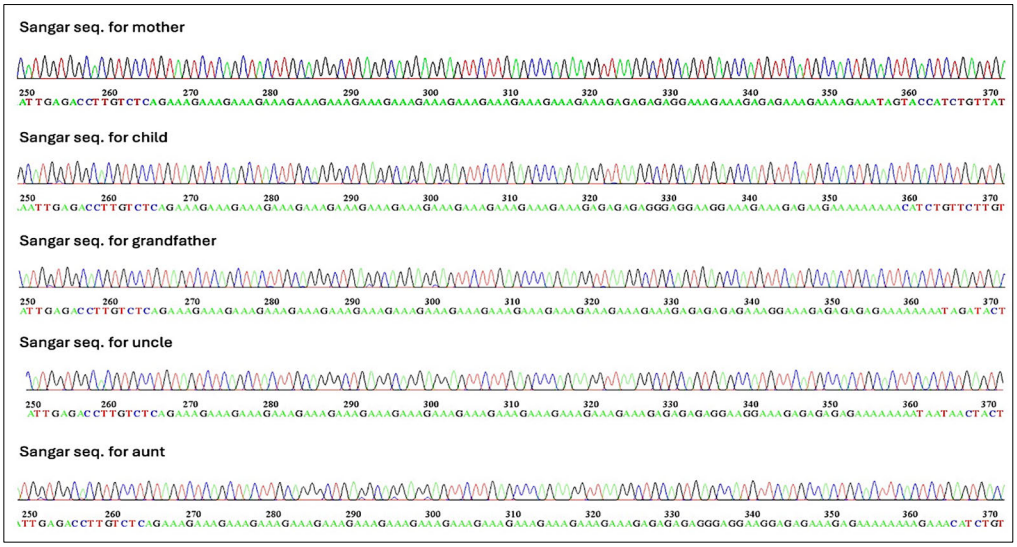

Figure 1: Representative Sanger sequencing Output for Case 1

Case 2 showed full agreement between the child and the inferred paternal profile at D18S51, TPOX, CSF1PO, and FGA, with the sequence pattern 12, 11, 10, and 22. Case 3 showed the same complete agreement, with the child and inferred paternal profile both carrying 16, 11, 10, and 21 at the four loci. Case 4 also showed complete agreement between the child and inferred paternal profile, with the sequence pattern 12, 11, 10, and 22. Case 5 showed full agreement across all four loci, with the child and inferred paternal profile both carrying 14, 8, 10, and 23. Case 6 showed complete identity across the four sequenced loci for the child, mother, uncles, aunts, and inferred paternal profile, all carrying 12, 8, 10, and 21. Case 1 differed from the other five cases because the child matched the inferred paternal profile at D18S51 and FGA only, whereas TPOX and CSF1PO showed different values.

Comparison between Sequence-Based Calls and STR Designations

Table 5 and Figure 2 compares the sequence-based values with the corresponding STR designations at the same four loci. That comparison involved 120 paired locus entries across the 30 individuals. Sequence results were recorded as single sequence-level values, whereas STR results were reported as allele pairs derived from fragment-length analysis. Direct one-to-one identity between the two formats therefore did not appear in every entry. For example, in Case 1 the maternal TPOX sequence value was 9, whereas the corresponding STR designation was 8,10. In Case 6 the maternal CSF1PO sequence value was 8, whereas the corresponding STR designation was 10,11. Similar format-related differences were visible in other rows across the table. Family-level interpretation nevertheless remained broadly consistent, because the sequence results tracked the same inheritance pattern at the four targeted loci in most cases.

Routine STR profiling generated a broad 20-locus dataset across all 30 individuals and served as the primary basis for kinship assessment in the six families. Targeted Sanger sequencing added 120 sequence observations across four loci and showed complete child-to-inferred-paternal agreement in five cases and partial agreement in one case. The combined results indicate that sequence-level analysis supported the interpretation obtained from routine STR profiling while providing locus-specific clarification in missing-father paternity assessment.

Table 5: Comparison of the Sanger Sequence-Based Calls and STR-Based Allele Designations

Parameters	Sanger sequence				STR profiling			
Sample code	D18S	TPOX	CSF	FGA	D18S	TPOX	CSF	FGA
Case 1								
Mother	14	9	13	24	13.14	8.10	10.13	19.24
Child	13	8	13	23	13.15	8.11	12.13	19.23
Grandfather	16	9	13	21	16.17	9.9	12.13	21.21
Uncle	15	9	12	22	15.17	9.11	12.12	22.24
Aunt	15	9	12	21	15.16	9.11	12.12	21.23
Case 2								
Mother	12	11	10	22	12.12	8.11	10.12	22.24
Child	12	9	10	22	12.17	11.11	10.12	22.23
Uncle	12	11	10	22	12.15	11.11	10.12	24.28
Aunt 1	12	9	10	22	12.15	11.11	10.10	23.24
Aunt 2	12	11	9	22	12.17	8.10	10.11	24.28
Case 3								
Mother	16	9	10	21	16.17	9.11	10.10	21.21
Child	16	11	10	21	16.16	11.11	10.12	21.21
Grandmother	16	11	10	21	11.16	11.12	10.12	21.21
Aunt 1	16	11	10	21	11.14	11.11	10.12	22.24
Aunt 2	16	9	10	21	14.16	9.12	10.12	21.24
Case 4								
Mother	12	12	10	22	12.17	12.12	10.10	22.23
Child	12	11	10	22	12.17	11.12	10.10	22.23
Sister	12	11	10	22	12.12	11.12	10.12	20.23
Grandfather	12	11	10	22	12.13	11.11	10.10	20.24
Grandmother	11	11	12	22	11.12	8.11	12.12	22.24
Case 5								
Mother	14	8	10	23	14.16	8.8	10.10	22.22
Child	14	8	10	23	14.14	8.8	10.10	22.25
Sister	14	8	10	23	14.16	8.8	10.10	22.25
Uncle	14	8	10	23	14.16	8.8	10.11	20.25
Aunt	15	8	11	23	15.16	8.8	11.11	20.21
Case 6								
Mother	12	8	8	21	13.17	8.8	10.11	23.24
Child	12	8	8	21	12.17	8.10	11.11	23.25
Uncle	12	8	8	21	12.15	8.10	11.12	20.25
Aunt 1	12	8	8	21	12.15	8.9	10.12	20.21
Aunt 2	12	8	8	21	13.15	8.9	10.10	20.25

Sequence results are shown as sequence-based calls, whereas STR results are shown as fragment-length allele designations. Commas separate the two alleles of each STR genotype, and decimal values indicate microvariant alleles

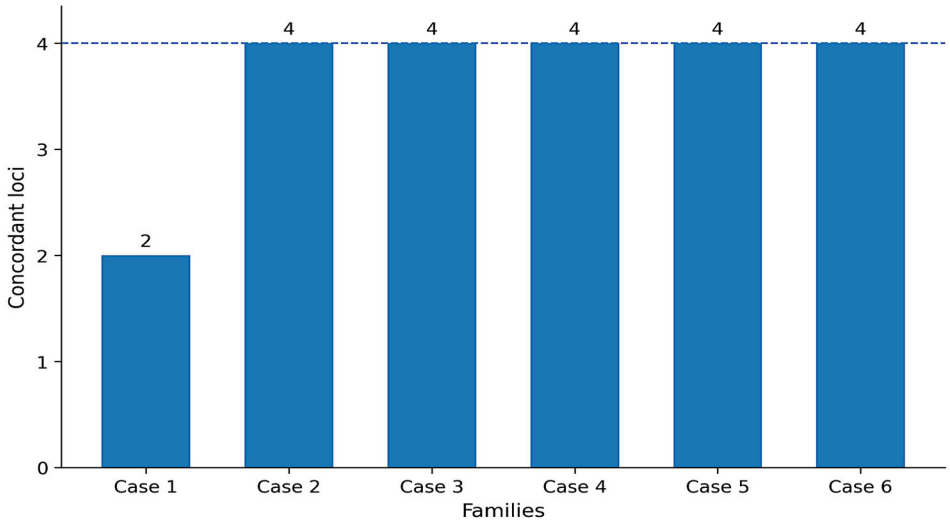

Figure 2: Locus-By-Locus Concordance between the Child and the Inferred Paternal Profile across D18S51, TPOX, CSF1PO, and FGA in the Six Families

DISCUSSION

The study examined the use of targeted Sanger sequencing as a supportive approach in six Iraqi families in which the alleged fathers were missing. Routine STR profiling provided the primary forensic framework through a 20-locus autosomal panel, whereas Sanger sequencing supplied sequence-level information for four selected loci, namely D18S51, TPOX, CSF1PO, and FGA. The results showed broad agreement between the family-level interpretation derived from STR analysis and the inheritance pattern observed at the sequenced loci. Five of the six cases showed complete child-to-inferred-paternal agreement across the four sequenced loci, whereas one case showed partial agreement, with concordance at two loci and discordance at two loci. Such pattern indicates that targeted Sanger sequencing can support conventional STR-based kinship assessment in missing-father cases, while routine STR profiling remains the principal method for paternity evaluation in such family structures.

Routine STR analysis retained its central role in the present work because autosomal STR markers continue to provide high discriminatory power, robust inheritance information, and broad forensic applicability in kinship testing. Their forensic value depends on high polymorphism, codominant inheritance, and compatibility with PCR-based workflows that can operate successfully even when sample quantity is limited [16-18]. The findings align with that principle as the 20-locus STR dataset generated the core evidence for the six families and allowed indirect paternal reconstruction through the child, the mother, and available paternal-side relatives. The Iraqi setting adds further relevance to that interpretation because forensic STR data from Iraq have already shown strong population-level discrimination and high paternity-testing utility, which supports the use of these loci in local kinship analysis [19]. Iraqi STR population data indicate that the analyzed loci are suitable for forensic identification and paternity testing in that population.

Missing-father cases remain analytically more complex than standard trio paternity testing because the paternal genotype must be inferred rather than measured directly. The present series included a mixture of grandparents, uncles, aunts, and siblings across the six families, which reflects the practical diversity of deficiency-case structures [20]. Such structures can reduce interpretive certainty because paternal relatives may share substantial portions of the missing father's genetic profile, thereby complicating the distinction between true paternal inheritance and broader familial similarity. Deficiency paternity studies have shown that incomplete family structures can increase the risk of ambiguous interpretation or misjudgment when the evidentiary model depends on limited markers or incomplete biostatistical framing [4]. International guidance from the ISFG likewise emphasizes that paternity testing in deficiency settings should be interpreted within explicit genetic and biostatistical frameworks, rather than through simple visual comparison alone.

Targeted Sanger sequencing added value by allowing sequence-level inspection of selected loci that were already informative in the STR dataset. That advantage becomes particularly relevant when locus-level clarification is required. The STR profiles in the present study included several decimal microvariants, such as 15.3, 16.3, 17.3, 9.3, 14.2, 16.2, 31.2, 32.2, and 33.2, which indicate structural complexity within the repeat regions. The presence of decimal microvariants in several loci is forensically important because the variants reflect sequence heterogeneity within repeat regions and can complicate allele designation, inheritance tracing, and comparison between fragment-length and sequence-based outputs. Sequence-based approaches are valuable in such contexts because fragment-length analysis alone may not fully capture the underlying variation within the repeat tract or its flanking regions [17,21]. Sanger sequencing, therefore, served a focused confirmatory role in the present study by adding nucleotide-level resolution at four loci without replacing the wider genomic coverage offered by the 20-locus STR panel.

Case 1 showed concordance at D18S51 and FGA and discordance at TPOX and CSF1PO. Possible explanations include locus-specific sequence variation, primer-binding site effects, allele dropout, or mutation [5,6,22]. Published human case reports have confirmed biological paternity despite multiple incompatibilities at analyzed loci, which demonstrates that isolated or limited mismatches should be interpreted cautiously and within a broader evidentiary context [6]. Recent human work has also shown that Sanger sequencing can identify primer-site variants responsible for dropout alleles in paternity testing, thereby explaining discrepancies that would otherwise appear inconsistent with inheritance expectations [22].

The comparison between the sequence-based results and the STR designations also requires careful interpretation. Sequence values and STR allele pairs do not represent identical forms of measurement. Fragment-length STR typing reports allele sizes, whereas Sanger sequencing reports nucleotide-level composition within the amplified region. Direct numerical identity between a sequence value and an STR allele pair is therefore not expected in every row. The meaningful comparison lies in the inheritance pattern and not in superficial similarity of notation. Sequence-based forensic studies have shown that underlying sequence variation, isoalleles, and flanking-region polymorphisms can remain hidden beneath apparently similar fragment-length calls, and that sequence-aware approaches can therefore improve resolution in kinship analysis [23,24]. MPS-based STR studies have likewise shown that sequence investigation reveals additional variation beyond conventional CE typing, which supports the broader interpretive principle behind the targeted sequencing strategy used here.

Our findings also need to be viewed within the wider transition from conventional CE-based STR typing to sequence-based forensic genetics. Massively parallel sequencing has expanded the analytical potential of parentage testing by increasing locus coverage and revealing sequence-based allele information that can improve resolution and statistical power [25-27]. MPS studies in paternity testing have shown that sequence-based STR analysis can strengthen inheritance tracking and reveal additional allelic detail beyond conventional CE-based systems. Such advances do not eliminate the relevance of targeted Sanger sequencing. A narrower Sanger-based approach remains useful when the laboratory goal is confirmatory analysis at a small number of loci rather than full high-throughput profiling. The present study supports that narrower role. Targeted Sanger sequencing functioned as an adjunctive tool that complemented routine STR analysis and helped strengthen interpretation in a resource-limited, case-oriented setting. MPS-based paternity studies and broader forensic STR reviews support that technological positioning.

The legal and social context of the present work adds practical importance to the analytical findings. Missing-father paternity cases can affect lineage, inheritance, family rights, and civil identification. A forensic approach that combines broad STR profiling with sequence-level clarification at selected loci may therefore provide stronger support for complex family decisions than either narrative comparison or a narrow locus set alone. The present study does not establish a universal need for Sanger sequencing in every deficiency case. The results do, however, support its use when locus-level clarification is required and when conventional STR analysis would benefit from targeted sequence confirmation.

Several limitations still shape the interpretation of the study. The series included six families only, and the sequencing component covered four loci only. The missing fathers were not sampled directly, so paternal profiles were inferred from relatives. Formal biostatistical measures such as likelihood ratios, paternity indices, or combined paternity indices were not reported, even though ISFG recommendations place strong emphasis on such measures in paternity testing and especially in deficiency settings [20]. International recommendations on paternity investigations and paternity biostatistics, therefore, support a more formal statistical framework for future work. Furthermore, formal pedigree-based likelihood modeling, paternity-index calculation, and combined paternity-index calculation were not reported.

Overall, the present findings indicate that routine STR profiling should remain the principal method for missing-father paternity analysis, whereas targeted Sanger sequencing can provide useful sequence-level clarification at selected loci. The study therefore contributes practical evidence from an Iraqi forensic context and supports a balanced interpretation of Sanger sequencing as a supportive confirmatory tool in complex kinship analysis.

CONCLUSIONS

The present case series showed that routine STR profiling provided the principal evidentiary framework for kinship assessment in six Iraqi families with missing fathers, whereas targeted Sanger sequencing of D18S51, TPOX, CSF1PO, and FGA added sequence-level clarification at selected loci. Family-level interpretation derived from the two approaches was broadly consistent, with complete child-to-inferred-paternal agreement across the four sequenced loci in five cases and partial agreement in one case. Case 1 showed that locus-level discrepancies may still arise even when the overall kinship pattern remains supportive, which underscores the value of sequence review in complex deficiency investigations. The combined findings indicate that targeted Sanger sequencing can strengthen interpretation of conventional STR results in missing-father paternity analysis, particularly when paternal inheritance must be reconstructed indirectly from relatives. Routine STR analysis should remain the primary forensic method, whereas targeted sequencing may serve as a focused confirmatory tool when additional clarification of selected loci is required. Broader studies with larger case series, wider locus coverage, and formal biostatistical evaluation would further define the practical role of this approach in complex kinship casework.

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Author Contributions

Conceptualization, I.A.A. and D.S.N.; methodology, I.A.A., D.S.N. and S.F.M.; validation, D.S.N., F.M.N. and S.F.M.; formal analysis, I.A.A. and D.S.N.; investigation, I.A.A. and D.S.N.; resources, D.S.N., N.N.M.A. and S.F.M.; data curation, I.A.A. and D.S.N.; writing, original draft preparation, I.A.A.; writing, review and editing, D.S.N., F.M.N., N.N.M.A. and S.F.M.; supervision, F.M.N., N.N.M.A. and S.F.M.; project administration, S.F.M. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

The data supporting the findings of this study are not openly available because the work involves sensitive human genetic and forensic case materials. Access to de-identified data may be considered by the corresponding author upon reasonable request and subject to approval by the relevant ethical and legal authorities, including the Medical-Legal Directorate, Baghdad, Iraq.

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