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CASE REPORT

Detection of sequence-tagged sites to reveal mechanisms of multi-step mutations at multi-copy Y-STRs in father-son pairs

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Abstract

The case report demonstrates that co-examining various forensic markers with different technologies can strengthen the connection between father-son pairs with multi-step mutations observed at multi-copy Y-chromosomal short tandem repeats (Y-STRs). We detect 33 autosomal STRs and 94 identity-informative single nucleotide polymorphisms (iSNPs) using capillary electrophoresis (CE) and/or next-generation sequencing (NGS) to confirm the relationship within pedigrees. Meanwhile, this study reveals that the mechanisms behind the mutations observed in these cases involve STR slippage (identified using NGS or Sanger sequencing methods) and/or chromosomal structure rearrangement (identified using sequence-tagged site analyses). Such rearrangement can result in one or more step mutations. Loci within the same rearrangement region will also be linked and 'mutate' simultaneously. The chromosomal structure rearrangement rate observed in this study is calculated as 0.0012 (95% confidence interval: 0.0002–0.0034) in Northern Han Chinese.

Keywords Forensic genetics · Y-STR · Multi-copy locus · Mutation · Sequence-tagged site

Introduction

The Y-chromosomal short tandem repeats (Y-STRs) located within the non-recombining region of the Y chromosome (NRY) serve as male-specific genetic markers. Y-STRs provide valuable investigative leads on male persons of interest in scenarios where the detection of autosomal short tandem repeats (A-STRs) is limited, such as in sexual assault cases involving male/female mixtures that present minor levels of male DNA amidst high female DNA backgrounds. In addition, the haplotypes of Y-STRs are stably inherited from father to son if there are no mutations, which benefits familial DNA searching and biogeographic ancestry inference. Thus, Y-STRs are recognized as one of the most important forensic genetic markers [1, 2].

In general, the commonly used Y-STRs exhibit an average mutation rate (μ) of approximately 10^{-3}, with a range spanning from 10^{-2} to 10^{-4} [3–8]. Neuhuber et al. [9] reclassified Y-STRs in function of their mutation rates in rapid mutating ($\mu > 1 \times 10^{-2}$), fast mutating (μ: 5×10^{-3}–1×10^{-2}), moderate mutating (μ: 1×10^{-3}–5×10^{-3}), and slow mutating ($\mu < 1 \times 10^{-3}$). The application of Y-STRs in forensics varies depending on the mutation rate. For instance, slow and moderate mutating Y-STRs are suitable for evolutionary, genealogical, and forensic studies [3, 9], while fast and

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rapid mutating Y-STRs are advantageous for distinguishing male relatives [10, 11].

A comprehensive pedigree study regarding the mutability of Y-STR demonstrates a more precise mutation rate compared to those derived from unrelated individuals. Our previous studies involved a pedigree analysis of 2548 Northern Han Chinese father-son pairs, wherein we identified that the mutation rates at DYF387S1a/b (4.9×10^{-3}), DYF404S1a/b (3.3×10^{-3}), DYS449 (9.0×10^{-3}), DYS518 (9.0×10^{-3}), and DYS570 (5.1×10^{-3}) were significantly lower than previously defined $>1\times10^{-2}$ [3]. Furthermore, we encountered mutational events affecting the gains or losses of 1 to 5 steps at 3 to 4 loci, which remain inadequately explained. In one case, four mismatches were found: a single-copy marker (DYS635) involving a one-step gain mutation alongside three multi-copy markers (DYS527a/b, DYF387S1a/b, and DYF404S1a/b) affecting multi-step gain or loss mutations. In two additional cases, three mismatches were recorded at DYS527a/b, DYF387S1a/b, and DYF404S1a/b, reflecting gains or losses of 1 to 3 steps. All the cases exhibited mutations at three multi-copy markers, but the potential connection between these mutational events has yet to be reported. Thus, we utilized different technologies in this study to gain insights into the mechanisms underlying these mutational events.

Materials and methods

Samples, DNA extraction, and quantification

In our previous studies, three pairs with three mismatches and one with four were observed from 2548 father-son pairs [4]. Samples were extracted using the Trace Evidence DNA Isolation Kit (AusBio, Yantai, China) on the Microlab STAR Liquid Handling System (Hamilton, Bonaduz, Switzerland). Genomic DNA was quantified on the Applied Biosystems ® QuantStudio 5 Real-Time PCR System using the Quantifiler ® Trio DNA Quantification Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's recommendations [12].

Length- and sequence-based STR and identity-informative SNP typing

Analyses of 29 A-STRs in the Goldeneye® DNA ID 30 A Kit (Peoplespot, Beijing, China) and 41 Y-STRs in the Goldeneye® DNA ID Y Plus Kit (Peoplespot) from father-son pairs were conducted on the Applied Biosystems™ 3730 DNA Analyzer (Thermo Fisher Scientific) according to the manufacturer's recommendations. Meanwhile, STR and identity-informative single nucleotide polymorphisms (iSNPs) were detected with the ForenSeq™ DNA Signature Prep Kit on the MiSeq FGx® Sequencing System (Qiagen, Hilden, Germany) or Sanger sequencing on the Applied Biosystems® 3130*xl* Genetic Analyzer (Thermo Fisher Scientific). All methods and materials were detailed in our previous studies [4].

Sequence-tagged site analysis

Sequence-tagged sites (STSs) were employed to detect potential deletions in the azoospermia factor c (*AZFc*) region. Primers of STSs (sY142, sY1161, sY1191, sY1291, sY1206, and sY1201) from [13] are listed in Table 1 for amplification and sequencing in this study. Sanger sequencing was performed on the Applied Biosystems® 3500XL Genetic Analyzer (Thermo Fisher Scientific) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Raw data were analyzed using the Sequencing Analysis Software v5.3.1 (Thermo Fisher Scientific). Figure 1 illustrates the information on the variance of STSs associated with the type of (partial) deletions in the *AZFc* region from published literature [14, 15].

Table 1 Primers of sequence-tagged sites (STSs) for PCR amplification and Sanger sequencing

STS	Forward Primer Sequence	Reverse Primer Sequence	GRCh38.p14 chrY Location	Amplicon Length (bp)
sY142	AGCTTCTATTCGAGGGCTTC	CTCTCTGCAATCCCTGACAT	21,831,728–21,831,923	196
sY1161	CGACACTTTTGGGAAGTTTCA	TTGTGTCCAGTGGTGGCTTA	22,092,892–22,093,221	330
			22,493,184–22,493,513	330
sY1191	CCAGACGTTCTACCCTTTCG	GAGCCGAGATCCAGTTACCA	22,729,473–22,729,857	385
sY1291	TAAAAGGCAGAACTGCCAGG	GGGAGAAAAGTTCTGCAACG	23,358,923–23,359,449	527
sY1206	ATTGATCTCCTTGGTTCCCC	GACATGTGTGGCCAATTTGA	24,380,299–24,380,692	394
			25,289,447–25,289,840	394
sY1201	CCGACTTCCACAATGGCT	GGGAGAAAAGTTCTGCAACG	26,311,169–26,311,845	677

Note: DYS527a/b (chrY:23739648–23739735 and chrY:25930428–25930349); DYF387a/b (chrY:23785261–23785600 and chrY:25884481–25884824); DYF404a/b (chrY:23807834–23808151 and chrY:25861938–25862247)

A

STS	Reference Sequence	b2/b4 deletion	gr/gr deletion	b1/b3 deletion	b2/b3 deletion
sY142	+	+	+	+	+
sY1161	+	+	+	–	+
sY1191	+	–	+	–	–
sY1291	+	–	–	–	+
sY1206	+	–	+	+	+
sY1201	+	+	+	+	+

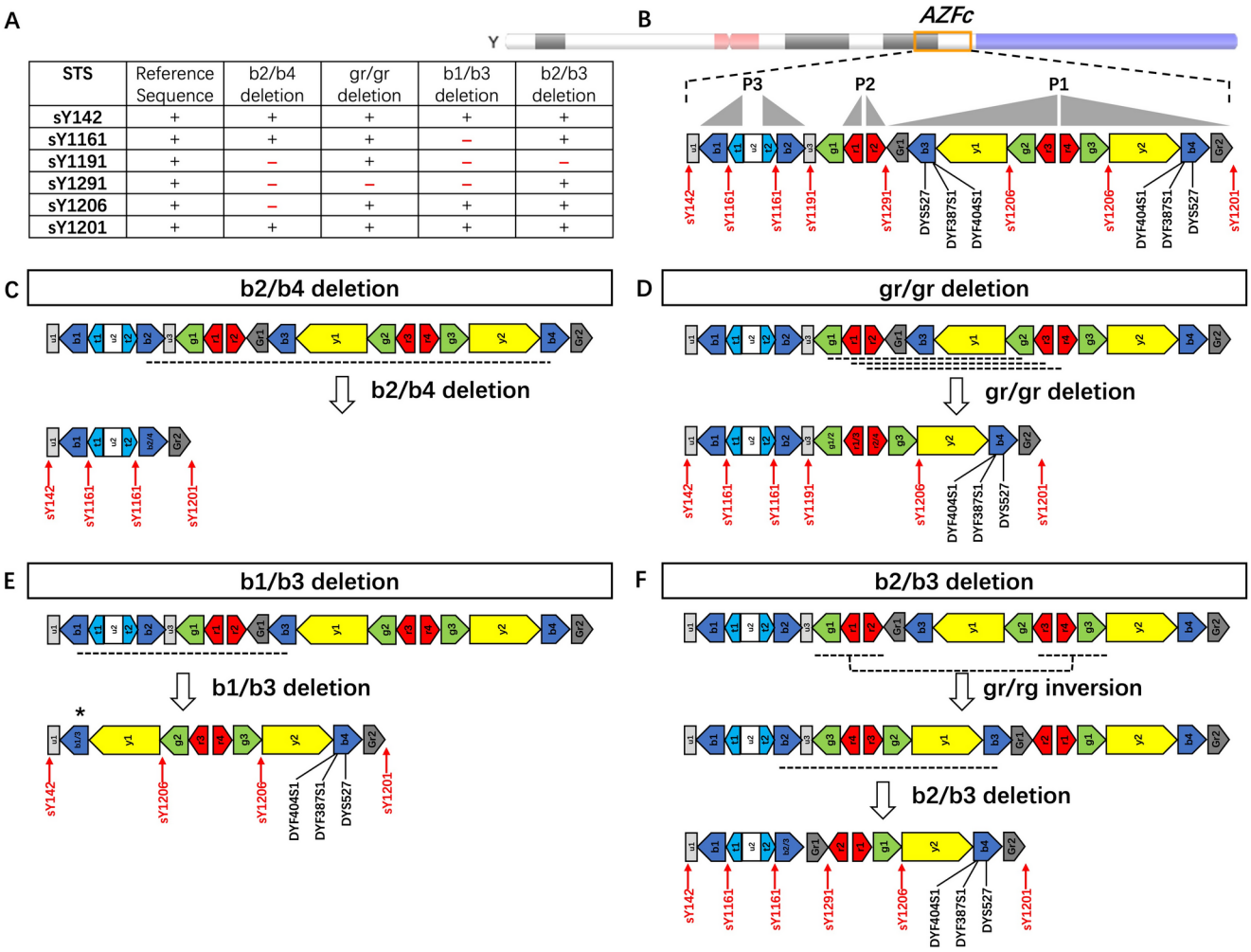


Fig. 1 Azoospermia factor c (*AZFc*) deletions and sequence-tagged site (STS) variants. **(A)** Six STSs are used to identify (partial) *AZFc* deletions. +: presence;–: absence. **(B)** Schematic diagram of STSs on the *AZFc* region. The light grey solid triangles indicate the transcriptional direction of the palindromes (P1 to P3). The *AZFc* region comprises numerous stretches of ampliconic sequences, which are classified into six color-coded sequence families (blue, turquoise, green, red, grey, and yellow), referred to as amplicons. The size and orientation of the colored arrows indicate the length and orientation of these sequences. Positions of STSs used to detect the deletion are indicated immediately below the arrow/bar. DYS527a/b, DYF387S1a/b, and DYF404S1a/b locate on b3 and b4 amplicons within P1 palindrome. **(C)** b2/b4 deletion: lacking sY1191, sY1291, and sY1206. The black dotted line depicts the approximate location of the deletion. **(D)** gr/gr deletions: including three variations (g1/g2, r1/r3, and r2/r4) and all lacking sY1291. **(E)** b1/b3 deletion: lacking sY1161, sY1191, and sY1291. The asterisk indicates the uncertain presence of the b3 amplicon, which includes DYS527a/b, DYF387S1a/b, and DYF404S1a/b. **(F)** b2/b3 deletion: lacking sY1191. It includes a gr/rg inversion followed by a deletion between b2 and b3 amplicons. The other pathway consists of a b2/b3 inversion followed by a rg/rg deletion (figure not shown)

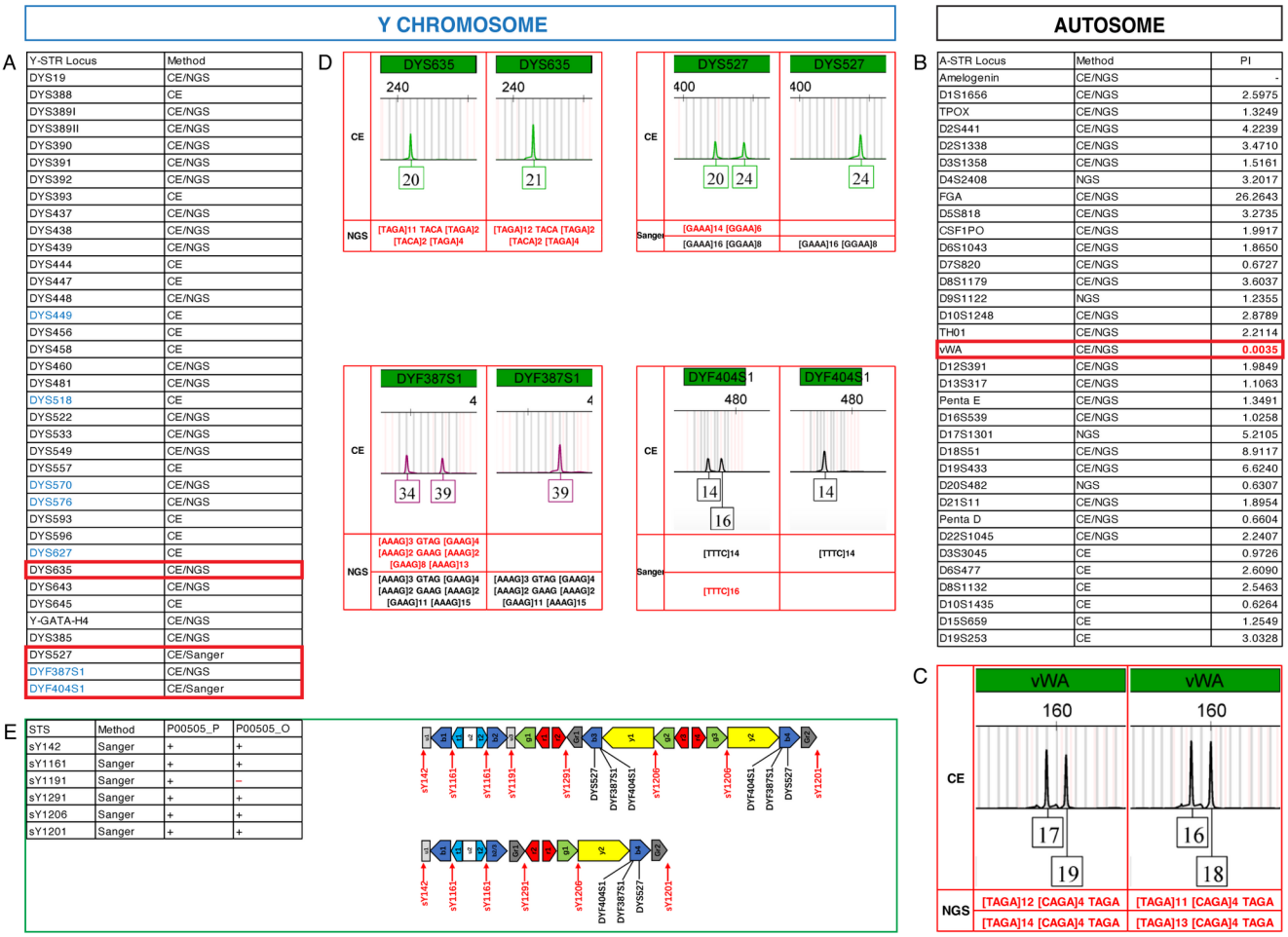
Statistical analysis

The paternity index for the father-son duo (PI_{duo}) was computed with EasyKin (https://forensicsysu.shinyapps.io/EasyKin/) [16] using default allele frequencies and a stepwise STR mutation model. The chromosomal structure rearrangement rate was calculated as the number of events divided by the number of father-son pairs, and the confidence interval (CI) was estimated using exact binomial probability distribution (https://statpages.info/confint.html).

Results and discussion

Case 1: four Y-STR and one A-STR mutations

Figure 2A lists the 41 Y-STRs detected in the capillary electrophoresis (CE) kit. Four mismatches were found: one single-copy marker (DYS635) involving a one-step gain mutation and three multi-copy markers (DYS527a/b, DYF387S1a/b, and DYF404S1a/b) affecting 2- to 5-step gain or loss mutations. In light of the complexity of these Y-STR mutations, we employed autosomal genetic markers to determine the relationship between P00505_P (alleged



Y-STR Locus	Method
DYS19	CE/NGS
DYS388	CE
DYS389I	CE/NGS
DYS389II	CE/NGS
DYS390	CE/NGS
DYS391	CE/NGS
DYS392	CE/NGS
DYS393	CE
DYS437	CE/NGS
DYS438	CE/NGS
DYS439	CE/NGS
DYS444	CE
DYS447	CE
DYS448	CE/NGS
DYS449	CE
DYS456	CE
DYS458	CE
DYS460	CE/NGS
DYS481	CE/NGS
DYS518	CE
DYS522	CE/NGS
DYS533	CE/NGS
DYS549	CE/NGS
DYS557	CE
DYS570	CE/NGS
DYS576	CE/NGS
DYS593	CE
DYS596	CE
DYS627	CE
DYS635	CE/NGS
DYS643	CE/NGS
DYS645	CE
Y-GATA-H4	CE/NGS
DYS385	CE/NGS
DYS527	CE/Sanger
DYF387S1	CE/NGS
DYF404S1	CE/Sanger

A-STR Locus	Method	PI
Amelogenin	CE/NGS	-
D1S1656	CE/NGS	2.5975
TPOX	CE/NGS	1.3249
D2S441	CE/NGS	4.2239
D2S1338	CE/NGS	3.4710
D3S1358	CE/NGS	1.5161
D4S2408	NGS	3.2017
FGA	CE/NGS	26.2643
D5S818	CE/NGS	3.2735
CSF1PO	CE/NGS	1.9917
D6S1043	CE/NGS	1.8650
D7S820	CE/NGS	0.6727
D8S1179	CE/NGS	3.6037
D9S1122	NGS	1.2355
D10S1248	CE/NGS	2.8789
TH01	CE/NGS	2.2114
vWA	CE/NGS	0.0035
D12S391	CE/NGS	1.9849
D13S317	CE/NGS	1.1063
Penta E	CE/NGS	1.3491
D16S539	CE/NGS	1.0258
D17S1301	NGS	5.2105
D18S51	CE/NGS	8.9117
D19S433	CE/NGS	6.6240
D20S482	NGS	0.6307
D21S11	CE/NGS	1.8954
Penta D	CE/NGS	0.6604
D22S1045	CE/NGS	2.2407
D3S3045	CE	0.9726
D6S477	CE	2.6090
D8S1132	CE	2.5463
D10S1435	CE	0.6264
D15S659	CE	1.2549
D19S253	CE	3.0328

STS	Method	P00505_P	P00505_O
sY142	Sanger	+	+
sY1161	Sanger	+	+
sY1191	Sanger	+	–
sY1291	Sanger	+	+
sY1206	Sanger	+	+
sY1201	Sanger	+	+

Fig. 2 Case 1: P00505 with three Y-STR and one autosomal STR (A-STR) mutations. **(A)** Genotypes at 41 Y-STRs are analyzed with capillary electrophoresis (CE), next-generation sequencing (NGS), and/or Sanger sequencing methods. The red boxes indicate the mutational loci. The rapidly mutating Y-STR is marked in blue. **(B)** Genotypes at 33 A-STRs are analyzed with CE and/or NGS methods. The paternity index for the father-son duo (PI_{duo}) at each locus is calculated with EasyKin. The red box indicates the mutational locus and its PI value. **(C)** CE-based and NGS-based genotypes at the mutational vWA. **(D)** Length-based and sequence-based genotypes at the mutational Y-STRs. **(E)** Schematic diagram of sequence-tagged site (STS) variants on the *AZFc* region. +: presence;–: absence

father) and P00505_O (son). After analyzing 33 A-STRs included in CE and next-generation sequencing (NGS) kits, 32 out of 33 markers showed that P00505_P and P00505_O shared half of their genetic information (Fig. 2B). One mismatch at vWA was observed with CE and confirmed with NGS (Fig. 2C). The PI_{duo} at vWA was calculated as 0.0035 using the stepwise STR mutation model. However, the combined PI (CPI) value of 1.32×10^8 supported H_0 (father and son) rather than H_1 (unrelated individuals) with the extremely strong weight of evidence. Under the proposed hypotheses, EasyKin simulation yielded a false positive rate of 1×10^{-8}. The posterior probability was estimated as >0.999999992 under the equal prior probability of H_0 and H_1. Given that the mutation rates of SNPs are much lower than STRs, this is also robust evidence for the father-son relationship. Results showed no genotypes violated the Mendelian inheritance rule among the 94 iSNPs. Table S1 demonstrates the CPI_{duo} value was 1.25×10^{10} across 94 iSNPs and 1.65×10^{18} across the combination of 33 A-STRs and 94 iSNPs, respectively, supporting the father-son relationship. Despite observing one A-STR mutation and four Y-STR mutations within the P00505 pedigree, we can still determine their father-son relationship by comprehensively analyzing CPI values.

We further investigated the causes of Y-STR mutations by utilizing Sanger sequencing or NGS to scrutinize the sequence variance. NGS confirmed that this one-step gain mutation occurred at a larger variable repeat unit (marked in bold) in compound repeats $\mathbf{[TAGA]_n}$ $[TACA]_n$ $[TAGA]_2$ $[TACA]_2$ $[TAGA]_4$ of DYS635 (Fig. 2D), resulting from the STR slippage mechanism. However, more analyses may be needed to clarify the 2- to 5-step mutations of multi-copy markers. A clear manifestation was that the offspring was all homozygous at DYS527a/b, DYF387S1a/b, and

DYF404S1a/b, while the parent was entirely heterozygous. Simultaneous deletions may be attributed to structural rearrangements caused by non-allelic homologous recombination (NAHR) events on the long arm of the Y chromosome. The supposition was verified through STS analysis, which revealed a b2/b3 deletion: P00505_O lacked sY1191 but possessed sY142, sY1161, sY1291, sY1206, and sY1201. Figure 2E displays that one of the two copies at these three loci was all located in the b3 amplicon, which was just within the b2/b3 deletion region. Thus, the mutations observed at DYS527a/b, DYF387S1a/b, and DYF404S1a/b resulted from the Y chromosomal rearrangement.

Case 2–3: three Y-STR mutations

In the pedigrees associated with P00702 and P02298, as detailed in Table S2 and Table S3, no mutations were detected among the 33 A-STRs, yielding CPI $_{duo}$ values of 2.43×10^{10} for P00702 and 3.16×10^{13} for P02298, respectively. These findings supported H_0 (father and son) over H_1 (unrelated individuals) with the extremely strong weight of evidence. Regarding the Y-STR markers, three mutations were observed at DYS527a/b, DYF387S1a/b, and DYF404S1a/b involving gains or losses of 1 to 3 steps. Both occurrences exhibited similarities to Case 1. Sanger sequencing or NGS was also conducted for these Y-STRs; however, additional analyses may be required to clarify the multi-step mutations of multi-copy markers. The STS analyses detected the b2/b3 deletion in P00702_O (son), leading to absent sY1191. Similarly, the gr/gr deletion was found in P02298_O (son), resulting in absent sY1291. Therefore, the mutations observed at DYS527a/b, DYF387S1a/b, and DYF404S1a/b were also attributed to Y chromosomal rearrangements.

Concluding remarks

In forensics, there is a high demand for family DNA searching with Y-STR profiling. However, when two Y-STR haplotypes have a few mismatched loci, it becomes challenging to determine if they are from the same male lineage due to the high mutation rate of Y-STRs. In practical scenarios, we usually have two options. One is to increase the number of loci for detecting genetic markers, which aids in diluting the impact of mutations. For example, Liu et al. [17] suggest that for Yfiler haplotypes with 17 Y-STRs, ≤ 2 mismatched loci (with ≤2 cumulative mutation steps) still indicate a high likelihood of two males originating from the same pedigree, whereas this policy for Yfiler Plus haplotypes with 27 Y-STRs changes to ≤4 mismatched loci (with ≤5 cumulative mutation steps). However, the increased loci are not always unlimited because the amplitudes of increases in Y-STR haplotype diversity and discrimination capacity are mild or very slow when exceeding 25 loci [4, 18]. Yet, this may not be the best option in our cases since we have already examined more than 27 Y-STRs.

The other option is to expand the types of genetic markers detected, such as A-STR, X chromosomal STR (X-STR), iSNP, Y-SNP, and mitochondrial DNA (mtDNA), to ensure the provision of results that can elucidate complex familial situations and thus clarify these disputes. A good case was reported in the recent literature [19]. In our cases, iSNPs are added to strengthen evidence when an A-STR mutation was observed in P00505. With the strengthened confirmation of the father-son relationships by CPI values, we further reveal the mutation mechanism in these three cases. Firstly, the origin of A-STR and Y-STR mutations involves the mechanism of STR slippage (identified using NGS and Sanger sequencing methods). Secondly, one or more step mutations observed at DYS527a/b, DYF387S1a/b, and DYF404S1a/b are connected due to chromosomal structure rearrangement (identified using STS analyses). The chromosomal structure rearrangement rate observed in this study is calculated as 0.0012 (3/2548) with the 95% CI of 0.0002–0.0034 in Northern Han Chinese.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00414-025-03506-1.

Author contributions Fei Guo, Fu Ren, and Shaobo Yu: Writing– review & editing, Conceptualization. Longnian Zhang: Writing– original draft, Design, Data analysis. Guangxin Jing: Methodology, Software, Data analysis. Ze Liu, Haiduo Wu, and Dahua Liu: Sample collection, Methodology.

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Data availability All data generated or analyzed during this study are included in this article and its supplementary information files.

Declarations

Ethical approval All participants signed the informed consent form and agreed to the results published in scientific publications. This study was approved by the Ethical Committee of Jinzhou Medical University (No. 2020016).

Conflict of 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.

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