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

Examination of the usefulness of next-generation sequencing in mixed DNA samples

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ARTICLE INFO

Keywords:
mixed DNA
short tandem repeat (STR)
next-generation sequencing (NGS)
single nucleotide polymorphisms (SNPs)
DNA typing

ABSTRACT

The identification of individuals from mixed DNA samples is an important application of DNA typing. Although the discriminatory power of DNA profiling has improved dramatically, a limiting factor is that individuals cannot be identified via short tandem repeat (STR) analysis. We used next-generation sequencing (NGS) to examine the mixed DNA samples. Our results showed that STR nucleotide sequences and single nucleotide polymorphisms (SNPs) analysis via NGS may enable the identification of each distinct subject from a DNA mixture containing DNA of the victim and suspect.

1. Introduction

The discriminatory power of DNA typing has dramatically improved with the increase in the number of loci tested using DNA amplification kits [1,2]. Short tandem repeat (STR) typing has low discriminatory power when used at one locus, which improves when multiple loci are examined. The frequency of appearance of the most commonly detected STR types in the Japanese population is about one in 565×10^{16} using the current GlobalFiler™ PCR Amplification Kit (Thermo Fisher Scientific) [3].

Although there are various interpretations of the results from the analysis of mixed samples [4–9], an important drawback of STR typing using such samples is the inability to identify the individuals. Semen is the only body fluid that can be isolated from mixed samples by two-step differential extraction [10], and the process cannot be used to isolate other fluids. Therefore, by using STR typing, we cannot exclude the mixture of a third-party DNA in the same sample. Multiple persons may have the same DNA type, even if the type of the suspect and the victim are not detected in excess or a deficient manner. This makes the identification of DNA from multiple persons difficult in a mixed appraisal (evidence) sample. Hence, it is difficult to consider the intervention of others by performing advanced statistical processing based on the number of detected types, peak height ratio, and appearance frequency [11–13].

Next-generation sequencing (NGS) methods can be used to analyse several test samples at a time, to obtain the nucleotide sequence in addition to the STR type, and detect single-nucleotide polymorphisms (SNPs) at various locations in the sequenced DNA [14–16]. In this study, we aimed to analyse STR nucleotide sequences and SNPs using NGS to identify individuals in mixed samples with high accuracy, in addition to STR typing. We sought to examine the utility of the method in DNA typing analysis and to examine whether SNPs analysis of DNA from decomposed samples can be used for re-appraisal.

2. Materials and methods

DNA was extracted from the oral swab samples of 50 individuals (men:42 individuals, women:8 individuals). All the individuals provided informed consent. Oral swabs were collected using clean DNA-free cotton swabs. The DNA was extracted using the PrepFiler Express™ Forensic DNA Extraction Kit (Thermo Fisher Scientific) and AutoMate Express™ Forensic DNA Extraction System (Thermo Fisher Scientific). The extract was quantified using the human genome quantification kit ver. 2 (TaKaRa) by the real-time PCR method using the 207 bp DNA sequence present at the D17Z1 locus as an index.

2.1. STR analysis

2.1.1. STR-NGS analysis of DNA extracted from oral swabs

DNA was amplified using the Precision ID GlobalFiler™ NGS STR Panel (Thermo Fisher Scientific), and a library was created using the Precision ID Library Kit (Thermo Fisher Scientific) and Ion Xpress™

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https://doi.org/10.1016/j.legalmed.2021.101874
Received 15 December 2020; Received in revised form 19 March 2021; Accepted 24 March 2021

Table 1
The tested loci and analysis items for STR-NGS and STR-CEP analyses.

	STR-NGS	STR-CEP
Test panel or kit	Precision ID GlobalFiler™ NGS STR Panel	GlobalFiler™ PCR Amplification Kit
Tested loci common	D3S1358, vWA, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, DYS391, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, D10S1248, D1S1656, D12S391, D2S1338, Y-indel	
Tested loci non-common	D12ATA63, D14S1434, D1S1677, D2S1776, D3S4529, D4S2408, D5S2800, D6S1043, D6S474, X-indel	SE33
Analysis items	STR types and nucleotide	STR types

Barcode Adapters 1–16 Kit (Thermo Fisher Scientific). The library was adjusted and was loaded to Ion 316 ™ Chip v2 BC (Thermo Fisher Scientific) using the Ion PGM™ Hi-Q ™ Chef Kit (Thermo Fisher Scientific) and Ion Chef™ Instrument (Thermo Fisher Scientific). The Chip was sequenced using the Ion PGM™ Hi-Q™ Sequencing Kit (Thermo Fisher Scientific) and Ion PGM™ Sequencer System (Thermo Fisher Scientific). The DNA types were determined using the HID STR Genotyper Plugin (Thermo Fisher Scientific). The amount of template used was 1 ng and the manufacturer's protocol were followed for the amplification cycles and PCR conditions used in library preparation [17]. Analysis settings were according to user guide [18]. Table 1 shows the tested loci and analysis items used with the Precision ID GlobalFiler™ NGS STR Panel.

2.1.2. STR-NGS analysis of DNA extracted from mixed samples

We selected DNA solution of three samples (X, Y, and Z) out of 50 samples and created mixed samples at 1:1 to 1:10 of DNA concentration ratios (10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10) using two of the samples (X + Z and Y + Z). The total amount of DNA in the mixed samples was 1 ng. STR nucleotide sequence analysis was performed using NGS. To compare with the result of STR-NGS analysis, the amplification reaction was also performed using the GlobalFiler™ PCR Amplification Kit using the prepared mixed samples, and the amplified product was analysed via capillary electrophoresis (CEP) using the 3500xL Genetic Analyzer (Thermo Fisher Scientific). GeneMapper ID-X Software v1.4 (Thermo Fisher Scientific) was used for electrophoresis data, the analytical threshold was analysed using 175 RFU, which is the default value of 3500xL Genetic Analyzer. Table 1 shows the tested loci and analysis items of the GlobalFiler™ PCR Amplification Kit. Samples A, B, and C had the same STR types but different nucleotide sequences at D8S1179, D21S11, and D2S1338.

2.2. SNPs analysis

2.2.1. SNP-NGS analysis for oral swabs

The samples were amplified using the Precision ID Identity Panel (Thermo Fisher Scientific) as described in section 2.1., and the type determination was performed using the HID SNP Genotyper Plugin (Thermo Fisher Scientific). Analyses for 90 autosomal and 34 Y chromosomal sites were performed. The amount of template used as input was 1 ng and the amplification cycles and PCR conditions used were according to the manufactureŕs protocol [17]. Analysis settings were according to user guide [19].

2.2.2. SNP-NGS analysis from mixed samples

SNP-NGS analysis was performed. In brief, mixed samples were prepared as in section 2.1.2., amplified using the Precision ID Identity Panel, and sequenced using the Ion PGM™ Sequencer System.

2.2.3. Utility of SNP-NGS analysis for decomposed DNA samples

For samples exposed to a poor environment or that have been degraded over time, the STR type may not be detectable. The DNA extracted from a single sample was irradiated in six stages using ultraviolet light from 50 mJ/cm^2 to 5000 mJ/cm^2 (50, 100, 500, 1000, 2500, 5000 mJ/cm^2) (Vilber Lourmat), and performed the SNP-NGS and the STR-CEP analyses.

3. Results

3.1. STR analysis

3.1.1. STR-NGS analysis of DNA from oral swabs

The identified STR types were homozygous and a difference in the nucleotide sequence was identified at six loci: D8S1179, D21S11, D2S1338, D12S391, D3S1358, and D4S2408. Among these, polymorphisms were frequently observed at the loci D8S1179, D21S11, and

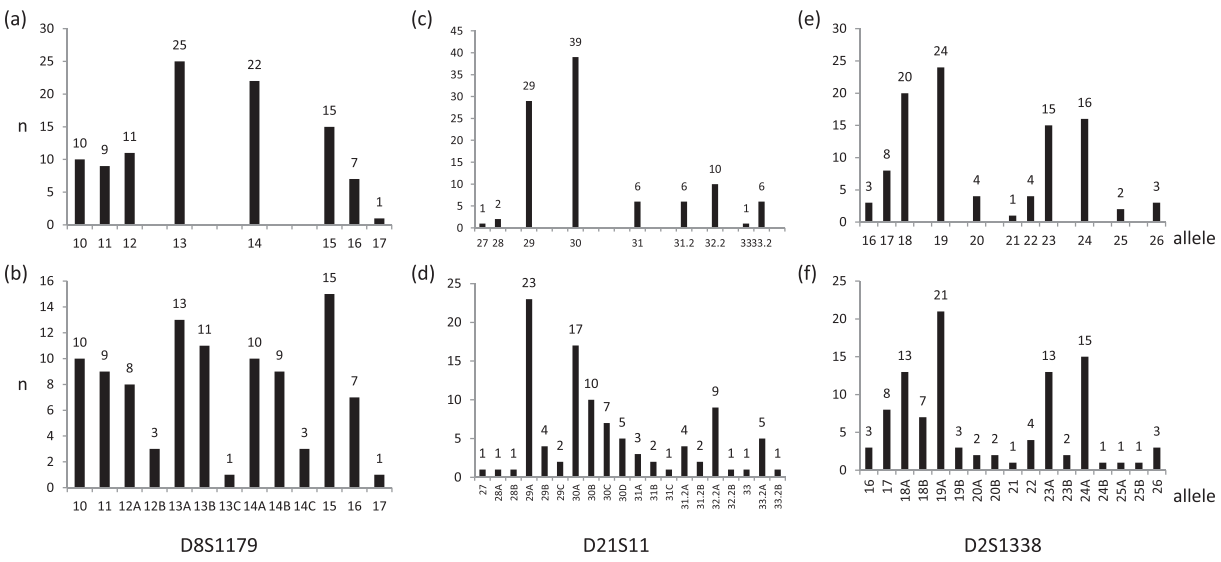

Fig. 1. Distribution of in 3 loci (D8S1179, D21S11, D2S1338) (n = 50) (a, c, e): STR-CEP analysis, (b, d, f): STR-NGS analysis.

D2S1338 (Fig. 1). Table 2 shows the nucleotide sequences of the STR type in which polymorphisms were observed in D8S1179, D21S11, and D2S1338. Polymorphisms were observed in the allele types 12, 13, and 14 in D8S1179, 28, 29, 30, 31, 31.2, 32.2, and 33.2 in D21S11, 18, 19, 20, 23, 24, and 25 in D2S1338. The nucleotide sequences are numbered from A in descending order of the number of observations.

3.1.2. STR-NGS analysis of DNA from mixed samples

Samples with the same STR type but different nucleotide sequences were used in subsequent experiments (samples X, Y, and Z). Table 3 shows the nucleotide sequences of D8S1179, D21S11, and D2S1338 for each sample. In the STR-NGS analysis, the nucleotide sequences were different despite the STR type being the same; therefore, new information can be added to the STR-CEP analysis (Fig. 2). Mixed samples were prepared from samples X, Y, and Z at ratios of 1:1 to 1:10, and STR-CEP analysis was performed. The results showed that it was possible to detect differences for a mixing ratio of approximately 1:5 to 1:10 (Table S1). When the DNA type of minor component is the same as the stutter peak of major component, it tends to be undetectable. In vWA, the type of sample X is 15, 16 and the type of sample Z is 16, 17, so the mixed sample type should be detected as 15, 16, 17, but 15 is judged to be 16 stutter peak, and 16, 17 is detected at ratio of 1:10.

To improve the accuracy of the analysis of the minor and stutter peaks in the mixed samples in the STR-NGS analysis, the initial settings were changed as shown in Fig. S1. The setting changes were not applied for D12ATA63 and D22S1045, which are repeated loci of three bases. No improvement was required for the single sample, but the settings were changed to correctly type the low peaks in the mixed samples. By changing the stutter offset from 0 to −4, the stutter ratio from 0 to 0.17, and the peak height ratio from 0.3 to 0.1, the allele that was judged to be "below PHR" is judged as sttuter and alleles derived minor components. Mixed samples were prepared using samples X, Y, and Z at ratios of 1:1 to 1:10, and the STR-NGS analyses were performed using the changed settings (Table S2). Our results showed that although the loci of the partial profile increased, the STR-NGS analysis was able to detect a mixing ratio of up to ~1:5. Additionally, the STR-NGS analysis with the same STR type having different nucleotide sequences at three loci (D8S1179, D21S11, and D2S1338) showed up to the same mixing ratio.

3.2. SNPs analysis

3.2.1. SNP-NGS analysis of DNA from oral swabs

The SNP-NGS analysis showed that none of the 90 autosomal sites had the same type in 50 samples. In contrast, 34 Y chromosomal sites in 42 samples were divided into six groups: C, D, O, O2, O3, and N. The distribution was similar to the results of the Y chromosome haplogroup study of 2,390 Japanese males [20] (Table S3).

3.2.2. Simulation analysis to confirm the discriminatory power of SNPs

Based on the sequencing results of 50 SNP-NGS analysis, we assumed that SNPs in which two DNAs were mixed out of 50 DNA (combination 1225 ways), and examined whether 48 other than two could not be mixed (58800 total comparisons). In order to examine whether or not the involvement of a person who is not involved in mixing is denied by this simulation, it is necessary to examine only the positions where a single base such as AA or TT (homozygotes) is detected in the mixed samples. Since the SNP-NGS kit used in this study detected two bases per position, for example, in the case of a position where A or G is detected, either AA, GG or AG is detected at this position. In the position where different bases are detected like AG (heterozygous) in mixed samples, any person with AA, GG, or AG cannot deny the mixing. Mixing can only be denied at positions where only a single base is detected, such as AA and GG. An example is shown in Table S4. The mixed sample of sample 1 and sample 2 is detected as GG at the position of rs1109037, but the SNP of sample 3 at the same position is AG, so the involvement of sample 3 in this mixed sample is denied.

Our simulation showed that in two DNAs of 50 samples, the remaining 48 were not found to be mixed by using 90 autosomal and 34 Y chromosomal sites.

Table 2
STR nucleotide sequences in which polymorphisms were observed in the loci D8S1179, D21S11, and D2S1338.

D8S1179 Allele	Nucleotide sequence	D21S11 Allele	Nucleotide sequence	D2S1338 Allele	Nucleotide sequence
12	A:(TCTA)12 B:(TCTA)1(TCTG)(TCTA)10	28	A:(TCTA)5(TCTG)5(TCTA)3TA (TCTA)3 TCA(TCTA)2TCCATA(TCTA)10 B:(TCTA)6(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)9	18	A:(TGCC)7(TTCC)11 B:(TGCC)6(TTCC)12
13	A:(TCTA)13 B:(TCTA)1(TCTG)(TCTA)11 C:(TCTA)2(TCTG)(TCTA)10	29	A:(TCTA)6(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)10 B:(TCTA)6(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)10 C:(TCTA)5(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)10	19	A:(TGCC)7(TTCC)12 B:(TGCC)6(TTCC)13
14	A:(TCTA)2(TCTG)(TCTA)11 B:(TCTA)1(TCTG)(TCTA)12 C:(TCTA)14	30	A:(TCTA)6(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)11 B:(TCTA)5(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)11 C:(TCTA)4(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)12 D:(TCTA)7(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)10	20	A:(TGCC)7(TTCC)2(TTTC)(TTCC)10 B:(TGCC)7(TTCC)13
		31	A:(TCTA)5(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)10 B:(TCTA)7(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)11 C:(TCTA)6(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)12	23	A:(TGCC)7(TTCC)13(GTCC)(TTCC)2 B:(TGCC)6(TTCC)14(GTCC)(TTCC)2
		31.2	A:(TCTA)5(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)11TA(TCTA)1 B:(TCTA)5(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)12TA(TCTA)1	24	A:(TGCC)7(TTCC)14(GTCC)(TTCC)2 B:(TGCC)5(TTCC)16(GTCC)(TTCC)2
		32.2	A:(TCTA)5(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)12TA(TCTA)1 B:(TCTA)5(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)13TA(TCTA)1	25	A:(TGCC)6(TTCC)16(GTCC)(TTCC)2 B:(TGCC)7(TTCC)15(GTCC)(TTCC)2
		33.2	A:(TCTA)5(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)13TA(TCTA)1 B:(TCTA)6(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)12TA(TCTA)1		

3.2.3. *SNP-NGS analysis for mixed samples*

Mixed samples were prepared from samples X, Y, and Z using ratios of 1:1 to 1:10, and SNP-NGS analysis was performed. The partial SNP profiles at the loci were increased compared to STR-CEP analysis but these could be detected from mixing ratios of 1:2 to 1:5 (Tables S5 and S6).

3.2.4. *The utility of SNP-NGS analysis in decomposed DNA samples*

Next, we irradiated DNA samples using six intensities of ultraviolet rays from 50 mJ/cm^2 to 5000 mJ/cm^2 (50, 100, 500, 1000, 2500, 5000 mJ/cm^2) per sample, and our results showed that all DNA types were detected via STR-CEP analysis for samples irradiated with 50 mJ/cm^2 of ultraviolet rays. In samples irradiated with 100 mJ/cm^2 of ultraviolet rays, all the SNPs were detected via the SNP-NGS analysis (Table S7).

4. Discussion

In this study, we found that the STR-NGS analysis was able to resolve mixed DNAs for a 1:5 mixing ratio, which is similar to that using STR-CEP analysis, and was achieved by partially changing the analysis conditions from the initial settings. As the nucleotide sequences may differ even in homozygous STR types, new information can be obtained

Table 3
STR nucleotide sequences in D8S1179, D21S11, and D2S1338 from the samples X, Y, and Z that were used to create the mixed samples.

Loci	sample X	sample Y	sample Z
D8S1179	13 (TCTA)(TCTG)(TCTA)11 13 (TCTA)13	10 (TCTA)10 13 (TCTA)13	12 (TCTA)(TCTG)(TCTA)10 13 (TCTA)(TCTG)(TCTA)11
D21S11	30 (TCTA)6(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)11 30 (TCTA)6(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)11	29 (TCTA)6(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)10 30 (TCTA)4(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)12	29 (TCTA)6(TCTG)5(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)10 30 (TCTA)5(TCTG)6(TCTA)3TA(TCTA)3 TCA(TCTA)2TCCATA(TCTA)11
D2S1338	23 (TGCC)7(TTCC)13(GTCC)(TTCC)2 23 (TGCC)7(TTCC)13(GTCC)(TTCC)2	23 (TGCC)7(TTCC)13(GTCC)(TTCC)2 23 (TGCC)7(TTCC)13(GTCC)(TTCC)2 (TCTA)6(TCTG)5(TCTA)3TA(TCTA)3	23 (TGCC)6(TTCC)14(GTCC)(TTCC)2 24 (TGCC)7(TTCC)14(GTCC)(TTCC)2

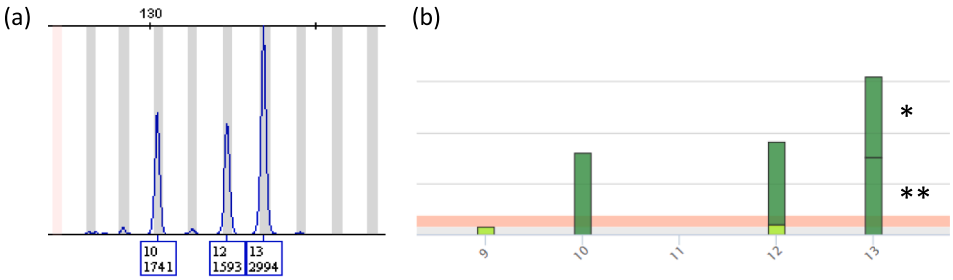

Fig. 2. Comparison of STR-CEP and STR-NGS analyses (an example showing the usefulness of STR-NGS analysis) STR-CEP analysis (a) and STR-NGS analysis (b) in mixed samples (samples Y and Z). sample Y: sample Z = 1 : 1, Locus : D8S1179. sample Y: 10 (TCTA)10, 13* (TCTA)13. sample Z : 12 (TCTA)(TCTG)(TCTA)10, 13** (TCTA)(TCTG)(TCTA)11.

by adding the STR-NGS analysis to the STR-CEP analysis for mixed samples.

A recent report showed a sequence error which indicated that the vWA locus was determined to be type 14 by STR-CEP analysis, but was identified as type 15 by STR-NGS analysis [21]. Although NGS has dramatically improved sequencing technology, several issues still need to be resolved. However, if results are interpreted taking into account these drawbacks, STR-NGS analysis may be considered useful. In addition to misreading, STR-NGS analysis has confounding factors such as high running costs, complicated procedure, and time consuming compared to STR-CEP analysis. In the future, it is expected that improvements to equipment and reagents will solve this problem.

In contrast, our simulation showed that if two DNAs were mixed, the mixing of 48 other than two was denied by the SNP-NGS analysis. Therefore, SNP-NGS analysis may enable the detection of the presence of DNA from a 3rd individual in mixed samples of DNA from 2 individuals. Certainly, like the birthday paradox, increasing the number of samples may make it impossible to deny the involvement of a 3rd individual. However, even our SNPs analysis of 90 autosomal and 34 Y chromosomal sites have such discriminating power. In this study, we used a kit that detects 2 bases per position, but if we select a position where 3 or 4 bases are detected, the discriminating power will be further improved [22–24]. Furthermore, if the use of reagents specialized for Japanese people and the number of analysis positions are increased, the possibility of denying the involvement of 3rd individual will increase.

Although the SNP analysis showed few polymorphisms at single sites, its usefulness was demonstrated in mixed samples by the analysis of multiple sites. The SNP-NGS analysis was able to detect SNPs of minor contributors in the sample mixing ratios of 1:2 to 1:5. Furthermore, SNPs may be detectable even in samples that have been exposed to a poor environment in which the STR is not detected, or in samples that have decomposed over time due to DNA degradation because our analysis in irradiated DNA samples. A re-appraisal of samples is often requested over the years, and the likelihood of sample degradation with time is increased. Although it is necessary to make a careful judgement as to whether the SNPs detected in the mixed samples are homozygous or only one of heterozygous in poor condition, SNP-NGS analysis may help in sample analysis effectively in such cases. The average amplicon size of the Precision ID Identity Panel used in the SNP-NGS analysis in this study was 138 bp, whereas the amplicon size of the GlobalFiler™ PCR Amplification Kit was ~ 70 bp to 420 bp, indicating that SNP-NGS analysis is effective. Further, research has shown that the number of individuals from mixed DNA can be accurately identified by performing mitochondrial DNA analysis using NGS [25,26]. By adding mitochondrial analysis to STR-NGS and SNP-NGS analyses, additional data will be available for analysis.

The detection method used in STR-NGS and SNP-NGS analyses is different from the peak height identification through fluorescence intensity in STR-CEP analysis, and off-scale results observed in STR-CEP analysis do not occur. Further, the possibility of detecting minor components in the mixed samples is increased. NGS is capable of analysing several test items concomitantly; however, its disadvantages are the higher cost and longer sample preparation time than those using the STR-CEP analysis. Despite these limitations, in a sample containing DNA of the victim and suspect, the addition of STR-NGS and SNP-NGS analyses to the STR-CEP analysis may aid in the conclusive identification of both.

Acknowledgements

The English used in this manuscript was revised by editage.

Ethical approval

This study was approved by the Ethics Committee of the Japanese Association of Forensic Science and Technology (The approval number is 29G12, 30GR3). Informed consent was obtained from all individual participating in this study.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.legalmed.2021.101874.

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