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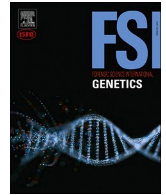

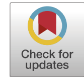

Performance comparison of a previously validated microhaplotype panel and a forensic STR panel for DNA mixture analysis

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ABSTRACT

Short Tandem Repeats (STRs) are the most widespread markers in forensic genetics. However, STR stutter peaks can mask alleles from a minor contributor when analysing mixtures, hindering the interpretation of complex profiles. In this study we compared the performance of a previously described panel of microhaplotypes (MHs), an alternative type of forensic marker, against a standard STR kit. The parameters evaluated included: capability of determining the minimum number of contributors in the mixture; percentages of allele drop-outs and drop-ins; retrieval of alleles belonging to the minor contributor, and estimation of likelihood ratio (LR) values. In addition, the capacity of EuroForMix software to estimate each donor's percentage of contribution was tested, as well as the impact on results when using manually, or automatically prepared libraries. The MH panel showed better performance than STRs for the detection of 2-contributor mixtures, but the lower degree of polymorphism per MH marker hindered the task of deconvolution with multiple contributors. MHs presented higher drop-in rates and lower drop-out rates, a higher capability to recover the minor contributor's alleles and provided higher LR values than STRs, likely due to the much higher number of loci combined in the panel. Estimations of contributor ratios using EuroForMix showed promising results and marginal differences were found in these values between manually and automatically prepared libraries. Overall, results showed that the mixture detection performance of the MH panel was better or equal to the standard forensic autosomal STR panel, indicating microhaplotypes are informative markers for this purpose.

1. Introduction

Short Tandem Repeat (STR) genotyping is established as the routine method for analysing forensic evidence containing DNA, as STRs are highly polymorphic and can be easily analysed by capillary electrophoresis. The use of these markers in criminal investigations has led to the creation of national DNA databases of STR profiles from convicted offenders or biological traces found at crime scenes, which allows fast identification of reoffending criminals and facilitates data exchange between countries [1].

One of the challenges of STR profiling is the analysis of mixed DNA, especially when they comprise highly imbalanced mixtures or involve multiple contributors [2–6]. Two factors have been identified as contributing to mixed profile misinterpretation: i) preferential amplification of small amplicon alleles and, ii) stutter peaks caused by slippage of DNA Polymerase during amplification, which can pose as, or mask, real alleles from the minor contributor. Moreover, STR profiling can be impacted by stochastic PCR effects when analysing degraded and low-level DNA.

Microhaplotypes (MHs) were proposed as forensic markers in 2014 [7], and are defined as loci spanning short amplicons (usually between 150 and 300 bps) that contain two or more Single Nucleotide Polymorphisms (SNPs) showing contrasting allele frequencies [7–10]. As sequence-based polymorphisms, microhaplotypes lack both preferential amplification and stutter peaks, each a factor that can facilitate the analysis of mixed DNA. However, MHs show less polymorphism than most forensic STR loci currently used, so more markers are required to achieve a similar discrimination power.

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MHs have been investigated as promising markers in forensic genetics, both on their own or as complementary loci to established STRs, and several panels have been proposed for different applications over the last ten years. Forensic applications evaluated include kinship testing [11,12], individual identification and mixture analysis [4, 13–15], as well as multi-purpose panels [16,17].

The panel used in this study was first implemented on the Illumina MiSeq and Thermo Fisher Ion S5 Massively Parallel Sequencing (MPS) platforms and has been successfully adapted for the MinION [16,18]. It comprises a total of 118 short-amplicon loci, 107 autosomal and 11 X-chromosome markers (<120 bps), initially dedicated to kinship and degraded DNA analysis. Follow up studies indicated the panel also provides biogeographical ancestry information [19] that can be used as a DNA intelligence tool in criminal cases.

Here, we compare the performances of the above described MH panel and a commonly used CE-based STR kit (GlobalFiler) by analysing artificial mixtures with different numbers of contributors and evaluating the following aspects of interest in mixture analysis: i) probability of misclassifying the mixtures according to the minimum number of contributors; ii) percentages of observed allele drop-out and drop-in; iii) percentage of alleles exclusive to the minor contributor detected, and; iv) Likelihood Ratio (LR) values obtained for the presence of the minor contributor.

Additionally, we tested the equivalence of allelic sequence read coverage in MHs with capillary-electrophoresis peak heights in STRs as a measure of each donor's contribution percentage to the mixed profile in a quantitative model. Finally, as MHs can only be studied with MPS technology, manual and automatic library preparation methods were assessed. An automatic MPS library preparation workflow reduces hands-on work and should prevent contamination of samples, providing increased replicability of results. In this study, we compared allelic recovery, drop-out and drop-in for two-contributor mixtures for both methods.

2. Material and methods

2.1. Sample collection, DNA extraction and mixture preparation

DNA samples were obtained from unrelated voluntary donors with informed consent approved by the Bioethics Committee of the Universidade de Santiago de Compostela (USC-30/2021).

DNA extraction was carried out using the AutoMate Express DNA extraction system (Applied Biosystems, AB) and PrepFiler Express Forensic DNA Extraction Kit (AB), following manufacturer's instructions. DNA quantification was performed with Quantifiler Trio DNA Quantification kit (AB) in a 7500 Real Time PCR System (AB) following manufacturer's instructions.

DNA mixtures of up to four different unrelated contributors were prepared at ~1 ng/μL from normalized DNAs, as outlined in Table 1.

Table 1
Quantified mixtures of 2, 3 and 4 contributors / donors (D).

Number of contributors	Proportion	D1	D2	D3	D4
2	1:1	1	1		
	1:3	1	3		
	1:9	1	9		
	1:14	1	14		
	1:19	1	19		
	1:24	1	24		
	1:29	1	29		
	1:39	1	39		
	1:79	1	79		
3	1:1:1	1	1	1	
	1:3:5	1	3	5	
	1:3:7	1	3	7	
	1:7:9	1	7	9	
	1:3:15	1	3	15	
4	1:1:1:1	1	1	1	1
	1:3:7:9	1	3	7	9
	1:3:5:11	1	3	5	11
	1:3:9:15	1	3	9	15

2.2. STR analysis by capillary electrophoresis

STRs were amplified in a Veriti 96-Well Thermal Cycler (AB) using the GlobalFiler PCR Amplification Kit (AB) with 0.2 ng of total input DNA amount and analysed in a 3500 Genetic Analyzer (AB) following manufacturer's instructions. Electropherograms were interpreted with GeneMapper ID-X v1.4 (AB) software, setting an analytical threshold of 85 RFUs for mixtures and 170 RFUs for single-source samples. The non-autosomal markers included in the kit (amelogenin, Y indel and DYS391) were removed from the study.

2.3. Microhaplotype analysis with Ion S5 MPS system

Microhaplotypes were sequenced with the Ion S5 MPS system (Thermo Fisher Scientific, TFS), herein Ion S5, as detailed in [16], using 1 ng of total input DNA. Additionally, a no template control was included. Libraries for 1:1, 1:3, 1:9, 1:14, 1:19, 1:24, 1:29 and 1:39 mixture ratios were prepared for input into two different protocols: manual and automatic using the Ion Chef (TFS) instrument and following manufacturer's protocols.

MH calling was performed using the Torrent Suite Server v.5.10.1 and the HID_Microhaplotype_Research_Plugin v.1.55 applying a relative threshold of 0.02 for mixtures and 0.1 for reference profiles and an absolute threshold of 5 reads per allele (see Table S1 for details on chip runs).

The 11 X-chromosome MH loci and the previously identified as underperforming in [16] autosomal MH loci 3pC, 5qD, 10qC, 12qA and 19qB, were removed from the bioinformatic analysis. Additionally, autosomal MH locus 6pB was removed due to low coverage in the reference profiles. The Integrated Genome Viewer (IGV [20]) was used to curate the reference profiles and, when necessary, to check sequence alignment to the reference genome.

The no template control was analysed to review the baseline noise, applying an absolute threshold of 1 read per allele, resulting in a mean of 3.47 reads across the 101 MH markers included in the study.

2.4. MH and CE-STR comparison for mixture analysis

Comparative analysis between CE-STRs and microhaplotypes was performed by using custom pipelines in R software v.4.0.2 [21].

The minimum number of contributors to a mixed profile was established by dividing the number of alleles found in the locus with the largest number of alleles by two and rounding up [2]. The probability of, having n contributors, finding 2(n-1) or less alleles or more than 2 n alleles in the genetic profile, i.e. the probability of misclassifying the minimum number of contributors (NOC) to the mixture, was estimated using Torben Tvedebrink's DNA mixtures web program accessible at http://apps.math.aau.dk/noa/ and described in [22]. Locuswise probabilities for finding 2 n and (2n-1) alleles were subtracted to 1 to find the probability of misclassifying the minimum NOC.

Likelihood Ratio (LR) estimations were performed with the R package EuroForMix v3.3.1 [23] under the quantitative model, contrasting the following prosecutor (Hp) and defendant (Hd) hypotheses: (Hp) all the donors have contributed to the mixture, and (Hd) all the donors except donor 1 (D1), plus a random individual from the same population and not related to the donors, have contributed to the mixture. Drop-out probability estimation was performed by sensitivity analysis and drop-in probability was set to the standard value of 0.05. The LR values obtained are represented by their 5 % percentile in all cases except for mixtures 1:1 and 1:3 for MHs, where the program returned infinite LR values that impeded further calculation. Population frequencies used for CE-STRs are described in [24,25], and those for MHs, in [16]. Ae values for

each marker can be consulted in Tables S2 (for STRs) and S3 (for MHs).

3. Results and discussion

3.1. Theoretical assessment of the number of contributors to the mixture

Table 2 shows the probabilities of misclassifying the minimum NOC for mixtures of 2, 3 and 4 donors for the whole set of STRs and MHs considered in this study. Values for each marker and number of contributors are shown in Table S4. The probabilities of misclassifying the mixture for 2, 3 and 4 contributors were of 1.89 E^{-15}, 5.21 E^{-4} and 0.19 for STRs, respectively, and 8.61 E^{-38}, 8.05 E^{-2} and 0.97 for MHs, respectively. Thus, MHs performed better than CE-STRs in mixtures of two contributors due to the higher number of loci included in the analysis but showed a greater chance of misclassification in 3 and 4 contributor mixtures.

Analysing mixtures of a high number of contributors presents a considerable challenge to the forensic community [2]. When estimating the number of contributors in 4-contributor mixtures using both the MH and STR panels, they presented a misclassification rate of 0.97 and 0.19, respectively. A close inspection of the MH panel reveals that only marker 22qB presents more than six haplotypes with a population frequency of at least 0.05.

The effective number of alleles (Ae) is considered a key factor when evaluating loci for potential inclusion in MH panels focused on mixture analysis; the higher the Ae, the better the panel's capability to differentiate contributors in complex mixtures. Microhaplotype loci with Ae > 3 are considered useful in routine forensic practice [8,10]. The Ae values for the MH and STR loci were estimated using the allelic frequencies from [16] and are fully displayed in Tables S2 and S3. Out of the 101 analysed autosomal markers of the MH panel, 33 have an Ae < 3, so lack the power to detect a high number of contributors to a mixture. Only 1 STR marker (TPOX) shows an Ae < 3. Of the 68 remaining MH loci in our panel, 17 reach an Ae > 4, while 17 out of 21 STR marker present an Ae above 4. The Ae for the autosomal MH markers used ranges from 1.95 to 5.06, with an average Ae of 3.37. In STRs, the range of Ae goes from 2.86 to 17.09, with an average of 6.00.

The limited values of Ae in the MH panel are a consequence of selecting target sequences in short amplicons required for the study of degraded DNA, leading to fewer SNPs forming the haplotype in the shortest MH sequences. Optimal Ae for complex mixture interpretation has been estimated to be between 15 and 20 [13], but this would require much longer haplotypes containing numerous SNPs and would compromise the ability of the panel to reliably analyse degraded DNA.

3.2. Comparisons of drop-out and drop-in in quantified DNA mixtures

Fig. 1 and Tables S5.1 and S5.2 show the comparison of the percentage of drop-out, drop-in and correctly called alleles in each of the quantified mixtures for MH and CE-STR analysis, respectively, when compared to the expected genotype and haplotype of the mixture, obtained by combining the reference profiles from the contributors. Should both contributors present two different alleles in each locus, a complete loss of the minor contributor would be expected to produce a 50 % mixture drop-out rate. However, drop-out rates do not reach this value due to homozygous genotypes and allele sharing. Allele-sharing percentages between donors 1 and 2 reached values of 24 % for CE-STRs and 31 % for MHs, as expected considering the higher degree of polymorphism from STRs. Homozygosity rates were estimated at 33 % (STRs) and 32 % (MHs) for donor 1 and 24 % (STRs) and 34 % (MHs) for donor 2.

Table 2
Theoretical probabilities of misclassifying the mixture as being of a different number of contributors from reality for GlobalFiler (STRs) and the 118 MH panel (MHs).

Mixture	STRs	MH
2 contributors	1.89 E^{-15}	8.61 E^{-38}
3 contributors	5.21 E^{-4}	8.05 E^{-2}
4 contributors	0.19	0.97

Both CE-STRs and MHs show similar drop-out levels across all DNA mixtures, the latter presenting slightly lower values. Drop-out increased towards the more imbalanced mixture ratios. Notably, drop-out reached the highest values in 2-donor mixtures from ratios 1:14, 1:19, 1:24, 1:29, 1:39 and 1:79 with drop-out rates of 29 %, 26 %, 34 %, 31 %, 37 % and 35 % for CE-STRs, respectively, and 15 %, 28 %, 25 %, 31 %, 31 % and 35 % for MHs, respectively. The recovery of exclusive alleles from the minor contributor is explored in Section 3.3. Drop-in rates were lower with CE-STRs than with MHs, reaching a mean drop-in rate of 0.34 % for CE-STRs and 1.5 %, for the MH panel across all quantified mixtures.

In multiple-contributor mixtures, it should be noted that for both drop-out and drop-in, the lower level of polymorphism of MHs compared to STRs can lead to the masking of these stochastic effects. This explains the higher drop-out rates seen in CE-STR analysis when compared to MHs, and the lower drop-in rates detected in MHs when compared to their respective 2-contributor mixtures.

Supplementary Fig. S1 shows a raster plot in which drop-out and drop-in per mixture and marker are detailed, visualizing whether these phenomena were stochastic across mixtures or indicating a pattern. Regarding drop-out, loci affected at a certain ratio tend to also show allele loss at more imbalanced ratios, which might be related to the amplification success of each marker within the multiplex PCR.

Drop-in does not follow a specific pattern in CE-STR markers, but it does in MHs, with six loci showing consistent drop-in events across all 2-contributor mixtures: 1pC, 6qB, 9qA, 9qB, 15qB and 17qC. Notably, markers 1pC, 6qB, 15qB and 17qC were described in [16] as requiring manual inspection for genotype correction, as they tend to present artifacts during sequencing. In that study, those markers were recognised to be sub-optimal for mixture analysis as misincorporations could be mistaken for the alleles from a minor contributor. Manual inspection with IGV revealed that 1pC, 6qB and 15qB all show clearly identifiable reading anomalies that could explain the drop-in genotypes in each case. These artefacts tend to appear when sequencing next to polynucleotide regions, which can cause misalignment of the sequence reads. Although no visible IGV anomaly can be seen in 17qC, in all samples sequence coverage for this marker was very low, leading to unreliable genotype calls. These results are in line with those obtained in [16].

Markers 9qA and 9qB were not initially described as requiring manual inspection or to be problematic, but in this study were severely affected by drop-in. This is likely to be caused by applying a lower analytical threshold in this study compared to previous analyses [16] (0.02 vs 0.1, respectively). Indeed, a close inspection of the reference profiles (Table S6) reveals no-called drop-in artefacts in donors 2 and 4 with frequencies below 4 %. IGV scrutiny of the sequence (Supplementary Fig. S2) shows that 9qA is adjacent to a poly-A tract that causes misalignment, and the SNP allele is called as an insertion. In marker 9qB, the allele is also aligned as an insertion; however, no clear unstable tract could be identified as a likely cause of misalignment (Supplementary Fig. S3).

An in-depth discussion on the effects of the different calling parameters on the stochastic artifacts and the detection of the minor contributor is presented in the following section.

3.3. Detection of minor contributor exclusive alleles in quantified DNA mixtures

Reference profiles of the different donors of each mixture were compared to identify alleles exclusive to the minor contributor, resulting in a total of 24, 14 and 14 alleles for STRs, and 90, 49 and 30 for MH in mixtures of 2, 3 and 4 contributors, respectively.

As shown in Fig. 2 and Tables S5.1 and S5.2, MHs showed a better

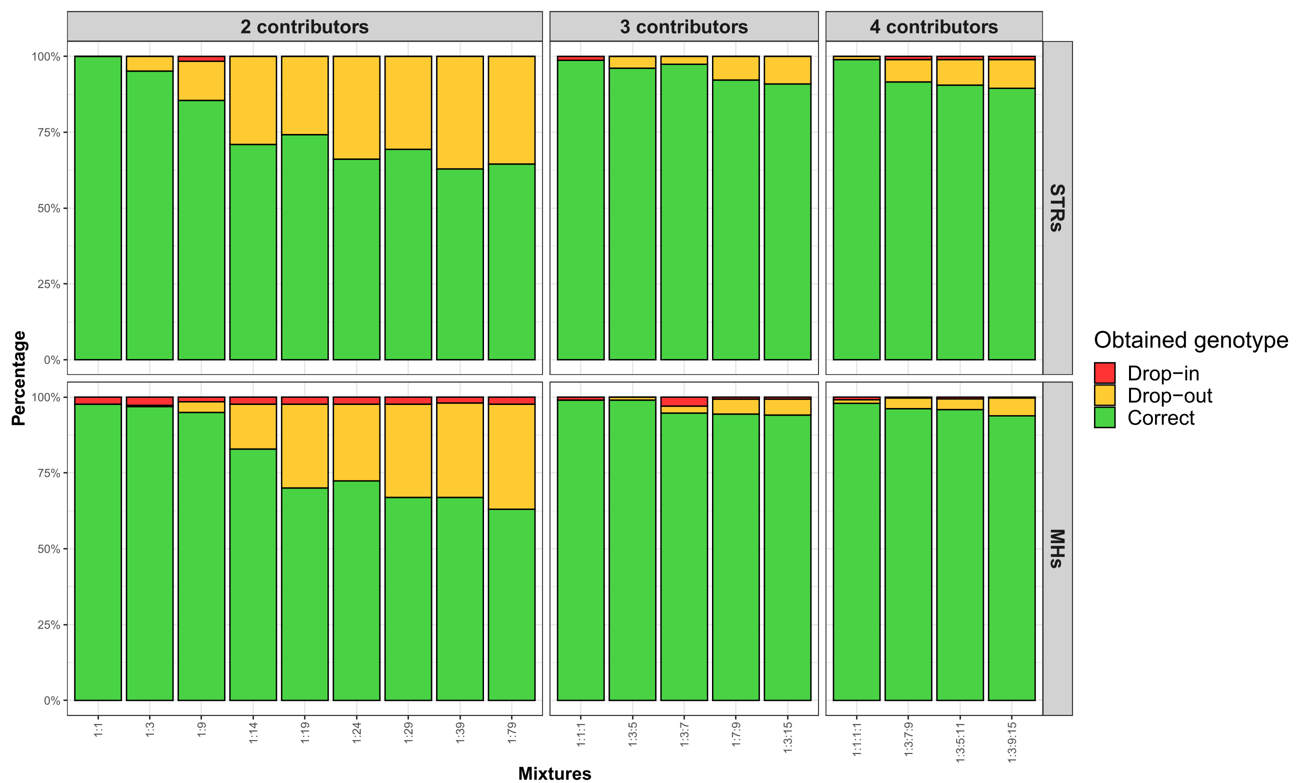

Fig. 1. Barplot showing the percentages of correctly identified alleles (green), drop-out (yellow) and drop-in (red) per mixture and type of marker (STRs and MHs).

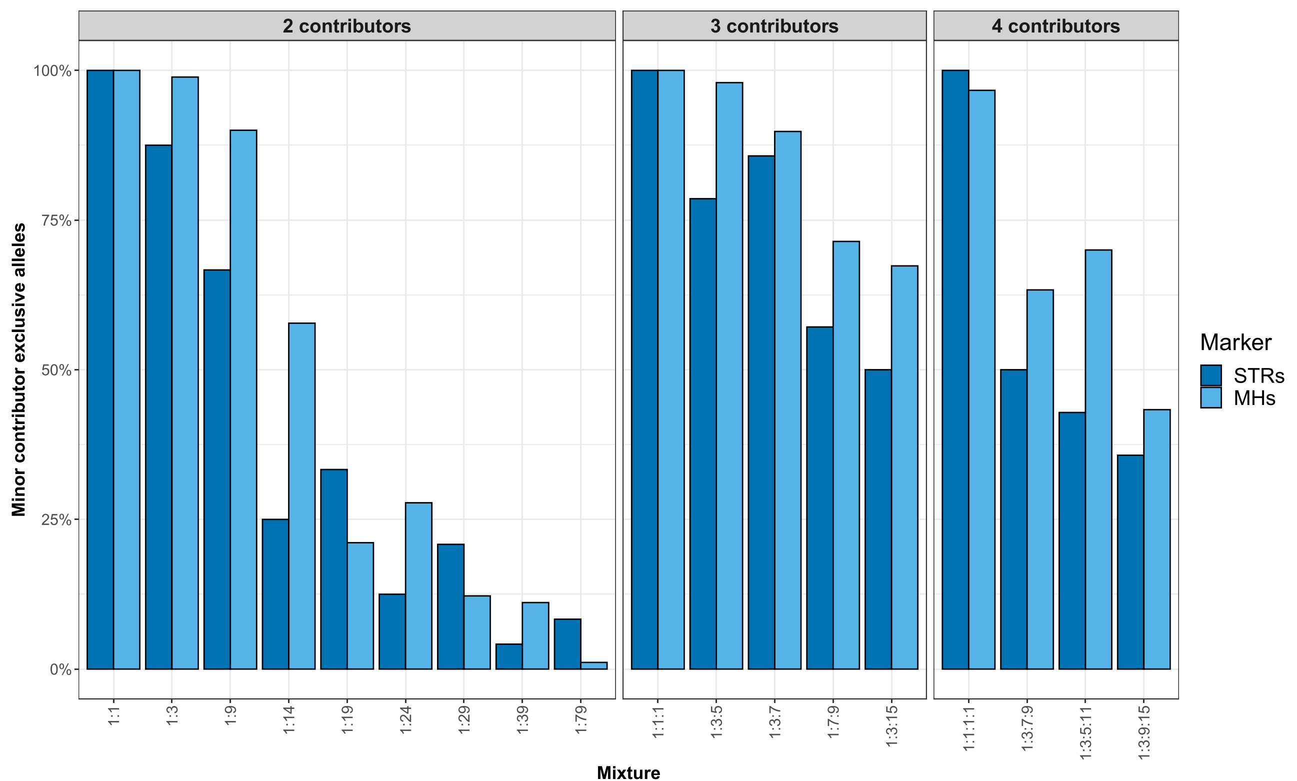

Fig. 2. Barplot showing the percentage of alleles that belong exclusively to the minor contributor for each mixture and type of marker. Dark blue: STRs; Light blue: MHs.

capacity to detect the minor contributor's exclusive alleles in mixtures of 2, 3 and 4 contributors, except for mixtures 1:19, 1:29, 1:79 and 1:1:1:1, in which CE-STRs detected a higher percentage of exclusive minor donor alleles. These results could be explained by the fact that the MH panel has a higher number of markers analysed in small amplicons, and therefore tend to be less affected by drop-out and DNA degradation.

The developmental validation of GlobalFiler evaluates the performance of the kit in the analysis of mixtures of imbalanced ratios up to 1:9 [3,26]. In the 1:9 mixture, CE-STRs detected 67 % of the minor contributor's exclusive alleles, while microhaplotypes detected 90 % of them, underlining their informativeness for mixture analysis in routine forensic analysis.

Other studies addressing comparisons between STR and MH panels reached similar conclusions regarding the ability of MHs to analyse mixtures. Microhaplotypes provide enhanced performance compared to CE-STRs when analysing complex and imbalanced mixtures [4,11,27,28]. However, a direct comparison between all these studies is not possible due to the following considerations: i) a lack of common analytical thresholds amongst studies; ii) ambiguity in the nature of the reported alleles from some studies; iii) consideration of different panel-performance measurements; iv) absence of information on drop-out and drop-in rates, and v) use of different MH and STR panels in other comparative studies.

In this study the absolute threshold was reduced from the 15 reads used in the original publication [16] to 5 reads per allele, the plugin's default value. This decision is supported by the low baseline noise detected in the study (3.47 reads per marker in comparisons to the 23.72 for the same platform in [16]). Table S7 shows that out of a total of 75 drop-ins observed in the study, only 7 of them (i.e. 9.33 % of the total) would disappear by maintaining a 15 reads threshold. Moreover, 6 out of these 7 drop-ins occur in locus 17qC, identified in [16] and Section 3.2 as unreliable due to its tendency to present low coverage, resulting in an increase of stochastic artifacts. This means that the majority of drop-in events surpass this absolute threshold (be it 5 or 15) and should therefore be filtered out by the relative threshold.

As for the relative threshold, the detection of alleles from a heterozygote minor contributor in the more imbalanced mixtures of each group, 1:79, 1:3:15 and 1:3:9:15 theoretically requires an analytical threshold below 0.012, 0.024 and 0.034, respectively. For this reason, we opted for the 0.02 threshold used in the original publication [16], equal to the Torrent Suite threshold for the variant calling of somatic mutations, that was not optimal for the analysis of the highly imbalanced 1:79 mixture but was sufficient for the 1:39 mixture (requiring a threshold below 0.024). Other authors have proposed a threshold of 0.05 for mixture analysis with MHs [11]. This value would filter out 60 out of the 75 drop-in alleles (80 %), as shown in Table S7; however, it would come at the cost of losing alleles from the minor contributor in highly imbalanced mixtures. Taking the mixture of ratio 1:39 as an example, and as detailed in Table S8, just 10 out of the 90 exclusive alleles from D1 are detected using a threshold of 0.02. Increasing the relative threshold to 0.05 would filter out 9 of those 10 exclusive alleles, resulting in a reduction of Likelihood Ratio (discussed in Section 3.4) in 27 degrees of magnitude, going from a log(LR) value of 26.36 to – 0.88, with a threshold of 0.02 and 0.05, respectively.

In conclusion, the 0.02 threshold allowed the recovery of alleles from the minor contributor even in cases of low sequence coverage, while maintaining minimal levels of drop-in that are considered in the statistical evaluation of the evidence. Even when the sequencing errors causing drop-ins tend to be marker-dependent and could be corrected manually (e.g., with IGV), a high number of markers in the MH panel are challenging to scrutinize in very complex mixtures. If this phenomenon is particularly frequent in MHs, the drop-in rate for the statistical evaluation using these markers will require adjustment accordingly.

3.4. Likelihood ratio and contributor proportion estimations

LR values computed for the quantified mixtures for both CE-STRs and MHs are outlined in Table 3. Values included in the range $|\log(LR)| < 3$ were considered inconclusive [29]. A value of $|\log(LR)| > 0$ is theoretically expected as donor 1 (D1) has contributed to all the analysed mixtures.

When using CE-STRs, mixtures 1:14, 1:24, 1:29, 1:39, 1:79, 1:3:5:11 and 1:3:9:15 produced log(LR) values recorded as inconclusive ($|\log(LR)| < 3$). Only mixtures 1:39 and 1:79 gave negative log(LR) values; supporting Hd. This is because these two mixture ratios are highly imbalanced and the detection of exclusive alleles from the minor contributor is minimal (see Fig. 2). When comparing single profiles, we find that the minor contributor (D1) presents 90 MH exclusive alleles, and 24 STR exclusive alleles. Only 10 MH alleles exclusive from the minor contributor are detected in mixture 1:39, and just 1 allele for the 1:79 ratio. With CE-STRs, the number of alleles exclusive to the minor contributor detected are 5 and 1, respectively.

Table 3
Adjustment of log(LR) values to the 5 % percentile of the sensitivity curve (conservative value) obtained in EuroForMix for MHs and STRs. Hp: contribution of D1 and other contributors. Hd: D1 does not contribute, instead another random and unrelated individual of the population does. A value is considered inconclusive when higher than $\log(LR) = -3$ and lower than $\log(LR) = 3$.

Mixture	log(LR) MHs	log(LR) STRs
1:1	Infinite	24.12
1:3	Infinite	18.39
1:9	166.54	10.24
1:14	160.59	1.63
1:19	49.48	3.68
1:24	75.56	0.51
1:29	25.45	1.06
1:39	26.36	−1.17
1:79	−1.03	−0.04
1:1:1	221.49	22.22
1:3:5	193.05	12.42
1:3:7	126.52	16.26
1:7:9	123.96	6.50
1:3:15	115.92	6.69
1:1:1:1	141.83	18.64
1:3:7:9	72.33	4.14
1:3:5:11	83.15	2.53
1:3:9:15	49.75	1.99

The MH panel produced much higher LR values than CE-STRs (Table 3), except in mixture 1:79, which was also the only mixture ratio to give an inconclusive log(LR) value. In all other cases log(LR) values were positive and conclusive, supporting Hp. The log(LR) values obtained in EuroForMix with the quantitative model were much higher for MHs than for CE-STRs (Table 3), as exemplified by the fact that for mixtures 1:1 and 1:3, the program returns "infinite" as the log(LR) value. These higher values in the MH panel's log(LR) estimations and its better performance in imbalanced mixtures is due to the panel comprising approximately five times more markers than the STR panel. As the LR values for the MH panel require the multiplication of more values than STRs, the LR values obtained with each panel are not directly comparable. This also suggests a need to expand the range of values where log (LR) is considered inconclusive when using MHs. Further studies into values of analytical thresholds and considerations for drop-out and drop-in are necessary to establish microhaplotypes in routine forensic analyses, including comparisons between different platforms and internal validation studies of the panels.

Alongside the estimation of LR values, the quantitative module of EuroForMix implements an estimation of the percentage of each donor's contribution to the mixture for quantified mixtures (Fig. 3 and Table S9).

EuroForMix predictions were closer to theoretical values in two-contributor mixtures, and MH predictions, more accurate than those from STRs. The greatest divergence was seen in three-contributor mixtures, where neither CE-STRs nor MHs are particularly informative. In four-contributor mixtures, donor 1 and donor 3 were very precisely predicted, with donor 2's contribution being overestimated, and donor 4's underestimated. In this case, CE-STRs provide a slightly closer prediction to the true ratio in the case of donor 2 and donor 4 (see Table 1 for mixture ratios).

Overall, donor 2's contribution tended to appear overestimated, and the other donors' contributions were regularly underestimated. This

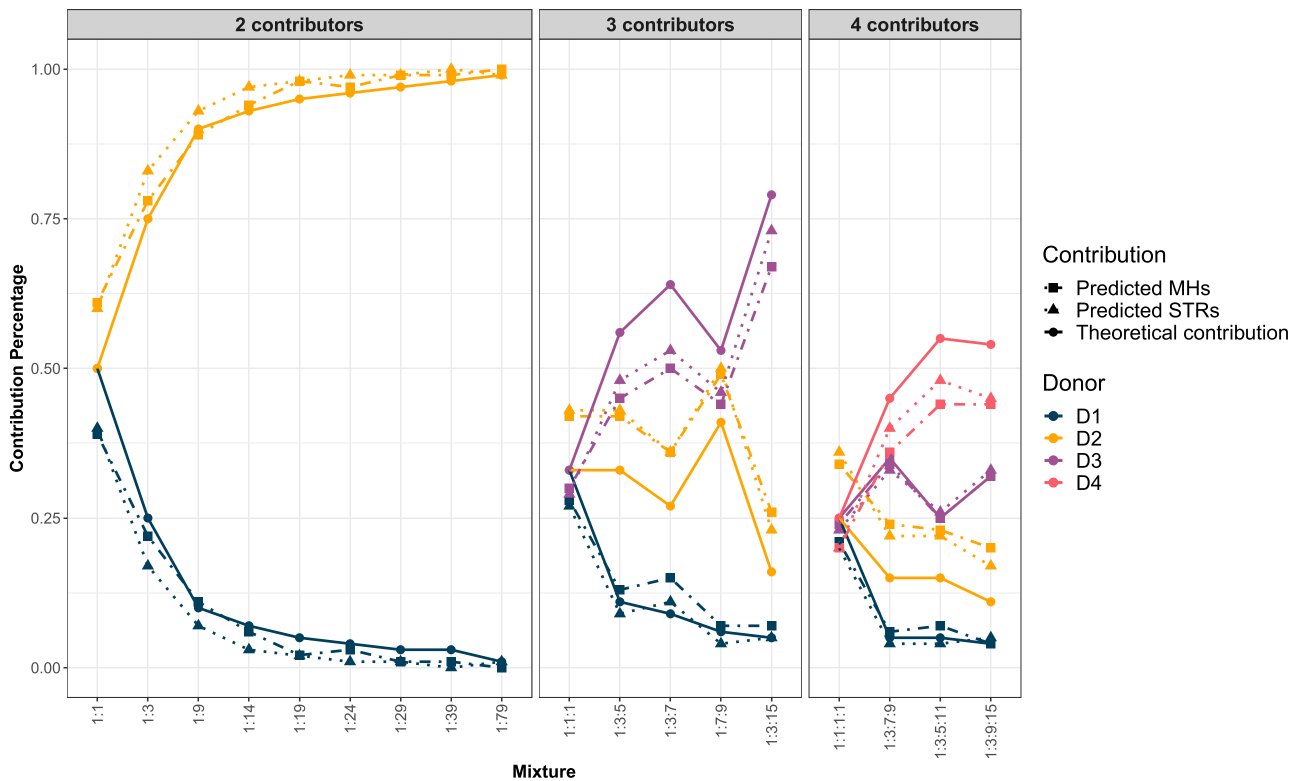

Fig. 3. Prediction of contributor percentages by EuroForMix quantitative model for STRs (triangle + dot line) and MHs (square + dot line). The theoretical value is also plotted (dot + solid line) for each contributor/donor (D) in each mixture. The donor 2 contribution is overestimated in most cases, while the rest of the contributors are slightly underestimated.

recurring phenomenon could be due to stochastic variation during mixture preparation or sample DNA quantification, leading to under- or overestimation of input DNA. This is especially noticeable for the 2-contributors' mixtures, that were prepared in a serial dilution manner, for which a systematic deviation is observed across the different ratios.

Based on our results, and bearing in mind that the mixture analysis tools implemented in EuroForMix are yet to be validated on a larger scale, the mixture predictions serve as a proof of concept for the precision of the EuroForMix's quantitative model when working with peak heights in CE-STRs and sequence read counts in MHs. It should be noted

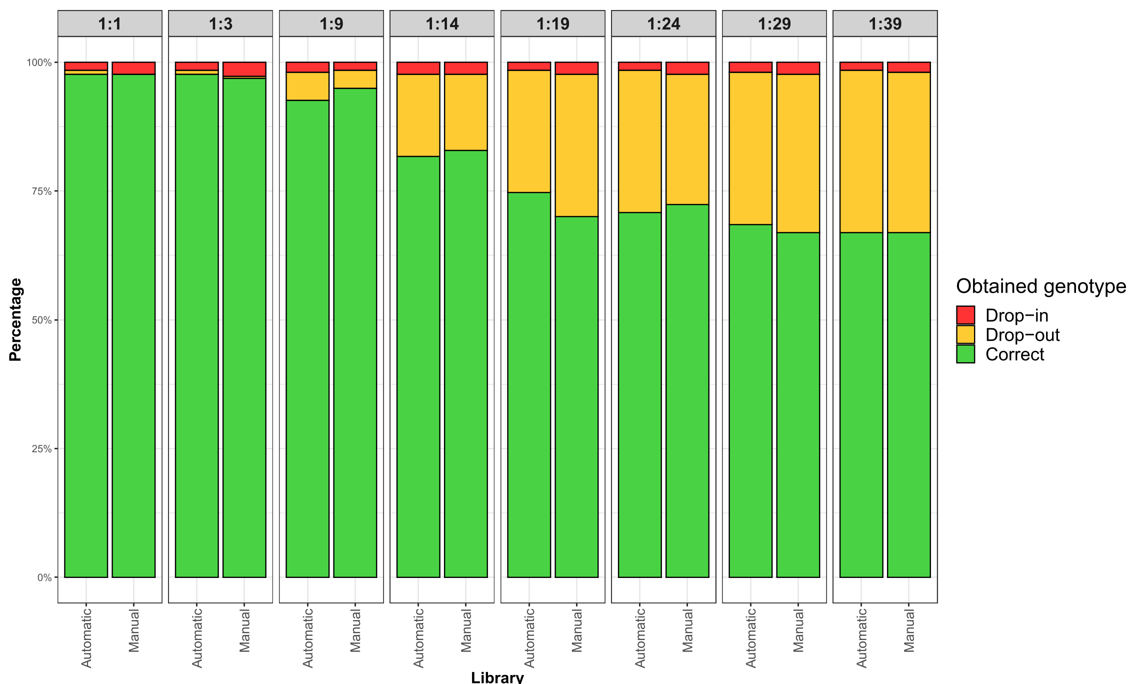

Fig. 4. Barplot showing the percentages of correctly identified alleles (green), drop-out (yellow) and drop-in (red) in mixtures of 2 contributors for MH libraries prepared manually and automatically.

that sequence read counts tend to show higher variability between markers due to differences in amplification efficiency, than variation between peak heights in electropherograms, but the model implemented in EuroForMix is suitable for both measurements. Our observations also add further support to the reliability of the LR values obtained with the quantitative model applied in this study.

3.5. Automatic and manual Ion S5 libraries sensitivity comparison

Fig. 4 and Tables S10.1 and S10.2 show a comparison in correctly called alleles, drop-out and drop-in between automatically and manually prepared MPS sequence libraries, respectively. In both cases we observed the general trend of increasing drop-out percentage with mixture imbalance, while drop-in levels remain more consistent through all mixtures. Differences were marginal between both methodologies: drop-out levels were slightly higher when using automatic libraries while drop-in levels followed the opposite trend.

Fig. 5, as well as Tables S10.1 and S10.2, show a comparison of the detection of the minor contributor's exclusive alleles for both library preparation approaches. Differences are more pronounced than in the previous case yet remain small. Only in mixtures 1:19 and 1:29 did the automatic library workflow detect a higher percentage of exclusive alleles than the manually prepared one. For mixtures 1:1, 1:3, 1:9, 1:14, 1:19, 1:24, 1:29 and 1:39 the percentages of alleles exclusive to the minor contributor (D1) are 97.78 %, 97.78 %, 84.44 %, 54.44 %, 32.22 %, 21.11 %, 15.56 % and 10 %, respectively, for automatically prepared libraries, and 100 %, 98.89 %, 90 %, 57.78 %, 21.11 %, 27.78 %, 12.22 % and 11.11 %, respectively, for manually prepared libraries.

It should be noted that when preparing automatic libraries, total input DNA is divided into two different PCRs, while the manual protocol performs a single PCR for the whole input amount. Moreover, manually prepared libraries can be quantified independently whereas automatic ones are pooled in batches of eight samples, impacting the normalization of libraries before templating and therefore the expected coverage. As previously shown, the automatic preparation of the libraries barely affected the performance of the panel for the 1 ng input DNA amounts used in this study.

While further research is required for lower DNA input amounts, the automatic library preparation method is a practical alternative that reduces both hands-on work and the probability of sample contamination, which is particularly important in compromised samples, which includes those requiring mixture analysis.

4. Concluding remarks

In this study the multipurpose MH panel described in [16] was evaluated for the analysis of DNA mixtures, comparing it with the standard GlobalFiler STR panel. MHs provide advantages over CE-STRs when analysing mixtures, as they do not produce stutter and have no size-dependent preferential amplification [7,8,10].

Drop-in affected MH analysis quite consistently, independently of the number of contributors and mixture imbalance. Some loci (1pC, 6qB, 15qB, 17qC) were previously identified as requiring manual inspection of the reads, and in our study 9qA and 9qB were additionally seen to require manual inspection, in part due to the use of a lower analytical threshold than in [16]. While drop-in can be reduced by increasing sequence coverage threshold levels for allele detection, this can lead to a failure to detect the minor contributor's exclusive alleles. Therefore, this study further cements the necessity of an internal validation of any one MH panel with the subsequent identification of misalignment issues in specific component loci.

Due to the higher number of loci, the MH panel is less affected by drop-out, which leads to the recovery of a higher percentage of minor donor exclusive alleles from imbalanced mixtures and provides greater LR values when statistically evaluating the contribution of a donor. Because of the disparity in the number of loci analysed, LR values obtained with the MH panel are not directly comparable with those obtained with CE-STRs. Moreover, the higher LR values of the MH panel might require an expansion of the range set for considering a result as inconclusive (currently $|\log(LR)| < 3$). The quantitative module of EuroForMix returned values of predicted percentage of each donor's contribution close to the actual values set up for each mixture tested, underlining the viability of using sequence read coverage as a proxy for

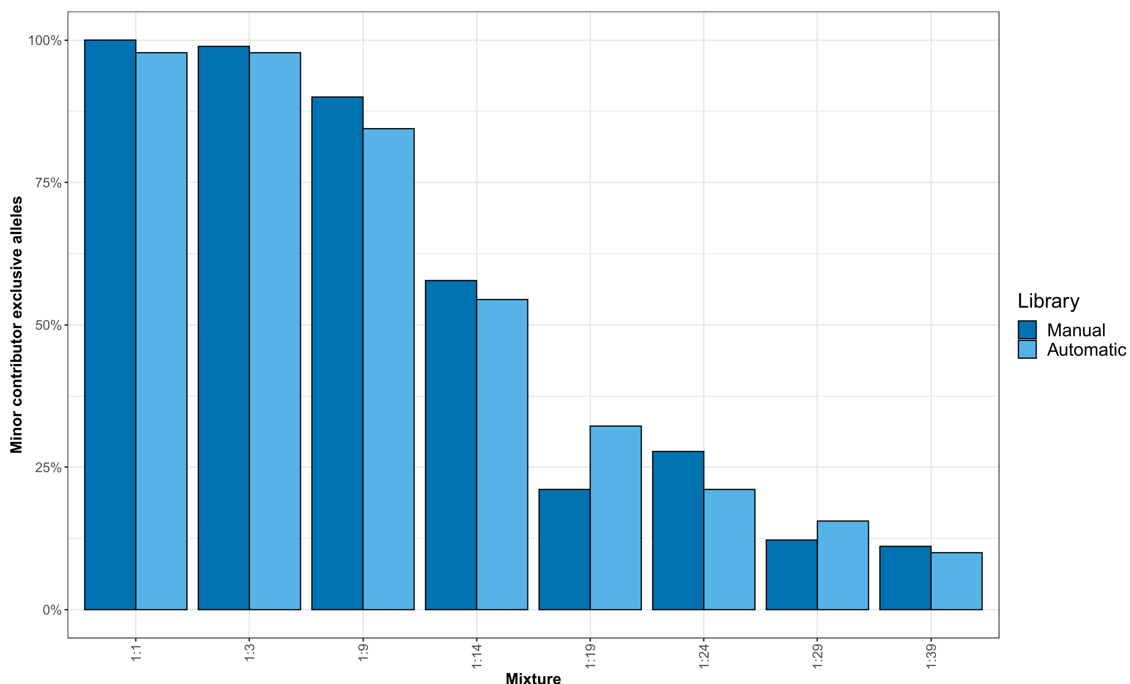

Fig. 5. Barplot showing the percentage of alleles that belong exclusively to the minor contributor in mixtures of 2 contributors for MH libraries prepared manually and automatically. Dark blue: manual; Light blue: automatic.

peak heights to estimate contributor ratios.

The alternative library preparation workflows did not significantly differ in performance, with both approaches showing similar levels of drop-out and drop-in, and the detection of minor donor exclusive alleles was almost equal. This is true even though the automatic method implemented with the Ion Chef divides the sample into two, halving the input DNA available for each reaction. Automatic library preparation still represents an improvement in forensic practice by reducing hands-on work and contamination, without compromising the quality of the samples.

It should be noted that the MH panel employed in this study was not designed as a mixture analysis microhaplotype marker set, focusing instead on analysing degraded DNA. This is reflected in the short amplicons of most loci in the panel (< 120 bps) [16]. Nevertheless, the MH panel applied here has proved to be suitable as a stand-alone solution for the analysis of simple mixtures. In multiple contributor mixtures, it could be combined with more polymorphic markers such as STRs or MHs with higher Ae values to correctly estimate the number of contributors.

This study supports the implementation of microhaplotype loci in routine forensic analysis as a powerful tool for the detection and statistical evaluation of DNA mixtures, especially when dealing with highly degraded DNA. Lessons learned for the design of MH panels dedicated to mixture analysis include: (i) the context sequence of potential MH markers should be carefully scrutinized to avoid misalignments, as confidence in the sequencing results is key to lowering the analytical thresholds and enhancing the recovery of the minor contributor's alleles; (ii) a high effective number of alleles (Ae) is required for the analysis of complex mixtures with multiple contributors, but a compromise solution must be reached between extending the amplicon length for increasing polymorphism and the sensitivity to degraded DNA; and (iii) MH markers should be selected to be well separated across the genome to ensure independence to maximize the informativeness of the LR values obtained from any one panel.

Declaration of Competing Interest

Authors RL and CR are employed by Thermo Fisher Scientific and a major part of this study centred on the adaptation and optimisation of the prototype version of Thermo Fisher Scientific's sequence analysis plug-in available for collecting microhaplotype data from the Ion S5 MPS system (HID_Microhaplotype_Research_Plugin v1.55). The plug-in is not a commercial product at the time of writing, and the other co-authors did not gain any financial benefits from the process of investigating the sequence analysis thresholds and parameter settings of the HID_Microhaplotype_Research_Plugin v1.55.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2024.103144.

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