Forensic Science International 303 (2019) 109938

Contents lists available at ScienceDirect

Forensic Science International

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

Brazilian forensic casework analysis through MPS applications: Statistical weight-of-evidence and biological nature of criminal samples as an influence factor in quality metrics

E. Avila[a,b,c,*], C.P. Cavalheiro[b], A.B. Felkl[b], P. Graebin[b], A. Kahmann[d], C.S. Alho[b,c]

[a] Setor Técnico-Científico, Superintendência Regional do Rio Grande do Sul, Polícia Federal, Porto Alegre, Brazil
[b] Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil
[c] Instituto Nacional de Ciência e Tecnologia INCT Ciências Forenses, Porto Alegre, Brazil
[d] Instituto de Matemática, Estatística e Física, Universidade Federal de Rio Grande, Rio Grande, Brazil

ARTICLE INFO

Article history:
Received 23 July 2019
Accepted 22 August 2019
Available online 12 September 2019

Keywords:
Massive Parallel Sequencing
SNP
Brazilian Federal Police
HID ion Ampliseq identity panel
Forensic
Quality metrics
Nature of biological evidence

ABSTRACT

Real forensic casework biological evidence can be found in a myriad of different conditions and presenting very distinct features, including key elements such as degradation levels, the nature of biological evidence, mixture presence, and surface or substrate deposition, among others. Technical protocols employed by forensic DNA analysts must consider such characteristics in order to improve the chances of successfully genotyping these materials. MPS has been used as a very useful tool for forensic sample processing and genetic profile generation. However, it is not completely clear how different features encountered with real forensic samples impact sequencing quality and, consequently, profile accuracy and reliability. In this context, the present study analyzes a set of 47 real forensic casework samples, obtained from semen, saliva, blood and epithelial evidence, as well as reference oral swabs, aiming to evaluate the impact of a sample's biological nature in profiling success. All DNA extracts from samples were standardized according to sample conditions, as assessed by traditional forensic profiling methods (real-time PCR quantitation and capillary electrophoresis-coupled STR fragment analysis). Samples were separated into groups according to their biological nature, and the resultant sequencing quality was evaluated through a series of well-established statistical tests, applied specifically to six different MPS quality metrics. The results showed that certain groups of samples, especially epithelial and (to a lesser extent) saliva samples, exhibited significantly lower quality in terms of some of the evaluated metrics. A number of reasons for such unexpected behavior are discussed. In addition, a series of calculations was performed to assess the weight of genetic evidence in Brazilian samples, and reflexes in data analysis and national allele frequency database construction are discussed. Overall, the results indicate that a unified national allele frequency database can be used nationwide. Besides this, MPS genetic profiles obtained from samples with particular biological origins may benefit from meticulous manual review, and visual inspection could be important as an additional step to avoid genotyping errors or misinterpretation, leading to more trustworthy and reliable results in real criminal forensic casework analysis.

1. Introduction

The highly variable aspects associated with biological evidence collected from crime scenes or other samples with forensic interest pose a potential challenge to efficient processing of such materials [1]. In order to generate DNA profiles with appropriate quality and necessary attributes to be admitted in court as genetic evidence for human identification or be inserted in genetic databases, forensic DNA analysis must deal with a huge variety of different biological tissues, fluids, secretions and materials, as well as a plethora of objects or deposition surfaces where such evidence can be collected [2]. All these factors can affect the levels and quality of obtained DNA to be used in genetic human identification methods as features like DNA levels can greatly vary among different biological materials [3]. Furthermore, real forensic casework analysis usually involves handling samples where phenomena, like low template DNA (also called low-copy numbers, or LCN), poor quality DNA owing to degradation or

* Corresponding author at: Laboratório de Genética Humana e Molecular, Escola de Ciências, Pontifícia Universidade Católica do Rio Grande do Sul. Av. Ipiranga, 6681 – Prédio 12C, Sala 221, 90619-900 Porto Alegre, Brazil.
E-mail address: e.avila@edu.pucrs.br (E. Avila).

http://dx.doi.org/10.1016/j.forsciint.2019.109938
0379-0738/

environmental exposure to elements, presence of inhibitors or occurrence of distinct contributor mixtures in biological evidence, can considerably impact genetic profile quality [4,5]. Hence, a proper appraisal of how these inconsistencies can influence genetic profiles is crucial, aspiring to satisfy quality standards in genetic data generation for forensic purposes.

In order to overcome technical issues limiting the ability to properly process biological evidence, especially those presenting critical conditions or other characteristics that notoriously hamper genetic profile yield or interpretation, or possibly leading to statistical weight of evidence decrease, alternative methodological solutions have been proposed as possible solutions for challenging samples [6]. When compared to traditional forensic human identification DNA methods, which employ Short Tandem Repeat (STR) evaluation coupled with Capillary Electrophoresis (CE) fragment analysis, possible approaches include analysis of different genetic markers sets, evaluation of alternative DNA polymorphisms types, improvements in kit chemistry leading to higher sensitivity or tolerance to inhibitors, as well as introduction of technological alternatives to standard methods, specifically regarding equipment for DNA sequencing or genotyping and software used in data processing and analysis [4,7]. In this context, the HID-Ion AmpliSeq Identity Panel (Thermo Fisher Scientific, Waltham, USA) commercial solution is presented as an option for criminal forensic casework processing and profiling. This reagent comprises simultaneous amplification and evaluation of 90 autosomal biallelic single nucleotide polymorphisms (SNPs) specially selected to provide enough discrimination power for individual identification based on presentation of high heterozygosity levels in different worldwide populations [8,9]. In addition, a set of 34 Y upper-clade SNPs was included in the kit, aiming to identify male lineages associated with masculine samples. This kit was designed to be utilized in Massive Parallel Sequencing (MPS) platforms, where sequence variations can be assessed on an individual nucleotide basis.

MPS technologies offer advantages for forensic use, such as high-throughput capacity, quick and efficient simultaneous typing of a large number of samples and forensic markers through barcoding approaches, and possibility of workflow automation, among others [10]. The HID-Ion AmpliSeq Identity Panel, in particular, features a peculiar design especially conceived to aid in human identification forensic procedures, where amplified fragments possess relative small sizes when compared to typical STR markers. This unique trait allows successful analysis of highly degraded, low-input, critical condition DNA, which might be, otherwise, unable to generate a robust quality genetic profile [11]. Several validation studies were conducted to appraise key features of this panel, as sensitivity, specificity, reproducibility, accuracy, and ability to handle samples with conditions such as low DNA input, mixtures or degraded DNA [12–14]. In addition, population data regarding genetic markers included in the panel were determined for a relevant number of worldwide populations [11,13,15–20]. Reported conclusions for the studies described indicate robust, efficient and reliable typing of forensic samples could be accomplished with different versions of this product. Intra- and inter-population analysis also determined a high degree of genetic variability among tested individuals, emphasizing the adequacy of this chemistry's use in forensic individual identification or parentage testing. However, most published results are based on data generated from pristine condition DNA, like commercially available control DNA, NIST standards or reference, single-source samples. In addition, investigations reporting mixture or degraded sample evaluations were designed through controlled environments, employing mixtures with known contributor ratios or artificially degraded DNA, to infer effects in amplification performance [12–14,21]. A limited number of studies actually describe results from real forensic casework [21] obtained with the present kit, but some questions related to the forensic origin of samples remain to be more thoroughly investigated - those related to the number of unaligned sequences (probably related to bacterial or other organisms from environmental contamination of real criminal samples) or variations associated with the biological evidence's place of collection (as the substrate or surface of deposition where such samples were deposited) [21].

In this study, we applied a series of well-known statistical tests to evaluate the effect of the nature of biological evidence and biological origin of real forensic criminal casework DNA in obtained results' quality, indicated by different sequencing or base-calling quality metrics associated with each produced genetic profile. To achieve this, criminal samples collected from real crime scenes and identified as blood, saliva, semen or epithelial evidence were grouped together, and effects of evidence type on obtained sequences were estimated. In order to eliminate or minimize effects of other possible interfering elements associated with samples, such as degradation levels or mixture occurrence, samples were normalized according to characteristics presented by their Real-Time PCR (RT-PCR) quantitation and degradation level results, as well as correspondent STR genetic profile, generated by fragment size analysis through traditional CE applications. All samples employed were submitted to identical procedures across all workflow phases as there is evidence that adoption of different methods and protocols in criminal evidence-sample processing can directly affect MPS results as evidenced by impact analysis in MPS quality results in steps as diverse as DNA extraction [22], amplification [23,24], chip-based sequencing [25,26] or data analysis [27]. Seeing that all investigated samples were actual criminal cases investigated by the Brazilian Federal Police (BFP) regional DNA laboratory, a basic evaluation of obtained results was also performed concerning genetic weight of evidence, taking into consideration recently reported population data and allele frequency for Brazilian national and regional populations [20]. In this analysis, generated profiles were used to estimate Random Match Probabilities (RMP) or Likelihood Ratios (LRs) of criminal samples, not only for Brazilian national frequencies but also regional and even worldwide populations. A discussion of the obtained results and their association with possible genetic stratification of the Brazilian population, in light of previously described Brazilian population features, is also presented.

2. Materials and methods

2.1. Ethical statement

All reference, known-origin, single-source samples analyzed in this study were voluntarily obtained following written informed consent from donors, and refer to a research-oriented human samples biobank at PUC/RS. Anonymous samples were randomly selected from this bank. This work follows the ethical principles stated in the Helsinki Declaration [28] of the World Medical Association, and was approved by Pontifical Catholic University of Rio Grande do Sul Institutional Review Board under number CAAE 52113715.9.0000.5336. Criminal samples were selected among unidentified, unknown-source specimens available in the BFP regional laboratory, where no suspects had ever been identified or presented as suggested sample origins. No information regarding case or sample identification was disclosed. Individual profiles or Y-chromosome haplogroups were not reported for criminal samples as they might express, at least to some extent, biogeographical ancestry and other genetic characteristics associated with an individual who originated criminal evidence [20].

2.2. DNA samples

2.2.1. Reference samples

Ten single-source, known-origin reference samples were employed as experimental controls (CT) for the present work. All samples belonged to the same regional population as criminal samples, and were collected from internal oral mucosa swabs. This collection method was selected owing to being the choice method for reference DNA collection according to Brazilian criminal forensic doctrine [29]. Two sterile, DNA-free dry swabs were utilized for sampling of internal oral mucosa, one for each cheek. DNA extraction was performed with a single swab only, per individual.

2.2.2. Criminal samples

Thirty-seven criminal samples were chosen among forensic casework counter-samples available in the BFP regional laboratory. Samples were randomly selected among those that fulfill the following criteria: a) no suspect or individual was proposed as connected to the case in question; b) the crime scene was processed over the period between 2014 and 2016; c) samples were previously evaluated by STR analysis using CE methods in the laboratory, specifically with commercial kits, and a DNA report was produced as a result; d) biological evidence generated a single-source, complete DNA profile based on STR markers; e) quantity of extracted DNA from evidence was sufficient to assure at least 0.5 ng of DNA per μL, as assessed by Real-Time PCR (RT-PCR) quantitation; f) samples should have minimal to null levels of degradation and PCR inhibition, according to applied techniques.

Samples satisfying the selection criteria were randomly selected according to their biological nature as follows: 10 samples each for blood (BL), saliva (SA) and epithelial or touch DNA (EP) evidence cases, and 7 samples containing semen (SE) biological evidence. Based on the national legal and criminal system structure (where a restricted number of violent crimes are considered federal jurisdiction), the BFP has a very limited number of cases involving rape, sexual assault or other criminal activity involving semen evidence. Therefore, a lesser number of semen evidence samples was available at the laboratory, all of them obtained from ejaculates collected from used condom interiors. The biological origin of each sample was determined according to information stated in the original crime scene documentation referring to each case, exactly as reported by the crime scene expert attending the scene or by the serology laboratory. No additional presumptive or confirmatory tests were performed to ratify the evidence considered in this study.

Following sample selection, analysis of STR profile obtained by CE was executed. STR profiles presenting overall lower *rfu* signals (classified by at least half of observed allele signals presenting peak heights inferior to 4x analytical thresholds (AT) settings) were categorized as low-signal samples. Samples presenting at least three discernible peaks with sizes between half AT and AT values (and, as such, not treated as real allele signals) were classified as low-level mixtures, where the major contributor is easily identified. Such samples were presented as mixture samples even though minor contributor allelic peaks did not reach AT, and, as such, not classified as an actual mixture for STR profiles. A comprehensive description of all selected samples, including the nature of biological evidence, collection method employed, surface or substrate where evidence was deposited and additional characteristics presented by samples is located in Table 1.

2.3. DNA extraction and quantitation

Genomic DNA was extracted from all samples using the automated platform, Automate Express Forensic DNA Extraction System (Thermo Fisher Scientific Inc., Waltham, USA) with the Prepfiler Automated Forensic DNA Extraction System (Thermo Fisher Scientific Inc.). DNA extracts were quantified using the Quantifiler Trio Human DNA Quantification Kit (Thermo Fisher Scientific Inc.) on a 7500 Real-Time PCR System (Thermo Fisher Scientific Inc.). All procedures followed the manufacturer's instructions, with elution volumes of 50 μL. A sample was considered not degraded/inhibited when the degradation index (DI) <1.5 and no IPCCT flag was triggered in Quantifiler Trio assay.

2.4. STR fragment analysis

Criminal samples were submitted to STR fragment analysis including 22 autosomal STR markers, one Y-chromosome STR locus and amelogenin for gender determination. STR analysis was executed using commercial reagent PowerPlex Fusion System (Promega Corp. Madison, USA). Fragment sizes were determined by CE in a 3500 Genetic Analyzer (Thermo Fisher Scientific Inc.) and GeneMapper Id-X v.2 software was used for DNA profile evaluation. All analyses followed the manufacturers' protocols. Quantity of extracted DNA from criminal samples used in each PCR reaction was equivalent to 0.5 ng.

2.5. MPS library preparation, emulsion PCR and sequencing

DNA libraries were constructed using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific Inc.) combined with the HID-Ion AmpliSeq Identity Panel (Thermo Fisher Scientific Inc.), following the manufacturer's suggested protocol (HID-Ion Ampliseq Library Preparation, Revision C.0). Briefly, PCR amplification was performed in a final volume of 20 μL that contained 1 ng of template DNA, 4 μL of 5x Ion AmpliSeq™ HiFi Mix and 10 μL of 2x Ion AmpliSeq™ primer pool (Identity Panel). Final volumes were adjusted with nuclease-free water. PCR was performed in a Veriti 96-well Thermal Cycler (Thermo Fisher Scientific Inc.) under the following conditions: enzyme activation for 2 min at 99 °C, 21 cycles of 15 s at 99 °C and 4 min at 60 °C, followed by a 10 °C hold. PCR amplicons were partially digested with 2 μL FuPa reagent, with incubation thereafter at 50 °C for 10 min, 55 °C for 10 min and 60 °C for 20 min, followed by a 1 h hold at 10 °C. Ligation of adaptors to the libraries was conducted based on the manufacturer's instructions using different barcodes (Ion Xpress Barcode Adaptors 1–96 Kit or IonCode Barcode Adapters 1–384 Kit) for each sample in the same run (Thermo Fisher Scientific Inc.), and resulting products were purified using Agencourt AMPure XP reagents (Beckman Coulter Inc., Brea, USA) according to the manufacturer's protocol. After purification, libraries were quantified using a 7500 Real-Time PCR System (Thermo Fisher Scientific Inc.) with the Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific Inc.). All samples generated high-quality libraries, which were pooled to a final concentration of 20 pM. Emulsion PCR (emPCR) was performed on the Ion OneTouch 2 instrument (Thermo Fisher Scientific Inc.) with the Ion PGM Hi-Q Template Kit (Thermo Fisher Scientific Inc.) following the manufacturer's protocol (Ion PGM Hi-Q Template Kit, Revision A.0). The emPCR products were enriched on the Ion OneTouch Enrichment System (Thermo Fisher Scientific Inc.). A final volume of 30 μL was loaded per chip. Sequencing was carried out on the Ion PGM™ (Thermo Fisher Scientific Inc.) Sequencer with the Ion PGM Hi-Q Sequencing Kit (Thermo Fisher Scientific Inc.) following the manufacturer's instructions (Ion PGM Hi-Q Sequencing Kit, Revision B.0) and using the Ion 318 Chip Kit v2 (Thermo Fisher Scientific Inc.). All samples were evaluated in a single run, in a chip loaded to maximum capacity (77 distinct samples, not all of them included in present study), as suggested by the manufacturer.

Table 1
Criminal samples used in this study and their descriptive data.

Sample Id	Evidence Features					
	Type	State	Method	Surface/Substrate	Location	Classif.
BL.07	Blood	Patent	SC	Cotton t-shirt	Dry stain in sleeve	Normal
BL.09	Blood	Patent	WS	Shattered Glass	Dry stain	Normal
BL.11	Blood	Patent	DS	Laptop plastic shell	Wet stain	Normal
BL.20	Blood	Patent	DS	Metalic car door surface	Wet stain	Normal
BL.22	Blood	Patent	WS	Rendered brick wall	Dry stain	Normal
BL.24	Blood	Latent*	SC	Car Internal rooftop	Stain on headliner	LS
BL.25	Blood	Patent	WS	Glass window	Dry stain	Normal
BL.26	Blood	Patent	DS	Basalt pavement	Wet stain on sidewalk	Normal
BL.27	Blood	Patent	DS	Keramic tiles	Wet stain on wall	Normal
BL.30	Blood	Patent	DS	Keramic pavement	Wet stain on floor	Normal
EP.01	Epithelial	Latent	SC	Sports cap	Inner sweatband	Normal
EP.08	Epithelial	Patent	WS	Glass Surface	Smudged fingerprint	Normal
EP.10	Epithelial	Latent	SC	Cotton male underware	Inner waistband	Normal
EP.13	Epithelial	Latent	WS	Survaillance camera	Body	Mix
EP.14	Epithelial	Latent	WS	Laptop power cable	Connector jack	Mix
EP.15	Epithelial	Latent	WS	Car door handle	Inner side	Normal
EP.17	Epithelial	Latent	WS	Metal padlock	Shackle	Normal
EP.18	Epithelial	Latent	SC	Cotton bath towel	Lining stitches	Normal
EP.21	Epithelial	Latent	DS	Metalic nail	Shank	Normal
EP.28	Epithelial	Latent	WS	Plastic surface	Smudged fingerprint	LS
SA.02	Saliva	Latent	WS	PET bottle	Inner and outter finish	Normal
SA.03	Saliva	Latent	SC	Cigarrete butt	Tipping paper	Normal
SA.04	Saliva	Latent	WS	PET bottle	Inner and outter finish	Mix
SA.05	Saliva	Latent	WS	Stainless steel mug	Mug edge	Mix
SA.06	Saliva	Latent	SC	Cigarrete butt	Tipping paper	Normal
SA.12	Saliva	Latent	WS	PET bottle	Inner and outter finish	Normal
SA.16	Saliva	Latent	SC	Cigarrete butt	Tipping paper	Normal
SA.19	Saliva	Latent	SC	Tootbrush bristles	Free extremity	LS
SA.23	Saliva	Latent	SC	Cigarrete butt	Tipping paper	Normal
SA.29	Saliva	Latent	WS	Dry chewing gun	External surface	LS/Mix
SE.02	Semen	Patent	DS	Used condom	Internal ejaculate	Normal
SE.03	Semen	Patent	DS	Used condom	Internal ejaculate	Normal
SE.07	Semen	Patent	DS	Used condom	Internal ejaculate	Normal
SE.22	Semen	Patent	DS	Used condom	Internal ejaculate	Normal
SE.26	Semen	Patent	DS	Used condom	Internal ejaculate	Normal
SE.27	Semen	Patent	DS	Used condom	Internal ejaculate	Normal
SE.30	Semen	Patent	DS	Used condom	Internal ejaculate	Normal

* Luminol revealed stain; SC = substrate cutting; WS = wet swab; DS = dry swab; LS = Low signal; Mix = Mixture.

2.6. MPS data analysis

Sequencing data were analyzed using the Torrent Suite Software v5.0 (Thermo Fisher Scientific Inc.)). Hg19 was employed as reference genome data. The number of mapped reads was calculated by the Torrent Coverage Analysis v5.0 plugin (Thermo Fisher Scientific Inc.). SNP genotypes were called by the HID SNP Genotyper v4.3.1 plugin, with target regions file (iiSNPv3_FP.hotspot) and the hotspot regions file (IISNPv2.20140429.Designed) under default analysis settings. Minimum coverage was set to 6 reads per base position, and a minor allele standard threshold of 10% was utilized for heterozygous calls. All SNP genotypes were independently reviewed by two different collaborators with manual corrections applied.

2.7. Statistical data analysis

2.7.1. Weight of genetic evidence

All statistical analysis was performed according to BFP internal routine procedures. Weight of genetic evidence is reported for criminal samples as likelihood ratios (LRs) for a traditional match hypothesis following a logical Bayesian approach [30] or RMP in reference to a specific population. Population substructure correction was applied for data generated in our laboratory, with a 0.01 value for theta index. LR calculations were executed with validated, internal use computational tools and confirmed with Familias software v.3.2.2 [31] for STR markers only. Population data for STR [32–34] and SNP [20] markers were used in accordance with current BFP technical protocols.

2.7.2. MPS performance metrics assessment

Statistical data analysis and reports concerning quality metrics results consolidation were performed according to previously reported techniques [18,20]. HID-Ion AmpliSeq™ Identity Panel sequencing performance was evaluated using six different descriptive parameters [13,18,20]: Read Depth (RD), reported as the number of reads for each marker; Locus Balance (LB), which assesses individual locus amplification efficiency and is calculated as the read depth of a locus divided by the mean read depth of all loci, per sample. As Y-chromosome markers are expected to have around half the autosomal read numbers, LB values were assessed independently for autosomal and Y SNPs; Locus Strand Balance (LSB) measures the balance between forward and reverse reads and is expressed as the number of forward reads divided by the total reads number; Heterozygote Balance (HB) estimates the ratio between two distinct allelic read counts in heterozygous samples, and it was plotted as the number of reads of the first base call in alphabetical order (A, C or G) divided by the number of reads of the alternate allele (C, G or T, respectively). For LB, LSB and HB, calculations were performed not with actual measured estimates, but with the absolute residual value of each read, where employed predicted value was defined as the ideal value for each metric in a fully balanced system (equivalent to 1, 0.5 and 1, respectively). Noise Level (NL) represents the amount of unspecific base call,

calculated as the ratio of non-allele calls divided by total read depth. Finally, QS (Quality Score) is a phred-scale marginal (unconditional) probability of the called genotype, and can be found in plugin results as a GQ (General Quality) measure. These six particular metric indexes were chosen among other possible options because they are either directly indicated by analysis and data reporting plug-in (some of them are even flagged when values are below selected thresholds) or easily assessed by simple calculations based on results presented in the plugin main tab. In addition, same measures were employed to evaluate panel performance in validation studies [12–14]. Additional accessory metrics were directly obtained from plugin reports, nominally Reads on Target (RT), equivalent to the ratio of total reads successfully mapped to any targeted sequence of the human reference genome, and Mean Read Length (MRL), described as the average size (in base pairs) of mapped amplicons, per sample.

For each quality metric, per sample data was consolidated in a single value, comprised by the mean value of a particular metric for all markers. Such an approach was opted for in order to aggregate the performance information into one single value. Once markers are expected to perform similarly among samples (considering sample conditions are similar and inter-marker variation seems to be dependent on panel design [12–14]), and heterozygosis occurrence probability for samples and markers is random within the boundaries of population allele frequencies, the mean value is expected to merge all markers' information with no significant bias.

2.7.3. Statistical analysis

All calculations were executed using IBM® Statistical Package for the Social Sciences (SPSS®), version 22. Single-factor analysis of variance (ANOVA) tests were performed for genetic weight-of-evidence in the form of samples' RMP values to verify if resulting values differ for regional Brazilian populations [20] and across worldwide frequency databases. The same statistical test was applied to evaluate accessory descriptive statistics data (nominally RMP and MRL) in order to identify difference among groups.

Quality metrics were analyzed by the Kruskal–Wallis (KW) and Median test, both coupled with Dunn-Bonferroni *Post-Hoc* analysis (where pairwise differences are evaluated for all groups with the goal of verifying which sample type presents significant difference from each other). These methods were employed to compare the distribution among samples and median measurement among samples, respectively. Results with $p\text{-values} < 0.05$ were considered statistically significant, except in *post-hoc* cases, where Bonferroni correction was applied. Outlier samples, classified as far-out extreme values (differing from median at least 3x the IQR (inter-quartile range) value) or out values (different from median in the 1.5x to 3x IQR range), were identified for each evaluated metric. This samples were not included in the correspondent statistical hypothesis test.

KW testing checks the null hypothesis of equal distribution among classes (grouped according to sample nature), while the median test verifies if groups' median are significantly different from the gran median (GM), i.e., the median of all combined samples. Although KW and Median testing may seem redundant, they both reflect distinct features, with both central tendency and variance established. In addition, both tests were applied once KW was more sensitive to outliers, but also more powerful in the absence of such anomalies. Therefore, this simultaneous analysis was performed to avoid bias owing to spurious data. Additionally, KW/Median tests were chosen rather than ANOVA once the obtained data did not have enough evidence of normal distribution among all variables. Bonferroni correction after the Dunn test constitutes a conservative approach regarding the rejection of the null hypothesis. That means our analysis is more prone to Type II errors (when the test does not reject a null hypothesis that should be rejected). Such course of action is employed based on the exploratory nature of this study, where findings require stronger evidence to refute the current understanding of the forensic interpretation of MPS genotyping results regarding real casework samples, where evidence of differences in performance for distinct types of criminal samples has not been previously systematically proposed.

3. Results and discussion

The HID Ion Ampliseq Identity Panel successfully generated genetic profiles for all 37 criminal Brazilian samples, as well as for 10 control reference samples included in the present study. All samples were both collected and analyzed in Brazil's southernmost state (Rio Grande do Sul), and, as such, were considered as belonging to the Southern Brazilian geopolitical regional population [20]. A complete description of samples evaluated in this study, including features like nature of biologic evidence, collection method, surface or substrate where evidence was found, as well as associated weight of genetic evidence related to obtained SNP and STR profile features, is presented in Table 1. Even though this kit was specifically designed to properly handle critical or difficult forensic samples, through a strategy of small-size amplicon usage, this study focused on regular forensic DNA cases, where appropriate amounts of low-degradation DNA were extracted from criminal biological evidence of different origins. All criminal casework samples generated genetic profiles where most genotypes were considered reliable and presenting enough quality to be used in forensic match comparisons. However, distinct performance in quality metrics was observed for different types of biological evidence, and implications of these findings will be discussed in this paper.

3.1. Comparative evaluation of statistical weight of evidence for Brazilian populations

Ninety autosomal SNP markers genotypes of all 47 Brazilian criminal and reference samples were used for determination of the statistical weight of genetic evidence generated with present panel use on the Ion PGM MPS platform. Results were compared with equivalent descriptive statistics obtained from traditional STR fragment analysis for profiles assessed through CE applications. When considered Brazilian national allele frequencies, hypothetical simulations of match exams between sample genotypes and their identical, fully equivalent profiles were calculated. Average LR for all criminal samples was estimated at 1.0715×10^{39} (s.d. = $\pm 2.1345 \times 10^{39}$) when the 90-SNP marker set was considered. These results show that SNP markers largely outperform alternative traditional STR method counterparts, where the obtained average LR for 22 autosomal microsatellite markers is equivalent to 8.6135×10^{30} (s.d. = $\pm 2.1282 \times 10^{31}$). Differences in LR for both marker sets, for each sample, ranged from less than 6 to over 13 orders of magnitude, with SNP markers consistently presenting superior LR values. Supplementary Table S1 presents individual SNP- and STR-simulated LR values for each criminal sample evaluated. It has been previously proposed that inclusion of SNP polymorphism results in addition to STR marker genotypes significantly improves statistical robustness of biological relatedness tests for variant kinship degrees [35]. In the present paper, the obtained results suggest that autosomal SNP markers included in the present panel are able to provide far superior genetic weight of evidence in criminal sample-suspect match cases than standard applied forensic methods (STR markers coupled with CE fragment analyses) in Brazilian criminal casework. Such results can even be improved in terms of resulting likelihood ratios, with inclusion of Y-haplotype matching probabilities in overall

calculations [36,37] as the Ion HID Ampliseq Identity Panel also includes 34 high-clade Y-chromosome SNPs which can also enhance statistical conclusions.

Brazilian national and regional allele frequencies were described in a previous study for SNP markers included in the panel discussed in this paper [20]. No evidence of strong regional genetic stratification in these particular SNP marker set was reported, as reflected by low fixation indexes (represented by F_{st} values and other descriptive statistics). In order to verify if significant disparities would be observed in statistical results when biological evidence is assumed to come from different territorial regions (possessing, therefore, alternative geographical origins), we evaluated RMP values considering allele frequencies for the whole country (national database) or specific geopolitical regions (regional databases). In addition, worldwide populations based on the 1000-Genomes Project [38] were used to leverage RMP results for populations with diverse continental biogeographical origin, also based on their individual allele frequencies. RMP values for these continental populations are depicted as presented by the plugin, and therefore might feature some levels of skewing owing to not using theta for substructure correction, as oppose to what is employed in Brazilian population calculations.

The resulting RMP values show that no significant differences in RMP values are obtained when the individual originating evaluated criminal evidence is assumed to come from Brazilian national or regional populations when considering allele frequencies proposed for such groups. Fig. 1 and Supplementary Table S2 display complete RMP estimates for all 37 criminal and 10 reference samples based on Brazilian and worldwide population frequency databases, including 88 autosomal SNP markers included with the HID Ion Ampliseq Panel. Fig. 1A depicts RMP values for worldwide and Brazilian national populations, while Fig. 1B portrays a similar scenario, including Brazilian national and regional populations exclusively. The average value for the difference between the largest and smallest RMP considering Brazilian populations (national and regional) is 2.6399×10^{-34}, which is over five orders of magnitude smaller than the same estimation based on RMP values available for worldwide populations, equivalent to 1.0876×10^{-28}. In a similar analysis, ratios between per sample largest and smallest RMP values, when Brazilian national and regional frequencies are considered, are all situated within three orders of magnitude, with only less than 8.5% of samples presenting ratios above the 10^2 range. However, when the same calculations are performed based on allele frequencies of worldwide populations,

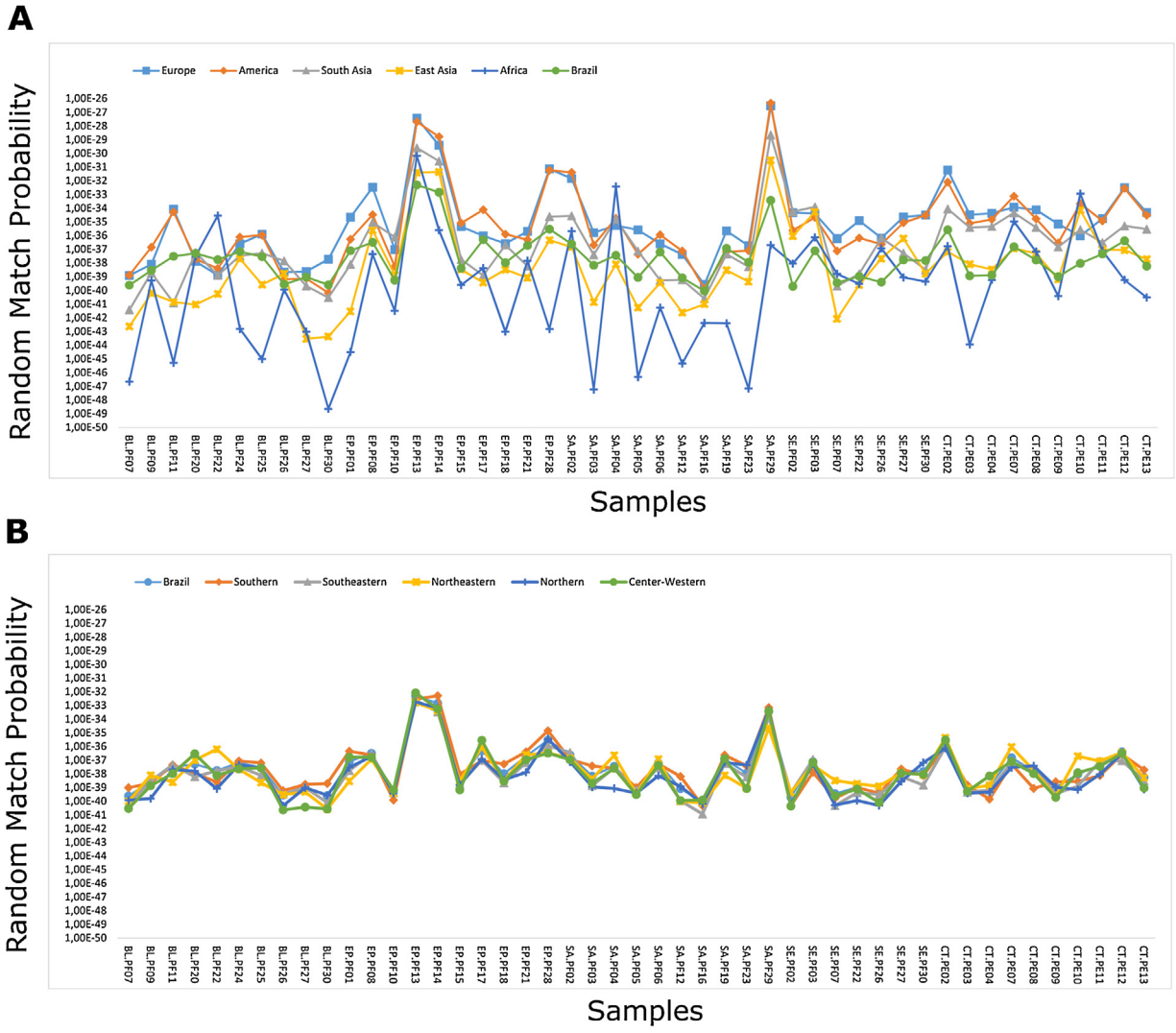

Fig. 1. Random Match Probabilities (RMP) for 47 Brazilian criminal and reference samples based on 88 autosomal SNPs included in HID Ion Ampliseq Panel. RMP values were obtained using distinct population frequencies databases (see Supplementary Table S2 for details on populations). Fig. 1A shows worldwide populations with distinct continental biogeographical origins, and Brazilian national database. Fig. 1B shows RMP values based on Brazilian national population (also included in Fig. 1A) and five distinct regional databases, equivalent to Brazilian five geopolitical regions.

differences between the smaller and largest RMP values per sample can reach over seven orders of magnitude, with 63.83% of samples showing results over 10^3. In addition, Fig. 1B depicts a very similar pattern for RMP obtained for all Brazilian populations, contrasting results presented in Fig. 1A for worldwide frequencies, where higher variation and a wider range of distribution is observed. These results show that variability of possible RMP values based on the Brazilian frequencies database is significantly smaller than observed in worldwide populations, suggesting an overall homogeneity in SNP polymorphism frequency distribution across national territory.

In order to further explore these results, variance analysis was carried out to compare RMP values for Brazilian, worldwide or totality of populations. Criminal samples included in the present study originate from cities belonging exclusively to the Southern Brazilian geopolitical region. Therefore, if a significant discrepancy in overall allele frequencies among regions can be distinguished, one would expect to observe differences in average RMP values for each region, or among RMP estimates calculated for a single sample using distinct frequency databases. In both cases, the largest RMP values are expected to be evidenced in the Southern Brazil database. Notably, smaller average RMP values for the current sample set were found to be reported for American populations. Such a fact is not surprising as modern American populations are similar to Brazilian ones, presenting high admixture levels and including a similar presence or contribution of ancestor populations [39]. One-way ANOVA for Brazilian populations resulted in no significant difference in RMP values among national and all five regional databases ($p\text{-}value = 0.827313$). The same conclusions were achieved for worldwide data ($p\text{-}value = 0.479719$) and all populations combined ($p\text{-}value = 0.418685$). Population-based evaluations of Brazilian and worldwide datasets for this particular kit have already reported appreciable differences in allele frequencies, which were robust enough to discern distinct continental origins [18,20]. Notwithstanding this, the bulk of observed genetic variation was still suggested to pertain to individual levels as expected in human identification forensic applications. Therefore, the results presented here are in agreement with such observations and support current BFP doctrine of a single national allele frequency database for use in forensic casework genetic analysis [32–34]. Even though, at the present time, statistical applications of a genetic national database have been limited to STR markers or (in rare cases) to uniparental lineages, it seems reasonable to propose that the same approach can be valid for SNP markers included in the HID Ion Ampliseq Panel. Applications of computational methods have been allocated to validate the use of a single national STR frequency database for the entire Brazilian territory, with evaluation of adjustment methods for population structure and sample size [40]. However, the proposed methods rely on extensive data availability, with specific information regarding allele frequency distribution regarding local, state, geopolitical regional or national levels. Present SNP marker frequencies should be further explored and spatially refined, including a necessary step of database expansion in both sample number and coverage area. This step is essential to appraise if proposed SNP markers for identification purposes can also be roughly considered as symmetrically distributed along Brazilian territory, and therefore suitable for unified database adoption. Further discussion on the adequacy and convenience of developing a Brazilian national SNP database has been presented in previous studies [20].

3.2. Evaluation of the nature of biological evidence's impact on quality metrics

A series of well-established statistical analyses were executed on six different quality metrics obtained through genotyping assays conducted with the Ion HID Ampliseq Identity Panel, and results for both Median and KW tests comprising all groups, as well as the *post-hoc* pairwise comparisons of all combined types of evidence are found in Table 2. Chosen metrics were selected among other possible criteria because they can be directly evaluated through analysis plugin results and provide an easily identifiable assessment of both sample and genotype quality for each genotyped polymorphism [11,12,14]. Plugin design includes quality thresholds, where sample or specific marker results not reaching previously defined values are flagged as low confidence data. In these cases, a thorough appraisal of obtained results and validation in a forensic context is necessary, including manual review of automatically generated findings for consistency verification [20,21].

Initial result evaluation of genotyping data of real criminal casework samples revealed differences in genotyping performance among samples based on their quality metrics as presented by the plugin. This result was somehow unexpected considering all samples selected for inclusion in the present research were specifically chosen to provide normalized DNA features presented by samples as revealed by their STR fragment analysis and quantitation results. Hence, all library preparation reactions included the same amount of non-degraded, non-inhibited, single-source human DNA, which was classified as such by previous examination of STR profiles generated with CE techniques, as well as quantitation procedures, which have also been

Table 2
Obtained p-values for statistical tests used in quality metrics comparison among groups of criminal evidences. Significant values in bold. Significance level was 0.05, with Bonferroni correction applied to post-hoc tests.

Metrics	LSB		HB		LB		NL		RD		QS	
Test	KW	Median	KW	Median	KW	Median	KW	Median	KW	Median	KW	Median
p-value	**0.030**	**0.032**	**0.001**	**0.004**	0.102	0.147	**0.009**	**0.016**	**0.000**	**0.000**	**0.003**	**0.005**
Pairwise Comparison	Post-Hoc p-value											
CT – SA	0.732	0.371	0.203	0.371	–	–	0.034	0.371	0.009	0.007	0.119	0.074
CT – SE	0.444	0.092	0.134	0.201	–	–	0.144	0.092	0.618	0.772	0.104	0.024
CT – EP	0.015	0.074	**0.004**	0.007	–	–	**0.000**	**0.000**	**0.000**	**0.000**	0.028	0.074
CT – BL	0.014	0.074	0.203	0.074	–	–	0.260	0.371	**0.001**	0.007	0.198	0.371
SA – SE	0.649	0.486	0.008	0.024	–	–	0.643	0.772	0.062	0.008	0.831	0.772
SA – EP	0.037	0.007	0.103	0.371	–	–	0.161	0.371	0.075	0.007	0.525	0.074
SA – BL	0.034	0.007	0.819	0.371	–	–	0.320	0.371	0.557	0.074	**0.004**	0.007
SE – EP	0.150	0.201	**0.000**	**0.001**	–	–	0.083	**0.001**	**0.000**	**0.000**	0.716	0.486
SE – BL	0.142	0.024	0.014	0.024	–	–	0.660	0.772	0.016	0.008	0.005	0.024
EP – BL	0.974	0.024	0.063	0.074	–	–	0.017	0.007	0.234	1.000	**0.000**	0.007

proposed as a reliable predictor of resultant STR profile quality [41]. As the main differences presented by samples refer just to the nature of biological evidence, as well as substrates or surfaces where such material was collected in crime scenes, obtained quality metric results were grouped according to the biological nature of evidence collected in crime scenes, and statistical tools were applied to evaluate these disparities. The results for all six quality metrics can be compared in Fig. 2, which features box plot graphics of each evidence group included in present study. Supplementary Tables S3-S8 contain data for all six appraised quality metrics, including all individual values for each SNP marker, obtained from all evaluated samples. Information regarding complete run performance for the chip was obtained from the results summary presented by Ion Torrent Suite and is reported in Supplementary Table S9.

3.2.1. Locus balance (LB) metrics

LB metrics results for each group are presented in Fig. 2A. Statistical analysis results show that no significant differences were observed among samples with a distinct biological nature for both median and distribution evaluations, indicating there is evidence that the present SNP panel performs similarly throughout a complete sample set in terms of this metric. Previous studies analyzing equivalent metrics in different biological tissues [11,12,14,20] have confirmed differences in amplicon efficiency for different SNP markers included with this reagent are related to panel design owing to primer construction features where sequence-related effects are important for amplification efficiency or pH-based nucleotide addition detection used for base calling and quality determination. Therefore, achievement of more balanced results would depend on changes in panel design itself as the observed imbalance among amplicons is an intrinsic feature of the present commercial solution. However, as this efficiency disequilibrium can be markedly significant to a specific number of SNP markers, it is important that manual inspection and results review are conducted by forensic analysts to verify data reliability and adequacy for inclusion in forensic sample analysis on a case-by-case basis as suggested in previous studies [12–14,20]. Alternatively, it has been suggested that low-quality markers should be excluded from results when analysis included criminal samples [12]. Removal of a few poorly performing SNPs should have limited impact in overall genetic weight of evidence as the

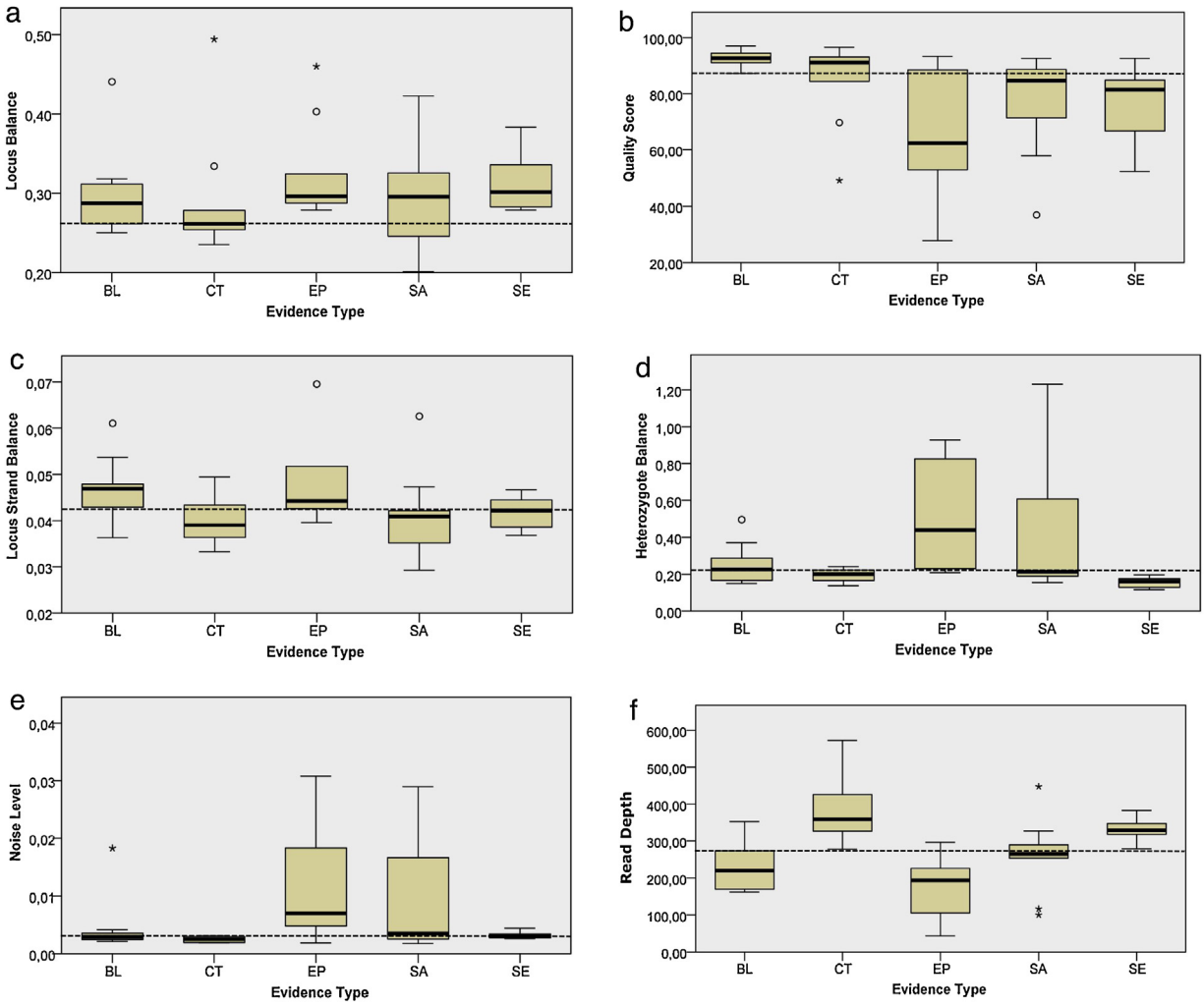

Fig. 2. Box plot distribution of six quality metrics for all tested evidence groups, with black line indicating median value and box representing 1st and 3rd quartiles. Upper and lower bars represent maximum and minimum values, respectively. Dotted line indicates gran median (GM), equivalent to the median value for complete sample set (all groups combined). Sample number is equal to 10 for all groups, except semen, where n = 7. Outlier results are indicated as stars (far out extreme values, differing from median at least 3x the IQR (inter-quartile range) value) or circles (out values, difference from median in the 1.5x to 3x IQR range). Outliers values are not included in any performed statistical hypothesis test. LB, LSB and HB values are plotted as the absolute residues for each value, as previously described, and as such indicates a target value of zero in Figs. 2A, 2C and 2D. BL: blood; CT: oral swabs (reference control); EP: epithelial; SA: saliva; SE: semen. 2A: locus balance, GM = 0.287; 2B: quality score, GM = 87.258; 2C: locus strand balance, GM = 0.043; 2D: heterozygous balance, GM = 0.217; 2E: noise Level, GM = 0.003; 2F: read depth, GM = 273.339.

elevated number of evaluated polymorphisms would still assure LR values above those obtained through traditional STR-CE techniques.

3.2.2. Quality score (QS) metrics

Concerning QS, analysis of the presented results (as in Fig. 2B) with respect to blood group determined it as the most consistent type of criminal evidence with a higher median and low variance. Owing to this exquisite behavior, the BL group yields significant distribution differences with both EP and SA samples, which displayed markedly lower performance in sequence quality. EP distribution shows several samples with overall lower QS, while SA and SE feature plainly bad sequencing performance for the obtained 50% lower values, located between the median and observed minimum. In contrast, the median among sample materials does not show a significant difference even though the obtained *p-values* approach significance levels in terms of pairwise comparisons for BL-EP and BL-SA evaluations (*p-value* for both comparisons was 0.07). Therefore, QS differs among sample groups mainly on with regard to weakly performing samples as low-quality events are markedly skewed in the first quartile and minimum values in SA and EP samples. No evidence of trends regarding individual sample features, as the surface where evidence was deposited, was found to be associated with QS metrics. Additionally, variations in library preparation or workbench manipulation do not seem to explain the findings as all libraries had high quality (as assessed by quantitation) and were diluted to the same concentration in the pool. As notable in Fig. 2B, poorly performing samples belonging to the SE group do not seem to significantly affect QS metrics for this tissue as visually identified differences were not supported by statistical hypothesis rejection.

QS metrics were found to have significantly different median values and distribution among all analyzed groups. Under this assumption, it is important to highlight that median values for all groups, except epithelial, were located above the 80 phred-score threshold. This has singular relevance as it suggests that, despite fluctuations in some of evaluated quality metrics presented by certain sample groups, overall confidence in base calling is still very significant with an estimated median accuracy equivalent to an error rate equivalent to a little less than one in a billion for called genotypes in all groups (gran median = 87.2 phred). Despite the fact particular specific markers consistently showed low QS, overall confidence in base calling for forensic samples is quite strong and seems to be appropriate for forensic applications. This suggestion is supported by the finding that roughly 68% of the total sample set presented median QS values equivalent to 99 when base calls for all markers are considered. The blood group displayed particularly robust results with a median value for the QS metric equivalent to the upper limit in all 10 analyzed samples. Thus, inter-sample evidenced variation in QS metrics was not shown to be associated with the nature or origin of biological evidence as all sample groups presented overall similar results for genotype reliability. Furthermore, no particular type of biological evidence was found to be more error-prone than others. However, outlier results were identified (with the EP.PF17 epithelial sample as the most extreme example with a median phred-score of 21, meaning a genotype base calling accuracy slightly above 99% for at least half of SNP marker set). Results suggest that base-calling efficiency seemed to vary among individual forensic samples. This finding emphasizes the need for execution of meticulous manual inspection of automatically generated genotypes by plugin. This step is recommended in order to assure low-quality results are not taken as adequate for genotype determination, and resulting genetic profiles are not effectively employed in real criminal casework.

3.2.3. Locus strand balance (LSB), heterozygote balance (HB) and noise level (NL) metrics

Besides LB index, all other metrics evaluated in the present study were found to have statistically significant differences among groups of biological evidence types. These variations were found in either median comparison or KW distribution tests, or in some cases, for both statistical tests, just as shown in Table 2. As for such metrics, LSB indicates that there is significant difference among groups of samples for both KW and median evaluations (see Fig. 2C). Therefore, at least one pairwise comparison was expected to display differences among tested groups. However, *post-hoc* analysis could not identify where proposed discrepancies lied. Without Bonferroni correction application, four significant differences would be highlighted. Owing to the conservative approach employed in this study, such differences were not considered significant, and therefore we conclude that, even though some level of association between LSB metrics and sample nature can be implied from the results, it is not clear how different groups contribute individually to this phenomenon.

Regarding the HB metrics, KW *post-hoc* analysis indicates that CT and SE yield significantly better results than EP. In the analysis featured in Fig. 2D, it can be seen that the epithelial group's first quartile approaches maximum value for the CT and SE groups. Median testing indicates that differences among CT and EP relies on the intragroup variability observed in EP samples, rather than on median values, while the discrepancy in the SE and EP results was strong enough to be detected by both statistical methods employed. Such results were considered sufficient to indicate EP samples presenting lower performance than all other kinds of evidence. Upon NL metrics analysis, again, we observed CT and SE performing better than EP, just as shown in Fig. 2E. Based on an employed conservative approach of Bonferroni correction, *post-hoc* analysis indicated a significant difference between SE and EP only on the median, mainly as a result of higher amplitude and maximum value of the SE distribution. On the other hand, CT-EP pairwise comparison indicates a significant difference for both median and distribution tests.

A simple examination of the results displayed in the box plot depicted in Figs. 2A-F shows that semen and control oral swab groups, followed by blood samples, usually had better quality median values and more balanced distributions for most of the measures included in analysis, with intragroup variability typically lesser than observed with other types of evidence (with the exception of QS distribution for semen samples, which displayed somewhat unexpected behavior). On the other hand, epithelial samples had lower performance for the same measures and, to a lesser extent, the same conclusion could be extended to the saliva group. Uniformity in the results presented by semen and control groups might be explained by low variation in overall conditions of such samples because of relatively low exposure to elements or environmental conditions, surfaces or substrates of deposit heterogeneity and low mixture probability. As semen sample availability in BFP laboratories is limited, all samples included belonged to cases where ejaculates were obtained from the interior of condoms, and therefore less subject to contamination or influence of idiosyncrasies with regard to substrate where these samples were collected. Result consistency obtained for both kinds of evidence indicate that such types of samples may be preferable for MPS-generated genetic profile analysis, when different tissues or biological fluids can be used as criminal DNA sources from a single individual. This fact may impact particularly known-source reference sample collection as results indicate that oral swabs provide more stable results. As such, there could be some advantages in choosing oral swabs as the choice method for reference material collection from suspects, victims or individuals involved in kinship tests. However, robust performance and low

variability displayed by the BL group in sequencing quality suggest that this biological material might also be appropriate for reference sampling. Previous studies employing this same SNP panel [42] reported that direct amplification of oral swabs preserved in FTA paper is possible, with results equivalent to extracted DNA from the same material. Authors also describe variations in RD, LB and HB between extracted or directly amplified DNA with a significant degree of intra-group variation. Such variations are linked with varying amounts of DNA in FTA punches, as well as a less precise fluorescence-based quantitation method for extracted DNA, and as such do not coincide with overall quality metric stability featured by control reference samples herein described. Relevant aspects regarding casework processing in forensic laboratories, such as per sample cost, throughput capacity and possibility of analytical process automation, are also affected by such identified features.

3.2.4. Read depth (RD) metrics

Read depth levels per samples, evaluated by RD metrics, is one of the evaluated indices that seems to be considerably influenced by the biological nature of evidence, as presented in Fig. 2F. Both median and distribution of samples vary among groups. Pairwise comparison among sample types demonstrates that, even though BL and SA also presented overall lower values than SE/CT, such differences are not statistically significant. However, RD analysis highlights again the difference between SE and CT with EP for both distribution and median tests - epithelial sample performance is markedly different than both high-quality groups. Without Bonferroni correction, several other post-hoc comparisons would indicate significant median difference, but this effect is not observed in distribution. This can be explained by high RD variance. Kruskal-Wallis testing shows that BL read depth distributions among samples also differed significantly from the CT group. Curiously enough, significant differences were also indicated between CT and BL results distribution. While presenting lower RD values, statistical evaluation elicited no difference in blood sample median from other groups, probably as a consequence of the conservative approach employed. Lower performance in depth metrics for blood samples have been previously reported for the present panel also in the form of reduced number of reads and allele imbalance [21] when compared to control 007 DNA. However, the same authors propose that such variation may be caused by inhibition during panel PCR steps or because of the presence of non-human DNA within the sample. Although hemoglobin and other *heme* compounds present in blood samples have been widely described as PCR inhibitors [43], such effects cannot fully explain these types of observations as analytical protocols include library quantitation and mixing in equimolar concentrations in a pool, where they are posteriorly co-amplified during the emulsion PCR phase. Inhibitions in primary PCR would be therefore attenuated by posterior PCR steps for library pool amplification, in which equal amounts of library for each sample is added. Moreover, if inhibitors present in blood samples are not removed during DNA extraction, we would expect them to impact not only blood samples during library preparation, but also affect library pool amplification in emulsion PCR steps. However, it is reasonable to expect that inhibition would affect all samples equally as they were all mixed together in the library pool. Non-human DNA presences in a sample, however, is a factor to be considered as a source of PCR bias and might be implicated in observed variations among groups. Nanopore membrane-based direct sequencing of unamplified total DNA extracted from forensic samples showed that latent forensic evidence can have as low as 10% of obtained sequences identified as possessing human origin, while almost half of the obtained sequences were classified as belonging to *Escherichia* genus [44]. In ancient DNA samples (which may present certain features resembling forensic evidence, including environmental or bacterial contamination), strong PCR bias favoring amplification of GC-rich bacterial sequences was reported in detriment to endogenous human DNA [23]. This event seems to be highly dependent on the employed polymerase, with a reduction of endogenous DNA fraction sequences to around half.

3.2.5. Sources of observed variation among groups

Several forms of error and bias introduction in MPS data is a widely known phenomenon, and its impact in forensic applications must be considered owing to the critical nature associated with these criminal evidence forms, which may include low-template copy numbers, high degradation levels or mixture occurrence in variable DNA donor contribution ratios [45]. Sequencing bias has been discussed and thoroughly studied primarily in clinical MPS applications as cancer and other pathology investigations (where only relatively small numbers of cells may be affected by mutational events leading to genetic polymorphisms) might be particularly interested in minor, less frequent genetic variants [46]. Forensic samples, however, still have been less explored as sources of genetic data for error evaluations.

MPS errors in general can be connected with experimental conditions, such as sample characteristics, PCR-introduced bias, analysis protocols or instrument-related technical limitations, but also to computational interpretation of generated data [46]. Variations in adopted post-sequencing pipelines include quality control of raw sequence reads, reference genome or sequence alignment, mutations or polymorphism detection (in variant-calling or genotyping steps), as well quality control procedures following each of the previous stages [27]. Sources of error in MPS applications as a result of PCR artifacts are well-known, and include phenomena like stochasticity, several forms of PCR bias (including amplicon length and CG content), polymerase errors and template switches [24]. Considering the employed sample set and its varying preservation or collection conditions, we considered the hypothesis that groups of samples might differ in amounts of non-human DNA present in extracts after evidence processing, which is expected to be highly variable among samples and not directly assessed by utilized RT-PCR quantitation methods. For such evaluation, the number of unaligned sequences were determined for each sample and differences among groups was evaluated. No statistical variation was observed for evidence groups in a single factor ANOVA test ($p\text{-}value = 0.459218$) with average values of on-target reads ranging from 91.84% for the semen group to 88.77% in epithelial samples (data not shown). Although a more detailed exploration of data regarding unaligned sequences associated with forensic samples is currently underway, inter- and intra-group overall homogeneity in the number of off-target reads with forensic samples suggests that bacterial contamination present in real casework forensic samples does not seem to be the main reason responsible for observed variation in quality metrics included in this study. It has been proposed in the literature that simultaneous analysis of reference and questioned criminal samples in a single run is not recommended in certain MPS applications as competition effects among specimens presenting varying DNA quality might favor sequencing of high-quality targets [47,48]. In forensic applications, this effect can have a significant impact on analysis owing to preferential amplification of reference samples in detriment to questioned criminal DNA, leading to overall lesser coverage (represented as average read counts) for the latter group. The current results seem to corroborate this hypothesis with higher quality biological evidence (represented by semen and reference oral swab groups) yielding better performance in sequencing quality metrics than other kinds of samples. Even though most of the previously reported impact is related to variations in read-depth values, other

metrics might also be concurrently affected by sample quality. Despite this, the executed analysis indicates genetic profiles can be simultaneously obtained for a large number of different samples (in this experiment, 77 distinct DNA extracts were included in a single run) through barcoding techniques with sufficient quality to support this method's use in real casework forensic applications. However, sequencing efficiency is still to be determined for critical real DNA evidence as highly degraded or low DNA template samples in order to fully assess potential and capability of the present panel in generating reliable, trustworthy genetic profiles to be employed in criminal justice procedures. If necessary, protocols can be adapted for low-quality criminal samples and smaller sample numbers, aiming for superior sequencing depth per genotyped marker.

Regarding LSB, HB and NL metrics, the performed analyses indicated there to be statistically significant differences among groups, for all three metrics, for both median and distribution comparisons. Curiously, LSB metrics' *p-values* indicated the null hypothesis was rejected for the test as a whole (including all groups), while pairwise evaluation of specific pairs of sample groups revealed no significant difference between all possible group combinations. For the remaining metrics, epithelial samples were found to have the most divergent results, with significance always associated with pairwise testing against semen and/or control groups. Even though no statistical significance was established, the saliva and, to a lesser extent, blood groups also displayed more erratic behavior in quality metrics measures than semen and control (oral swab) groups. This suggests an effect associated with environmental contamination as samples belonging to referred groups were collected in less stable or controlled conditions. It is not clear if this highly variable pattern presented by some quality metrics is caused by factors associated with conditions in which criminal biological evidence was collected (including but not limited to exposure to elements as UV radiation or high humidity, presence of PCR inhibitors, human or other species contaminating DNA presence, elements associated with surface or substrates where evidence was deposited, and others). Nonetheless, the observed results suggest these factors cannot be clearly identified or inferred through traditional forensic DNA analytical tools as all sample conditions were normalized according to RT-PCR quantitation and STR-CE fragment analysis results. Certain samples presented small-sized peaks in terms of STR genetic profiles, which could be associated with minor contributors in a mixture (described in Table 1). However, this pattern was found across almost all sample type groups, and manual inspection of the results suggested that the presence of minor mixture contributors in forensic samples does not present a marked impact in any of the investigated metrics, especially in those where low-level mixture impact is expected to have particular relevance (like NL and HB metrics). In addition, no association was found between the described results and the substrate or surface where biological evidence composing a sample set was collected, or even the collection method, although the evaluated sample number was not sufficient to provide an adequate number of observations for each evaluated deposition spot owing to the large variety of places or objects where evidence was located in respective crime scenes. Considering that these factors are highly diverse among real casework samples evaluated in forensic genetic laboratories, their features are associated with specific crime scenes and processing, and therefore cannot be controlled or managed by DNA laboratory staff. Notwithstanding, as the described elements might have significant repercussions in terms of quality aspects of DNA genotyping, their influence must be taken into consideration by forensic DNA analysts in results interpretation and profile generation.

A different approach to explain the differences observed among groups in quality metrics results might be related not only to variable environmental conditions or structural integrity of DNA obtained from such samples, but the very biological nature of human tissues and fluids from where this evidence originates and its intrinsic features. Thus, epithelial samples displayed a persistently inconsistent and erratic behavior with regards to quality metrics and were characterized as the only evidence type to differ with statistical significance from other sample groups (mainly to semen and/or reference oral swabs) for more than a single metric, both for median and distribution values. A recent review on trace DNA deposits [49] discusses factors influencing DNA recovery from "touch DNA" evidence and cellular composition of epithelial deposits. While circumstances surrounding the amounts of trace DNA (where shedders classification is discussed), transfer and persistence of genetic material, timing and place of deposition are considered, and such factors could have a limited impact on samples used in the present study as included cases comprise only DNA evidence where traditional methods (RT-PCR quantitation and STR-CE fragment analysis) were employed with success. As an example, considering touch DNA is widely reported as degraded [49], this effect was minimized in our sample set by selecting only evidence where degradation levels were undetectable. A possible explanation for the observed fluctuation might refer to the fraction of DNA present in touch deposits not related to cellular components deposited by contact, but to cell-free DNA (cfDNA) existent in epithelial evidence [50]. While the presence of cfDNA was reported for all different types of human tissues or fluids included in the present study (and therefore would be a component present in all evaluated samples) [51], its relevance may be more pronounced in epithelial deposits, whereas cfDNA can account for most recoverable DNA in manipulated objects, ranging from 84 to 100% of total DNA [52]. cfDNA presence in human fluids is believed to be mostly derived from hematopoietic apoptotic cells, with variable quantities according to tissue and deep sequencing of these DNA fractions featuring fragments subject to severe DNA damage and containing variant levels of sequence bias, introduced during programmed cell-death processes, leading to necessity of protocol modification for enhanced library preparations [53,54]. As most studies focus on degradation levels and fragment size of cfDNA components [49], it is not clear if this fraction of total epithelial DNA components would be more subject to the effects impacting PCR or sequencing quality. However, if such effects associated with DNA damage are potentially dependent on cfDNA fractions derived from criminal evidence, it is expected that large-sized amplicons would present lower amplification efficiency because of highly damaged patterns presented by this fraction. To test this hypothesis, a Kruskal-Wallis test was executed for all five sample groups with MRL additional metrics, or mean read length of sequenced amplicons (presented in Supplementary Table S10). It is important to notice that MRL metrics, as presented by the plugin, are provided as a unique number for the whole sample. Considering panel design prioritizes smaller amplicons, the effect of larger amplicon degradation can be harder to identify as smaller amplicons are not as affected by degradation as larger ones. Therefore, differences in samples for this metric are expected to be restricted to just a few base pairs. Yielded results confirm that distribution of amplicon size is not the same among all groups ($p\text{-}value = 0.0051$), and pairwise analysis showed that, after Bonferroni correction, significant differences are observed between epithelial-control ($p\text{-}value = 0.0037$) and saliva-control ($p\text{-}value = 0.0005$) groups. ANOVA testing also indicates differences in variance among groups ($p\text{-}value = 0.0483$). It is expected that control groups have higher overall values as collection processes (internal oral mucosa swabbing) promotes enrichment of cellular fractions in biological material used for DNA obtention through mechanical abrasion of oral mucosa. The described results are in accordance with the hypothesis of contribution of damaged cfDNA fraction of forensic evidence to the observed lowering of quality

metrics identified in specific groups tested. In addition to this, there is also a possibility that DNA quality in forensic evidence is being affected by external factors acting on DNA evidence only subsequent to its deposition. As such, additional environmental effects might increase DNA damage already present in cfDNA fractions or impacting overall conditions of genetic material present in evidence. It is possible that cfDNA fractions are more susceptible to diverse types of chemical alterations of nucleotide bases as a result of tautomeric isomerism (which are highly dependent on surrounding pH) [55], temperature, UV radiation or oxidative agent-mediated covalent bonds breaking/formation and other forms of structural changes to DNA, which seem plausible to a more exposed, cell-free fraction of total DNA found in criminal evidence. Despite being speculative, this hypothesis' verification would require additional experiments under controlled conditions in order to determine the molecular and/or chemical mechanisms involved in such DNA modifications, and how they would impact posterior steps in DNA genotyping of forensic samples.

Finally, we considered the possibility that variation observed in quality metrics for some sample types, which was markedly evident in epithelial criminal evidence, is a result of endogenous factors or particular features presented by this precise human tissue. As an example of such factors, accumulation of post-zygotic mutations in cells belonging to normal human tissues is a factor to be considered, especially in highly proliferative tissues, such as skin [56]. Somatic mosaicism manifests as single nucleotide variations (SNVs), epigenetic alterations, copy number variations (CNVs), different forms of *in-del* polymorphisms or even chromosomal variations that can be found widespread in human tissues and compatible with normal development and ageing [57]. Healthy tissue evaluation by deep-coverage sequencing revealed an average of 1035 benign SNVs found for each human fibroblast cell [56]. Extensive intra-individual variation has been described among different types of tissues, and experiments in skin cells suggest high levels of somatic mosaicism with at least 30% of cells presenting CNV polymorphisms not originally observed in early developmental phases [58,59]. Conceptually, it seems reasonable to assume somatic mosaicism would have at least some kind of impact on quality metrics, with measures such as NL and HB being particularly sensitive to these mutations' presence. However, considering the nature of groups included in the present study, one would expect the control group (constituted by oral mucosa swabs) also display the same susceptibility to such somatic variations taking into account the similar features presented by this kind of tissue and human skin. Nucleotide epigenetic differences between both groups of samples could explain this phenomenon as an alternative form of somatic natural variation as both tissues are subjected to discrepant environmental exposure to diverse external factors that can differentially affect distinct tissues in a single individual, with elements as aging or sun exposure already posited to alter methylation profiles of human skin cells [60,61]. Such an explanation is, however, very unlikely as it is not clear which molecular mechanisms might be involved in such an outcome considering no evidence of repercussions on PCR quality of efficiency based on methylated nucleotide presence in PCR templates is available. Therefore, lower performance in certain quality metrics presented by epithelial and, to a lesser extent, saliva and blood criminal casework are more likely to be associated with factors like environmental contaminants, as well as background DNA presence (as an element of relevance in DNA transfer studies) [62] than to variant features presented by distinct human tissues.

4. Conclusion

The results obtained in this study indicate there is enough evidence to propose the biological nature of criminal samples as a source of variation in certain MPS quality metrics considering the technical solutions employed in an analytical workflow. Such variations seem to be difficult to foresee as they are not directly apparently by analysis of criminal sample features using traditional profiling techniques. Epithelial samples were found to present relatively higher variation for certain parameters and overall lower sequencing performance than other types of criminal evidence. On the other hand, samples like oral swabs, semen and even blood presented overall robust sequencing quality and uniformity among samples. Some level of intragroup discrepancies was also detected, suggesting individual features presented by distinct samples might have a certain level of impact on sequencing quality metrics. A number of possible reasons for such variations were presented, albeit no conclusive explanations were proposed for intra- or inter-group result discrepancies. While different types of evidence were found to differ in their ability to generate high-quality genetic profiles, it seems plausible to propose group disparities in metrics do not prevent this technology's use in forensic cases, but rather draws attention to the need for human intervention in results interpretation and validation through a careful, meticulous expert review of reported results and considering variability. This particular follow-up step may be facilitated by technical modifications, improvement in analysis protocols or appropriate data processing, all seeking to minimize need for human intervention, yet such seems necessary under current technical conditions.

The present work also supports the previously proposed [20] nationwide use of a single allelic frequencies database for the whole Brazilian population through genetic weight-of-evidence evaluation in a forensic context. Once again, no evidence of a significant stratification for the Brazilian population was found taking into account the human identification-oriented SNP markers included with this reagent and its associated regional distribution frequencies. This observation is in agreement with current practices adopted by BFP and other Brazilian regional law enforcement agencies for data regarding STR frequency distributions [63]. Conservative approaches are employed for all statistical calculations, including corrections for substructures within populations [64], and it is highly recommended that such concepts are also adopted in SNP-based genotyping procedures.

It is possible that the distinct behavior of different types of criminal evidence for evaluated quality metrics may merely consist of mathematical artifacts, specifically resulting from nature or characteristics presented by the studied variables and employed calculation methods. However, the strict, conservative approach applied during statistical analysis supports the conclusion that at least some degree of asymmetry in quality metrics among groups is associated with the nature of human tissue where evidence originates. The present analysis was proposed as an exploratory, preliminary survey of this dataset, and additional investigation is necessary in order to ascertain proposed diversity in tested indices among groups. Thus, further scrutiny in real forensic casework is necessary, including evaluation of DNA transfer effects, impact of the substrate or deposition surface for each sample and other particular aspects of individual samples' nature. In order to achieve this objective, a significant increase in sample number is necessary with the objective of encompassing as much individual sample variability as possible. A proposed increased in sample number can reduce the influence of external factors and mathematical, spurious or stochastic effects, leading to a reliable, comprehensive appraisal of the nature of biological evidence's meaning in MPS genotyping of real criminal casework samples.

Declaration of Competing Interest

Authors declare they have no conflict of interest.

Financial support

Present work was funded by grants provided by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Edital 25/2014 – Pró-Forenses), CNPq (Conselho Nacional de Pesquisa Científica – Chamada n° 16/2014 – INCT) and FAPERGS (Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul).

CRediT authorship contribution statement

E. Avila: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Visualization, Project administration. **C.P. Cavalheiro:** Validation, Formal analysis, Investigation, Writing - review & editing. **A.B. Felkl:** Validation, Formal analysis, Investigation, Writing - review & editing. **P. Graebin:** Investigation, Resources, Data curation. **A. Kahmann:** Formal analysis, Resources. **C.S. Alho:** Conceptualization, Resources, Data curation, Writing - review & editing, Project administration, Supervision, Funding acquisition.

Acknowledgments

Authors would like to thank collaborators DB Silva, F Sawitski, CP Nunes and CEI Gonçalves for assistance with sample processing and data acquisition; G Chemale, C Lindholz and A Gastaldo for manuscript review and suggestions.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.forsciint.2019.109938.

References

[1] J. Butler, The future of forensic DNA analysis, Philos. Trans. B 370 (2015) 20140252, doi:http://dx.doi.org/10.1098/rstb.2014.0252.
[2] R.A. Wickenheiser, Trace DNA: a review, discussion and theory, and applications of the transfer of trace quantities of DNA through skin contact, J. Forensic Sci. 47 (3) (2002) 442–450, doi:http://dx.doi.org/10.1098/rstb.2014.0252.
[3] C. Martins, G. Lima, M.R. Carvalho, L. Cainé, M.J. Porto, DNA quantification by real-time PCR in different forensic samples, Forensic Sci. Int. Genet. Suppl. Ser. 5 (2015) e545–e546, doi:http://dx.doi.org/10.1016/j.fsigss.2015.09.215.
[4] E. Murphy, Forensic DNA typing, Annu. Rev. Criminol. 1 (2018) 497–515, doi:http://dx.doi.org/10.1146/annurev-criminol-032317-092127.
[5] S.K. Alketbi, The affecting factors of touch DNA, J. Forensic Res. 9 (3) (2018) 1000424, doi:http://dx.doi.org/10.4172/2157-7145.1000424.
[6] L. Roewer, DNA fingerprinting in forensics: past, present, future, Investig. Genet. 4 (1) (2013) 22, doi:http://dx.doi.org/10.1186/2041-2223-4-22.
[7] National Institute of Justice, The Future of Forensic DNA Testing: Predictions of the Research and Development Working Group, U.S Department of Justice, Office of Justice Programs, Washington DC, 2000 90 pp.
[8] K.K. Kidd, A.J. Pakstis, W.C. Speed, E.L. Grigorenko, S.L. Kajuna, N.J. Karoma, S. Kungulilo, J.J. Kim, R.B. Lu, A. Odunsi, F. Okonofua, J. Parnas, L.O. Schulz, O.V. Zhukova, J.R. Kidd, Developing a SNP panel for forensic identification of individuals, Forensic Sci. Int. 164 (1) (2006) 20–32, doi:http://dx.doi.org/10.1016/j.forsciint.2005.11.017.
[9] J.J. Sanchez, C. Phillips, C. Børsting, K. Balogh, M. Bogus, M. Fondevila, C.D. Harrison, E. Musgrave-Brown, A. Salas, D. Syndercombe-Court, P.M. Schneider, A. Carracedo, N. Morling, A multiplex assay with 52 single nucleotide polymorphisms for human identification, Electrophoresis. 27 (9) (2006) 1713–1724, doi:http://dx.doi.org/10.1002/elps.200500671.
[10] K.M. Canturk, R. Emre, K. Kınoglu, B. Başpınar, F. Sahin, M. Ozen, Current status of the use of Single-Nucleotide Polymorphisms in forensic practices, Genet. Test. Mol. Biomarkers (2014), doi:http://dx.doi.org/10.1089/gtmb.2013.0466.
[11] C. Børsting, S.L. Fordyce, J. Olofsson, H.S. Mogensen, N. Morling, Evaluation of the Ion Torrent™ HID SNP 169-plex: a SNP typing assay developed for human identification by second generation sequencing, Forensic Sci. Int. Genet. (2014), doi:http://dx.doi.org/10.1016/j.fsigen.2015.07.011.
[12] M. Eduardoff, C. Santos, M. de la Puente, T.E. Gross, M. Fondevila, C. Strobl, B. Sobrino, D. Ballard, P.M. Schneider, A. Carracedo, M.V. Lareu, W. Parson, C. Phillips, Inter-laboratory evaluation of SNP-based forensic identification by massively parallel sequencing using the Ion PGM, Forensic Sci. Int. Genet. 12 (2014) 144–154, doi:http://dx.doi.org/10.1016/j.fsigen.2015.04.007.
[13] A. Buchard, M.L. Kampmann, L. Poulsen, C. Børsting, N. Morling, ISO 17025 validation of a next-generation sequencing assay for relationship testing, Electrophoresis. 37 (21) (2016) 2822–2831, doi:http://dx.doi.org/10.1002/elps.201600269.
[14] K.A. Meiklejohn, J.M. Robertson, Evaluation of the precision ID identity panel for the ion torrent™ PGM™ sequencer, Forensic Sci. Int. Genet. 31 (48) (2017) 56, doi:http://dx.doi.org/10.1016/j.fsigen.2017.08.009.
[15] F. Guo, Y. Zhou, H. Song, J. Zhao, H. Shen, B. Zhao, F. Liu, X. Jiang, Next generation sequencing of SNPs using the HID-Ion AmpliSeq™ identity panel on the ion torrent PGM™ platform, Forensic Sci. Int. Genet. 25 (2016) 73–84, doi:http://dx.doi.org/10.1016/j.fsigen.2016.07.021.
[16] O. García, A. Soto, I. Yurrebaso, Allele frequencies and other forensic parameters of the HID-Ion AmpliSeq™ Identity Panel markers in Basques using the Ion Torrent PGM™ platform, Forensic Sci. Int. Genet. 28 (2017) e8–e10, doi:http://dx.doi.org/10.1016/j.fsigen.2017.03.010.
[17] R. Li, C. Zhang, H. Li, R. Wu, H. Li, Z. Tang, C. Zhen, J. Ge, D. Peng, Y. Wang, H. Chen, H. Sun, SNP typing using the HID-Ion AmpliSeq™ Identity Panel in a southern Chinese population, Int. J. Legal Med. 132 (4) (2018) 997–1006, doi:http://dx.doi.org/10.1007/s00414-017-1706-3.
[18] J. Liu, Z. Wang, G. He, X. Zhao, M. Wang, T. Luo, C. Li, Y. Hou, Massively parallel sequencing of 124 SNPs included in the precision ID identity panel in three East Asian minority ethnicities, Forensic Sci. Int. Genet. 35 (2018) 141–148, doi:http://dx.doi.org/10.1016/j.fsigen.2018.05.002.
[19] S. van der Heijden, S.J. Oliveira, M.L. Kampmann, C. Børsting, N. Morling, Comparison of manual and automated AmpliSeq™ workflows in the typing of a Somali population with the Precision ID Identity Panel, Forensic Sci. Int. Genet. 31 (2017) 118–125, doi:http://dx.doi.org/10.1016/j.fsigen.2017.09.009.
[20] E. Avila, A.B. Felkl, P. Graebin, C.P. Nunes, C.S. Alho, Forensic characterization of Brazilian regional populations through massive parallel sequencing of 124 SNPs included in HID ion Ampliseq Identity Panel, Forensic Sci. Int. Genet. 40 (2019) 74–84, doi:http://dx.doi.org/10.1016/j.fsigen.2019.02.12.
[21] E. Salata, A. Agostino, I. Ciuna, S. Wooton, L. Ripani, A. Berti, Revealing the challenges of low template DNA analysis with the prototype Ion AmpliSeq™ Identity panel v2.3 on the PGM™ Sequencer, Forensic Sci. Int. Genet. 22 (2016) 25–36, doi:http://dx.doi.org/10.1016/j.fsigen.2015.07.011.
[22] X. Zeng, K. Elwick, C. Mayes, M. Takahashi, J.L. King, D. Gangitano, B. Budowle, S. Hughes-Stamm, Assessment of impact of DNA extraction methods on analysis of human remain samples on massively parallel sequencing success, Int. J. Legal Med. 133 (2019) 51–58, doi:http://dx.doi.org/10.1007/s00414-018-1955-9.
[23] J. Dabney, M. Meyer, Length and GC-biases during sequencing library amplification: a comparison of various polymerase-buffer systems with ancient and modern DNA sequencing libraries, BioTechniques. 52 (2012) 87–94, doi:http://dx.doi.org/10.2144/000113809.
[24] J.M. Kebschull, A.M. Zador, Sources of PCR- induced distortions in high-throughput sequencing data sets, Nucleic Acids Res. 43 (21) (2015)e143, doi:http://dx.doi.org/10.1093/nar/gkv717.
[25] D.L. Apaga, S.E. Dennis, J.M. Salvador, G.C. Calacal, M.C.A. De Ungri, Comparison of two massively parallel sequencing platforms using 83 Single Nucleotide Polymorphisms for human identification, Sci. Rep. 7 (2017) 398, doi:http://dx.doi.org/10.1038/s41598-017-00510-3.
[26] A.E. Woerner, A. Ambers, F.R. Wendt, J.L. King, R.S. Moura-Neto, R. Silva, B. Budowle, Evaluation of the precision ID mtDNA whole genome panel on two massively parallel sequencing systems, Forensic Sci. Int. Genet. 36 (2018) 213–224, doi:http://dx.doi.org/10.1016/j.fsigen.2018.07.015.
[27] I. Abnizova, R.T. Boekhorst, Y.L. Orlov, Computational errors and biases in short read Next Generation Sequencing, J. Proteomics Bioinform. 10 (1) (2017) 1–17, doi:http://dx.doi.org/10.4172/jpb.1000420.
[28] WMA, World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, J. Am. Med. Assoc. 310 (20) (2013) 2191–2194, doi:http://dx.doi.org/10.1001/jama.2013.281053.
[29] BRASIL, Ministério de Justiça e Segurança Pública. Comitê Gestor da Rede Integrada de Bancos de Perfis Genéticos. Resolução n° 10, de 28 de fevereiro de 2019. Dispõe sobre a padronização de procedimentos relativos à coleta obrigatória de material biológico para fins de inclusão, armazenamento e manutenção dos perfis genéticos nos bancos de dados que compõem a Rede Integrada de Bancos de Perfis Genéticos, Diário Oficial [da República Federativa do Brasil] 50 (1) (2019) 60.
[30] J.S. Buckleton, J.A. Bright, D. Taylor, Forensic DNA Evidence Interpretation, 2nd edition, CRC Press, Boca Raton, FL, 2016 496 pp.
[31] D. Kling, A.O. Tillmar, T. Egeland, Familias 3-Extensions and new functionality, Forensic Sci. Int. Genet. 13 (2014) 121–127, doi:http://dx.doi.org/10.1016/j.fsigen.2014.07.004.
[32] V.R.C. Aguiar, E.V. Wolfgramm, F.S.V. Malta, A.G. Bosque, A.C. Mafia, V.C.O. Almeida, F.A. Caxito, V.C. Pardini, A.C.S. Ferreira, I.D. Louro, Updated Brazilian STR allele frequency data using over 100,000 individuals: an analysis of CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, Penta d, Penta e, TH01, TPOX and vWA loci, Forensic Sci. Int. Genet. 6 (2011) 504–509, doi:http://dx.doi.org/10.1016/j.fsigen.2011.07.005.
[33] L.A. Poiares, P.S. Osorio, F.A. Spanhol, S.C. Coltre, R. Rodenbusch, L. Gusmão, A. Largura, F. Sandrini, C.M. Silva, Allele frequencies of 15 STRs in a representative sample of the Brazilian population, Forensic Sci. Int. Genet. 4 (2) (2010) e61–63, doi:http://dx.doi.org/10.1016/j.fsigen.2009.05.006.
[34] P.E. Raimann, G. Hirschfeld-Campolongo, S. Schumacher, S.E.M. Almeida, C.M. Silva, R. Rodenbusch, A.S. Bassini, L.F. Souza, E.C. Nascimento, E.S. Oliveira, V.R. Santos, E.C. Oliveira, E.A. Auler-Bittencourt, R.C.R. Hirschfeld-Campolongo, M.

L. Sousa, H.P. Figueiredo, B.B. Figueiredo, R. Silva, R.S. Moura-Neto, Population genetic analyses of the AmpFlSTR® NGM™ in Brazil, Int. J. Legal Med. 126 (2) (2012) 337–341, doi:http://dx.doi.org/10.1007/s00414-011-0606-1.
[35] C. Phillips, M. García-Magariños, A. Salas, A. Carracedo, M.V. Lareu, SNPs as supplements in simple kinship analysis or as core markers in distant pairwise relationship tests: when do SNPs add value or replace well-established and powerful STR tests? Transfus. Med. Hemotherapy 39 (3) (2012) 202–210, doi:http://dx.doi.org/10.1159/000338857.
[36] W. Parson, L. Gusmão, D.R. Hares, J.A. Irwin, A.R. Mayr, N. Morling, E. Pokorak, M. Prinz, A. Salas, P.M. Schneider, T.J. Parsons, DNA commission of the international society for forensic genetics: revised and extended guidelines for mitochondrial DNA typing, Forensic Sci. Int. Genet. 13 (2014) 134–142, doi:http://dx.doi.org/10.1016/j.fsigen.2014.07.010.
[37] M.M. Andersen, D.J. Balding, How convincing is a matching Y-chromosome profile? PLoS Genet. 13 (11) (2017)e1007028, doi:http://dx.doi.org/10.1371/journal.pgen.1007028.
[38] 1000 Genomes Project Consortium, et al., An integrated map of genetic variation from 1092 human genomes, Nature 491 (2012) 56–65, doi:http://dx.doi.org/10.1038/nature11632.
[39] R.R. Moura, A.V. Coelho, V.Q. Balbino, S. Crovella, L.A. Brandão, Meta-analysis of Brazilian genetic admixture and comparison with other Latin America countries, Am. J. Hum. Genet. 27 (5) (2015) 674–680, doi:http://dx.doi.org/10.1002/ajhb.22714.
[40] T. Hessab, R.S. Aranha, R.S. Moura-Neto, D.J. Balding, C.G. Schrago, Evaluating DNA evidence in a genetically complex population, Forensic Sci. Int. Genet. 36 (2018) 141–147, doi:http://dx.doi.org/10.1016/j.fsigen.2018.06.019.
[41] S. Vernarecci, E. Ottaviani, A. Agostino, E. Mei, L. Calandro, P. Montagna, Quantifiler® Trio Kit and forensic samples management: a matter of degradation, Forensic Sci. Int. Genet. 16 (2015) 77–85, doi:http://dx.doi.org/10.1016/j.fsigen.2014.12.005.
[42] M.L. Kampmann, A. Buchard, C. Børsting, N. Morling, High-throughput sequencing of forensic genetic samples using punches of FTA cards with buccal swabs, BioTechniques. 61 (2016) 149–151, doi:http://dx.doi.org/10.2144/000114453.
[43] M. Sidstedt, J. Hedman, E.L. Romsos, L. Waitara, L. Wadsö, C.R. Steffen, P.M. Vallone, P. Rådström, Inhibition mechanisms of hemoglobin, immunoglobulin G, and whole blood in digital and real-time PCR, Anal. Bioanal. Chem. 410 (2018) 2569–2583, doi:http://dx.doi.org/10.1007/s00216-018-0931-z.
[44] J. Sim, B. Chapman, In-field whole genome sequencing using the MinIon nanopore sequencer to detect the presence of high-prized military targets, Aust. J. Forensic Sci. (2019), doi:http://dx.doi.org/10.1080/00450618.2019.1568562 in press.
[45] M.G. Ross, C. Russ, M. Costello, A. Hollinger, N.J. Lennon, R. Hegarty, C. Nusbaum, D.B. Jaffe, Characterizing and measuring bias in sequence data, Genome Biol. 14 (2013)r51 doi: 0.1186/gb-2013-14-5-r51.
[46] C. Xu, A review of somatic single nucleotide variant calling algorithms for next-generation sequencing data, Comput. Struct. Biotechnol. J. 16 (2018) 15–24, doi:http://dx.doi.org/10.1016/j.csbj.2018.01.003.
[47] K.T. Wai, M. Barash, P. Gunn, Performance of the early access AmpliSeq™ Mitochondrial Panel with degraded DNA samples using the Ion Torrent™ platform, Electrophoresis. 39 (21) (2018) 2776–2784, doi:http://dx.doi.org/10.1002/elps.201700371.
[48] D. Sims, I. Sudbery, N.E. Ilott, A. Heger, C.P. Ponting, Sequencing depth and coverage: key considerations in genomic analyses, Nat. Rev. Genet. 15 (2014) 121–132, doi:http://dx.doi.org/10.1038/nrg3642.
[49] J. Burrill, B. Daniel, N. Frascione, A review of trace "Touch DNA" deposits: variability factors and an exploration of cellular composition, Forensic Sci. Int. Genet. 39 (2019) 8–18, doi:http://dx.doi.org/10.1016/j.fsigen.2018.11.019.
[50] I. Quinones, B. Daniel, Cell free DNA as a component of forensic evidence recovered from touched surfaces, Forensic Sci. Int. Genet. 6 (2) (2012) 26–30, doi:http://dx.doi.org/10.1016/j.fsigen.2011.01.004.
[51] M. Vandewoestyne, D. Van Hoofstat, A. Franssen, F. Van Nieuwerburgh, D. Deforce, Presence and potential of cell free DNA in different types of forensic samples, Forensic Sci. Int. Genet. 7 (2) (2013) 316–320, doi:http://dx.doi.org/10.1016/j.fsigen.2012.12.005.
[52] C.E. Stanciu, M.K. Philpott, Y.J. Kwon, E.E. Bustamante, C.J. Ehrhardt, Optical characterization of epidermal cells and their relationship to DNA recovery from touch samples, F1000 Res. 4 (2015) 1360, doi:http://dx.doi.org/10.12688/f1000research.7385.1.
[53] K.-A. Hyun, H. Gwak, J. Lee, B. Kwak, H.-I. Jung, Salivary exosome and cell-free DNA for cancer detection, Micromachines (Basel) 9 (7) (2018) 340, doi:http://dx.doi.org/10.3390/mi9070340.
[54] M.W. Snyder, M. Kircher, A.J. Hill, R.M. Daza, J. Shendure, Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin, Cell 164 (2016) 57–68, doi:http://dx.doi.org/10.1016/j.cell.2015.11.050.
[55] B. Bax, C.W. Chung, C. Edge, Getting the chemistry right: protonation, tautomers and the importance of H atoms in biological Chemistry, Acta Crystallogr. D73 (2017) 131–140, doi:http://dx.doi.org/10.1107/S2059798316020283.
[56] A. Abyzov, L. Tomasini, B. Zhou, N. Vasmatzis, G. Coppola, M. Amenduni, R. Pattni, M. Wilson, M. Gerstein, S. Weissman, A.E. Urban, F.M. Vaccarino, One thousand somatic SNVs per skin fibroblast cell set baseline of mosaic mutational load with patterns that suggest proliferative origin, Genome Res. 27 (2017) 512–523, doi:http://dx.doi.org/10.1101/gr.215517.116.
[57] S. De, Somatic mosaicism in healthy human tissues, Trends Genet. 27 (6) (2011) 217–223, doi:http://dx.doi.org/10.1016/j.tig.2011.03.002.
[58] M. O'Huallachain, K.J. Karczewski, S.M. Weissman, A.E. Urban, M.P. Snyder, Extensive genetic variation in somatic human tissues, PNAS. 109 (44) (2012) 18018–18023, doi:http://dx.doi.org/10.1073/pnas.1213736109.
[59] A. Abyzov, J. Mariani, D. Palejev, Y. Zhang, M.S. Haney, L. Tomasini, A.F. Ferrandino, L.A.R. Belmaker, A. Szekely, M. Wilson, A. Kocabas, N.E. Calixto, E.L. Grigorenko, A. Huttner, K. Chawarska, S. Weissman, A.E. Urban, M. Gerstein, F. M. Vaccarino, Somatic copy number mosaicism in human skin revealed by induced pluripotent stem cells, Nature. 497 (20) (2012) 438–445, doi:http://dx.doi.org/10.1038/nature11629.
[60] E. Grönniger, B. Weber, O. Heil, N. Peters, F. Stäb, H. Wenck, B. Korn, M. Winnefeld, F. Lyko, Aging and chronic sun exposure cause distinct epigenetic changes in human skin, PLoS Genet. 6 (5) (2010)e1000971, doi:http://dx.doi.org/10.1371/journal.pgen.1000971.
[61] F. Bormann, M. Rodriguez-Paredes, S. Hagemann, H. Manchanda, B. Kristof, J. Gutekunst, G. Radtaz, R. Haas, L. Terstegen, H. Wenck, L. Kaderali, M. Winnefeld, F. Lyko, Reduced DNA methylation patterning and transcriptional connectivity define human skin aging, Aging Cell 15 (2016) 563–571, doi:http://dx.doi.org/10.1111/acel.12470.
[62] A. Gosch, C. Courts, On DNA transfer: the lack and difficulty of systematic research and how to do it better, Forensic Sci. Int. Genet. 40 (1) (2019) 24–36, doi:http://dx.doi.org/10.1016/j.fsigen.2019.01.012.
[63] BRASIL, Ministério de Justiça e Segurança Pública. Manual de procedimentos operacionais da Rede Integrada de Bancos de Perfis Genéticos, aprovado pela Resolução n° 08, de 24 de outubro de 2017, Diário Oficial [da República Federativa do Brasil] 2235 (1) (2017) 63.
[64] SWGDAM - Scientific Working Group on DNA Analysis Methods. Addendum to "SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories" to Address Next Generation Sequencing. Available online at https://www.swgdam.org/publications.