Levenshtein Distance as a Measure of Accuracy and Precision in Forensic PCR-MPS Methods

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ABSTRACT

Accuracy and precision determinations are standard components of method validations where they help to describe the performance of methods. Despite their importance, a standard approach to calculating these parameters is not available for forensic PCR-MPS methods that detect sequence-based alleles. In this paper, we describe a method based on the Levenshtein distance metric which aptly summarizes method accuracy in terms of the closeness of read sequences to reference sequences, and method precision in terms of the agreement among read sequences. Inaccuracy or imprecision in forensic methods can lead to wrong allele calls. By expressing method performance in terms of a distance metric, this method places PCR-MPS on equal footing with distance-based measures in PCR-CE methods. Summary statistics based on the Levenshtein distance can be used to compare performance of different kits, markers, sequencers, or methods.

KEYWORDS

Accuracy, Precision, Massively Parallel Sequencing

1. Introduction

Performance evaluations of quantitative analytical methods used in forensics often includes consideration of accuracy and precision [1]. These measures are particularly useful when describing the suitability of a method in an intended application or when comparing the performance of alternative methods [2]. Method accuracy and precision are core components of forensic method validations under SWGDAM and ENFSI guidelines [3-5]. In this context, accuracy has been defined as the degree of conformity of measurements to the true value; and precision has been defined as the degree of mutual agreement among a series of individual measurements [3].

1.1. Accuracy and Precision in PCR-CE Methods

The goal of PCR-CE methods is to determine the true length of PCR-targeted markers in the genome. In these methods, the measurements of interest are electrophoretic retention times from which the lengths of PCR amplicons can be derived. Electrophoretic retention times are routinely transformed into

fractional base pair units. Even though "base pair" is a discontinuous measure, the fractional units are essentially continuous. Means and standard deviations are calculated in units of fractional base pair usually under an assumption that Normal distributions fit the data [6, 7]. To avoid length-miscalls, PCR-CE methods must be accurate to retention times that are within ± 0.5 base pairs of the true amplicon length [5, 8]. It is not possible to measure accuracy at the individual PCR amplicon level. Rather, the entity measured is the mode of retention times of an aggregate of same-length amplicons that migrate together under electrophoresis [9]. Precision is commonly described in terms of a standard deviation of measurement error of the aggregate [10, 11]. That is, the standard deviation of replicate electrophoretic peak retention times.

In addition to error present in the electrophoretic process, PCR-CE methods can introduce error in PCR amplicons due to PCR misincorporation and PCR stutter. PCR misincorporation possibly contributes to variation in electrophoretic retention times. Stutter error holds a special status in forensic STR analysis. Stutter peaks are expected in electropherograms, and presence (or absence) of stutter contributes to visual assessments of the sensitivity of the method. Stutter artifacts are not typically considered in measures of accuracy or precision in PCR-CE methods.

1.2. Accuracy and Precision in PCR-MPS Methods

In contrast to PCR-CE methods, the goal of PCR-MPS methods is to determine the nucleotide sequence of PCR-targeted markers in the genome. Experimental determination of a marker sequence results in a sequencer read (hereafter simply called a read) expressed as a string of letters following the IUPAC coding convention [12] for the four nucleotides in the DNA backbone (A, C, G, T)[1]. Like PCR-CE, the genomic segments targeted by the assay are defined by the PCR primers, which for most STR markers, includes flanking sequence that may contain additional polymorphisms. Targeted segments can be bioinformatically trimmed to shorter extents, and typically at least the PCR primer sequences are trimmed away. The read sequence that remains becomes the sequence-based allele under consideration. Like PCR-CE, accuracy and precision should be sensitive to single nucleotide differences[2] because authentic allele sequences can differ by a single nucleotide due to point polymorphisms [13, 14]. PCR-MPS methods generate a read sequence for each individual PCR amplicon, and a base call for each nucleotide position. Thus, accuracy and precision can be determined at several levels: nucleotide, read, and allele.

Consensus has not yet been reached for reporting accuracy and precision for forensic PCR-MPS methods. Accuracy of PCR-MPS methods has been reported either as a call rate [15] or as a concordance to CE allele numbers. Precision is often not reported at all [16-21] or has been reported as a modified call rate [15].

[1] The IUPAC ambiguity code N can appear in sequencer output but reads containing N characters are typically not considered in forensic analysis and will be disregarded here.

[2] Our use of the term nucleotide is functionally equivalent to the term base pair commonly used in forensics. The term nucleotide is preferred because the object of most bioinformatic analysis is the sequencer read, which represents just one DNA strand.

1.3. Distance Measures

Accuracy and precision are often expressed in distance units. The measurement result of a PCR-MPS method is a read string which may be close to or distant from the expected sequence. The distance between two DNA sequences is called an edit distance. The concept of edit distance is tightly associated with sequence alignment, which can be thought of as a transcript of the edits (nucleotide substitutions, insertions, and deletions) required to convert one sequence string into another [22] (p 125). An optimized alignment may not correspond to the minimum possible number of edits due to weightings that may be assigned to mismatches and gaps in the aligned sequences. The minimum number of equally-weighted edit operations is called the Levenshtein distance [22, 23].

Levenshtein distance has been shown to satisfy [24] the axioms [25] (p.147) required of distance metrics (Appendix A). Distance metrics imply the existence of an underlying metric space. The concept of a metric space where the elements of the space are DNA (or protein) sequence strings has a well-established foundation. Maynard Smith [26] first described sequence spaces in the context of natural selection, while Eigen [27] provided a formal topological/geometrical interpretation of sequence spaces. In short, Levenshtein distance is a distance metric that can be used in mathematical and statistical calculations. By employing the Levenshtein distance metric, accuracy and precision can be expressed in accord with SWGDAM guidance. That is, accuracy in amplicon sequencing can be expressed as the degree of conformity of read sequences to the true DNA sequence, and precision can be expressed as the degree of mutual agreement among a series of individual read sequence determinations.

Here, we define concepts for accuracy and precision in sequence-based PCR-MPS methods that are 1) compliant with SWGDAM and ENFSI guidelines, 2) analogous to the concepts of sizing accuracy and precision currently used for PCR-CE methods [28], and 3) can be expressed in units that can be used in statistical calculations.

2. Material and Methods

2.1. Samples and Sequencing

One ng aliquots of four DNA standards were sequenced in triplicate: 2800M (Promega, Madison, WI), and NIST standard reference material 2391d components A, B, and E (NIST, Gaithersburg, MD). Sequencing was performed at Intermountain Forensics (Salt Lake City, UT) using the ForenSeq™ kit and MiSeq™ sequencer (Verogen, San Diego, CA). Raw FASTQ data files were analyzed using MixtureAce™ (NicheVision Forensics, Akron, OH) with an analytical threshold of zero. Read sequences were bioinformatically trimmed to match the Verogen recommended analyzable regions [29] and exported to comma separated value (CSV) files along with their read count intensities (RCI). Stutter artifacts identified by MixtureAce were deleted from the CSV files. A custom Python script was used to modify the CSV files for statistical analysis such that each data row corresponded to an individual sequencer read (RCI = 1). Sixteen alleles were selected for analysis based on being homozygous in one of the DNA standards (Table 1). Selecting homozygous alleles allowed unambiguous association of erroneous sequences to parent alleles.

Table 1. Alleles selected for analysis.

Standard[1]	Locus	Allele ID[1]	STRSEQ[2] Accession No.
2800M	CSF1PO	12 PD	MH085189.1
	D5S818	12 RR	MH167007.1
	D22S1045	16 AW	MH167279.1
	TPOX	11 UN	MF044255.1
NIST SRM 2391d Component A	D2S441	11 RC	MH167320.1
	D3S1358	17 ZU	MH166980.1
	D4S2408	9 ZI	MH039589.1
NIST SRM 2391d Component B	D7S820	10 TO	MH167034.1
	D13S317	11 AQ	MH167219.1
	TH01	7 CS	MH085116.1
NIST SRM 2391d Component E	D6S1043	11 BZ	MH166925.1
	D9S1122	11 KZ	MH085132.1
	D10S1248	14 TJ	MH167062.1
	D19S433	14 WU	MH174820.1
	D20S482	15 QX	MH085169.1
	PentaD	14 WB	MH232690.1

[1] Allele identifiers consist of the CE-equivalent allele number and the first two digits of the SID label [30].
[2] https://www.ncbi.nlm.nih.gov/bioproject/380127

Reads exhibiting Levenshtein distances > 10 were omitted from the data set. Reads with extreme Levenshtein distances may arise from trace level contamination, or PCR mis-priming of non-human sequence, and therefore are not representative data. Of 125,994 reads in the dataset, only 107 exhibited Levenshtein distances of > 10 from the expected sequences. The maximum Levenshtein distance was 78. The truncated data set included 125,887 reads. Levenshtein distances were calculated using a custom Python script employing the Jellyfish module (https://github.com/jamesturk/jellyfish). All other data analysis was performed using custom R scripts.

3. Results

3.1. Distribution of Levenshtein Distances

Histograms of Levenshtein distances of the individual reads generated by a PCR-MPS method exhibit a characteristic shape independent of locus (Figures 1-2; Appendix B). Most reads match the expected sequence and therefore, exhibit a Levenshtein distance of zero from the expected sequence. The most abundant error type consists of reads exhibiting a single point error and a corresponding Levenshtein distance from expected equal to one. Read frequencies tend to decline exponentially with increasing Levenshtein distance (Figures 1-2). If point errors in reads arise randomly, read count distributions would be expected to be Poisson. However, it is known that sequencing error rates increase over the length of DNA templates, and that other error processes including PCR misincorporation contribute to the variance of the distribution. These factors contribute to overdispersion. Tests for overdispersion (not shown) indicate the Poisson does not fit the data. This is consistent with the observation that variances exceed means at all loci tested (Table 2). In addition, the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) values consistently show that the negative binomial fit is better than the Poisson fit (Table 2).

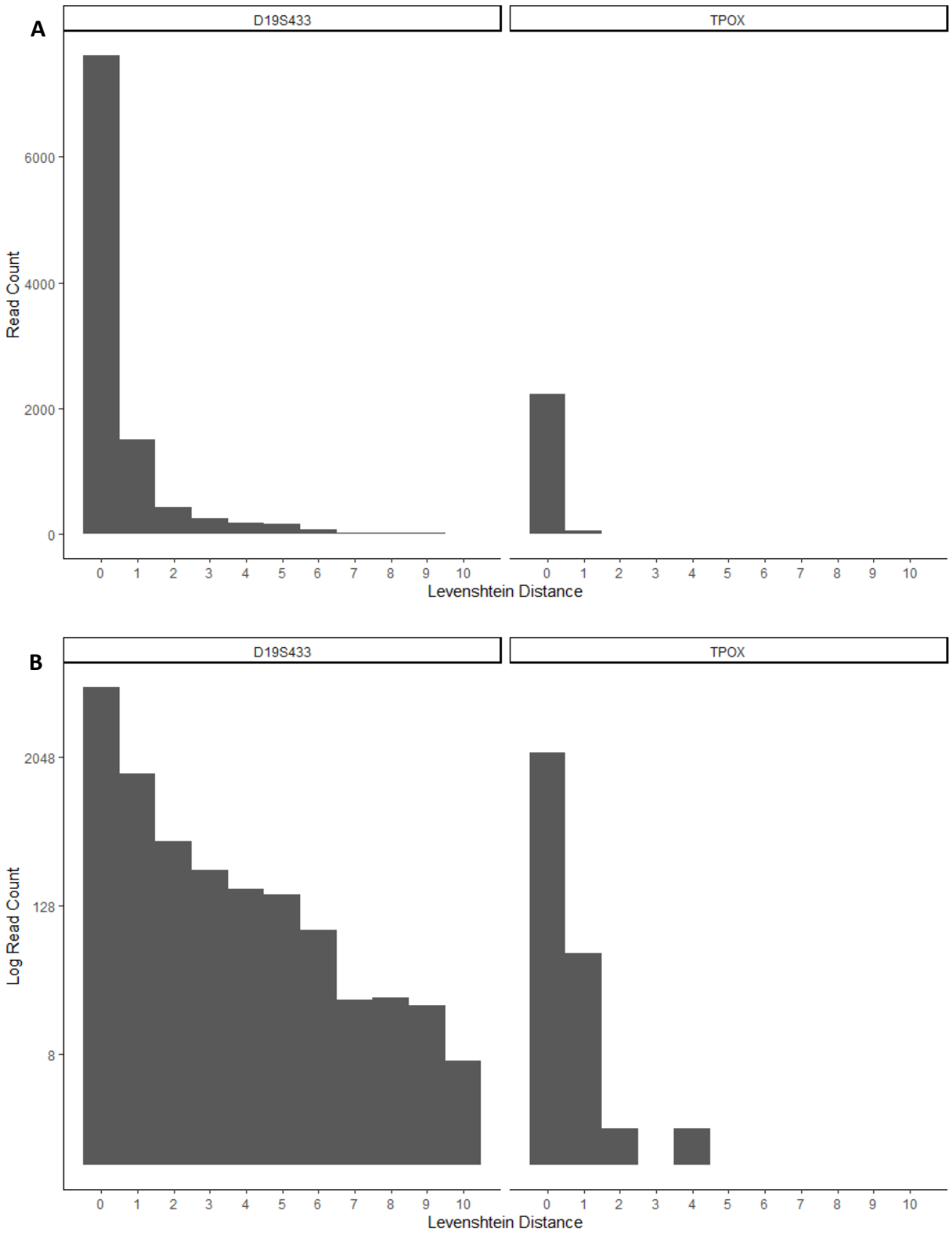
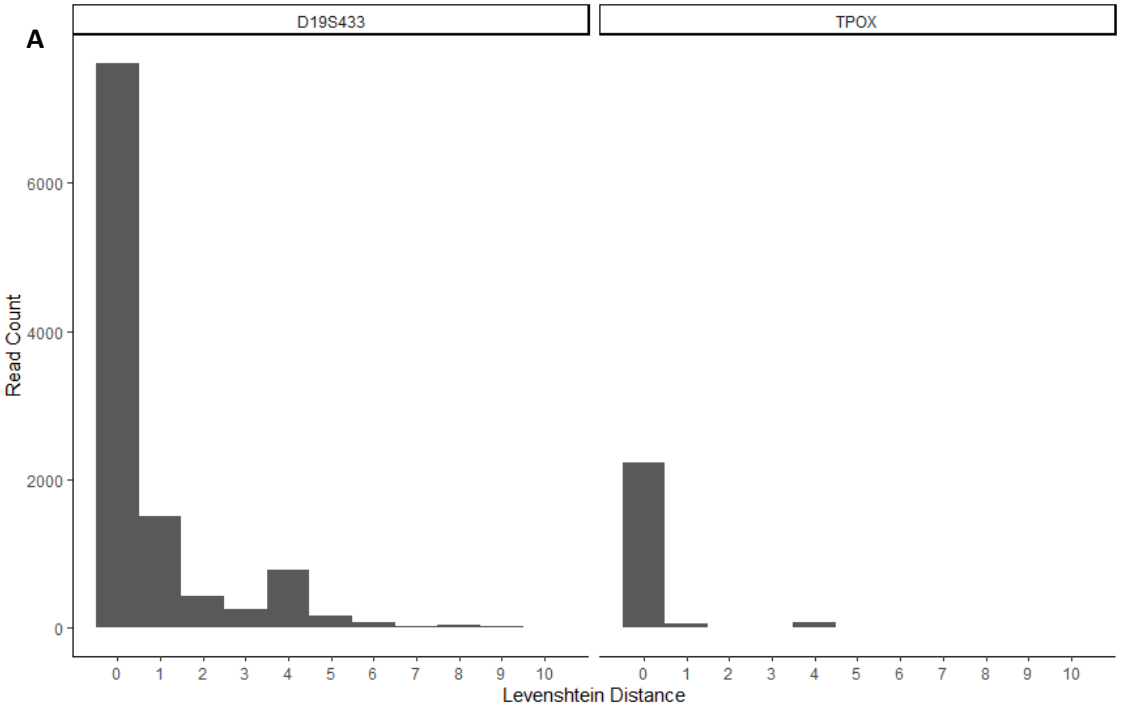


Figure 1. Histograms of read counts from selected loci after filtering stutter. Data was truncated to Levenshtein distances ≤ 10. A) Raw read counts. B) Read counts on log scale to aid visualization of low counts.



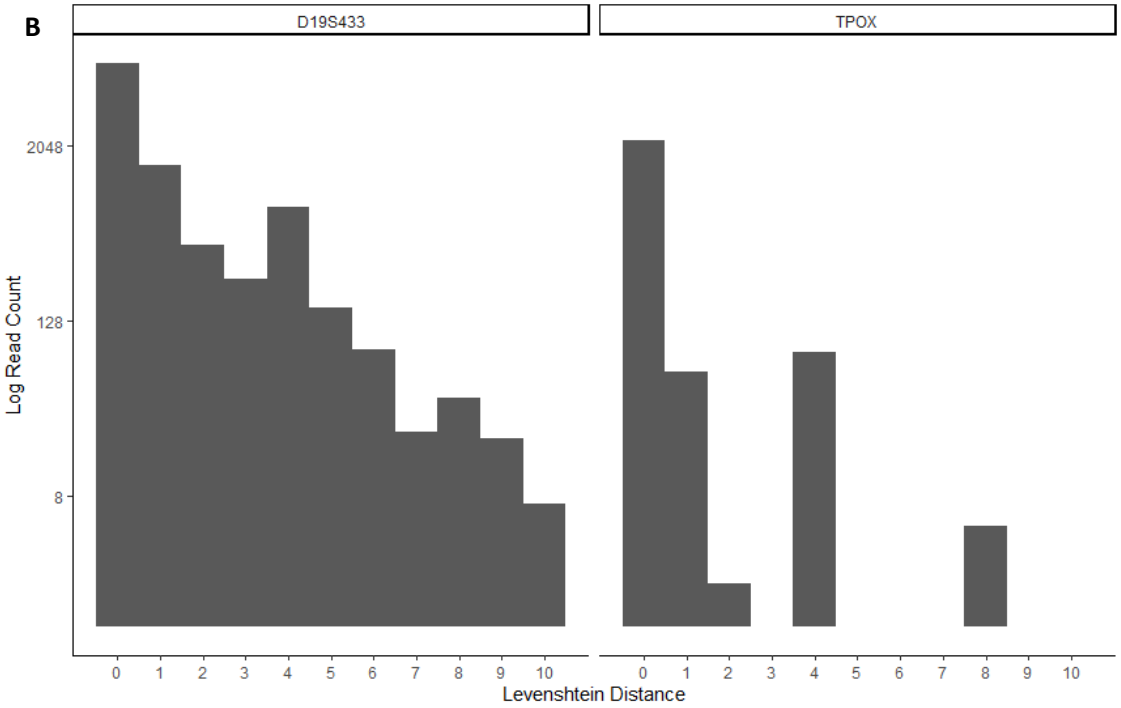


Figure 2. Histograms of read counts from selected loci with stutter included. Data were truncated to Levenshtein distances ≤ 10. A) Raw read counts. B) Read counts on log scale to aid visualization of low counts.

148 **Table 2. Read count statistics for 16 loci that were homozygous in the data set.**

Locus	Reads (N)	Mean	Variance	Standard Deviation	Poisson		Negative Binomial	
					AIC	BIC	AIC	BIC
TPOX	2,296	0.02918	0.04052	0.2013	655	661	606	617
D2S441	5,809	0.06335	0.1110	0.3332	2,918	2,925	2,699	2,713
D3S1358	10,822	0.1351	0.3811	0.6173	10,088	10,095	8,092	8,106
D4S2408	4,881	0.03176	0.04878	0.2209	1,430	1,436	1,334	1,347
CSF1PO	1,170	0.06838	0.1782	0.4221	653	658	531	541
D5S818	1,062	0.06309	0.1213	0.3483	603	608	493	503
D6S1043	15,335	0.1111	0.3090	0.5559	12,355	12,363	9,985	10,001
D7S820	3,747	0.1703	0.4354	0.6598	4,074	4,080	3,347	3,359
D9S1122	8,670	0.03956	0.09382	0.3063	3,134	3,141	2,605	2,619
D10S1248	6,407	0.1313	0.3294	0.5739	5,741	5,748	4,824	4,837
TH01	24,670	0.07937	0.1824	0.4271	15,112	15,120	12,967	12,983
D13S317	4,185	0.1101	0.2065	0.4544	3,185	3,192	2,906	2,918
D19S433	10,271	0.5516	1.643	1.282	24,843	24,850	19,489	19,504
D20S482	18,769	0.09414	0.2498	0.4998	13,340	13,348	10,760	10,776
PentaD	2,292	0.2133	0.3834	0.6192	2,731	2,737	2,567	2,578
D22S1045	5,501	0.1736	0.4270	0.6534	655	661	606	618
TOTAL	**125,887**							
Average		**0.1291**	**0.3213**	**0.5668**				

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150 ### 3.2. Accounting for Elevated Frequencies of Reads with Levenshtein Distances Equal to The Length
151 ### of Stutter Motifs After Filtering Stutter

152 Even when stutter artifacts have been filtered from the data, these artifacts can still contribute to
153 overdispersion in a cryptic way. Frequencies of reads with Levenshtein distances in multiples of tandem
154 repeat motifs are slightly elevated relative to the overall pattern. For example, counts of reads with
155 Levenshtein distances equal to four and eight tend to be slightly elevated for loci with tetramer repeat
156 motifs (Figure 1). This phenomenon is a consequence of defining stutter as a pure motif shift; combined
157 with how Levenshtein distances are calculated. In PCR-MPS, stutter artifacts formed during the PCR
158 step are subject to sequencing error during the sequencing step. If a point sequencing error occurs in a
159 stutter artifact, then a doubly mutated read results where the total number of nucleotide changes is five
160 (assuming a tetramer repeat motif). If the sequencing error occurred in the same repeat motif that was
161 increased or decreased in number due to stutter, then the minimum number of edit operations required
162 to convert the doubly mutated sequence to the correct parent allele sequence may be only four. In the
163 case of N+1 stutter the number of tandem repeats increased by one. When a point error occurs
164 somewhere in the tandem repeat that increased by one repeat, then only the repeat motif containing
165 the sequencing point error need be deleted for an edit distance equal to four. In the case of N-1 stutter,
166 the structure of the repeat motif may be such that the conversion of the doubly mutated sequence to
167 the parent allele sequence can also be solved using only four edits (Appendix C). Elevated frequencies
168 can also be observed for reads with Levenshtein distances of 5 and 9 (assuming a tetramer repeat
169 motif). These elevated frequencies can be attributed to the presence of sequencing errors outside the
170 STR repeat motif of stutter artifacts. In this case, the stutter error necessitates four (or eight) edits, and
171 a point sequencing error necessitates an additional edit. Mechanisms other than those described here
172 may also be involved.

173

3.3. Levenshtein Distance as an Estimator of Error Rates

Locus and profile error rates can be estimated from Levenshtein distances. The mean Levenshtein distances for the sixteen loci studied here ranged from 0.58 errors per 1,000 nucleotides (D9S1122) to 3.73 errors per 1000 nucleotides (D19S433, Table 3). Error rates for most loci were less than 1 per 1,000 nucleotides, which is consistent with published error rates for Illumina sequencing [31]. However, different loci exhibit different error rates, and these differences can be quantified using Levenshtein distances.

Table 3. Inference of error rate using Levenshtein distance statistics.

Locus	Sample	Allele	Allele Length	Mean Levenshtein Distance	Inferred Error Rate	
					Per nt	Per 1000 nt
TPOX	2800M	11 UN	49	0.02918	5.96×10^{-4}	0.596
D2S441	2391d_A	11 RC	96	0.06335	6.60×10^{-4}	0.660
D3S1358	2391d_A	17 ZU	134	0.1351	1.01×10^{-3}	1.01
D4S2408	2391d_A	9 ZI	50	0.03176	6.35×10^{-4}	0.635
CSF1PO	2800M	12 PD	68	0.06838	1.01×10^{-3}	1.01
D5S818	2800M	12 RR	68	0.06309	9.28×10^{-4}	0.928
D6D1043	2391d_E	11 BZ	129	0.1111	8.61×10^{-4}	0.861
D7S820	2391d_B	10 TO	95	0.1703	1.79×10^{-3}	1.79
D9S1122	2391d_E	11 KZ	68	0.03956	5.82×10^{-4}	0.582
D10S1248	2391d_E	14 TJ	97	0.1313	1.35×10^{-3}	1.35
TH01	2391d_B	7 CS	72	0.07937	1.10×10^{-3}	1.10
D13S317	2391d_B	11 AQ	122	0.1101	9.02×10^{-4}	0.902
D19S433	2391d_E	14 WU	148	0.5516	3.73×10^{-3}	3.73
D20S482	2391d_E	15 QX	102	0.09414	9.23×10^{-4}	0.923
PentaD	2391d_E	14 WB	228	0.2133	9.36×10^{-4}	0.936
D22S1045	2800M	16 AW	174	0.1736	9.98×10^{-4}	0.998
Overall Average				**0.1291**	**1.13×10^{-3}**	**1.13**

3.4. PCR-MPS Method Accuracy and Precision

PCR-MPS method accuracy can be expressed as the mean Levenshtein distance of reads relative to zero. The mean Levenshtein distance is an expression of the degree of conformity of experimentally determined sequences to the expected sequences. In the data presented here, the overall method accuracy is 0.1291 distance units. The standard deviation of Levenshtein distance values is an expression of the degree of mutual agreement among a series of individual sequence determinations. In the data presented here, the overall method precision is the overall standard deviation of 0.5668.

3.5. Comparison to Other Measures of Accuracy and Precision

Currently published methods for expressing accuracy and precision in PCR-MPS methods involve either allele call rates [15] or standard deviations of allele read count intensities [32]. These measures are indicators of PCR efficiency and variability but are less informative for whole method accuracy and precision. In many cases where inhibition or low template is not involved, percent call rate can be an insensitive measure (Table 4, Figure 3). Levenshtein distance is more closely related to total method error and is sensitive to differences in method error rates. In this study, we demonstrated the utility of Levenshtein distance-based accuracy and precision by discriminating performances at different loci

199 within a single forensic kit. However, this same method can be employed in the comparison of kits,
200 sequencers, technicians, laboratories, etc.

201

202 **Table 4. Comparison of call rates and standard deviations of read count intensities with**
203 **Levenshtein distance statistics.**

Locus	Call Rate (%)	Read Count Intensity			Levenshtein Distance	
		Mean	Standard Deviation	Standard Error	Mean	Standard Deviation
TPOX	100	722	149	60.8	0.02918	0.04052
D2S441	100	1,884	421	172.0	0.06335	0.1110
D3S1358	100	3,040	817	333.4	0.1351	0.3811
D4S2408	100	1,561	507	206.8	0.03176	0.04878
CSF1PO	100	352	104	42.3	0.06838	0.1782
D5S818	100	426	146	59.5	0.06309	0.1213
D6D1043	100	4,745	621	358.7	0.1111	0.3090
D7S820	100	1,204	182	74.5	0.1703	0.4354
D9S1122	100	2,809	92	53.1	0.03956	0.09382
D10S1248	100	1,948	142	82	0.1313	0.3294
TH01	100	7,571	1,378	562.4	0.07937	0.1824
D13S317	100	1,482	559	228.3	0.1101	0.2065
D19S433	100	2,537	892	515.0	0.5516	1.643
D20S482	100	5,876	687	396.8	0.09414	0.2498
PentaD	100	639	315	181.8	0.2133	0.3834
D22S1045	100	1,942	420	171.5	0.1736	0.4270
Average	**100**	**2,421**	**464**	**218.7**	**0.1291**	**0.3213**

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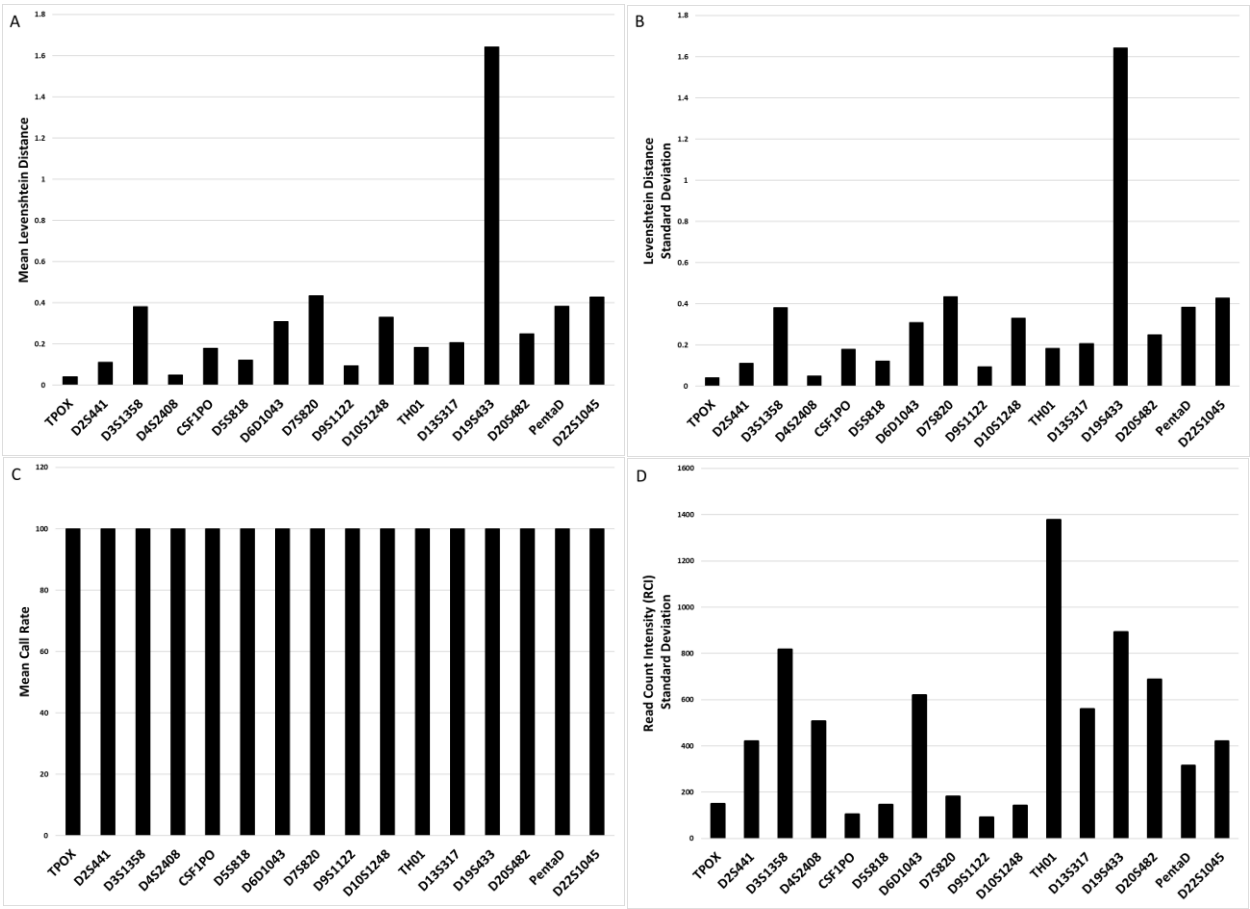

Figure 3. Alternative measures of accuracy and precision in PCR-MPS methods. Each plot represents data pooled across triplicate analyses of DNA standards. A) The mean Levenshtein distance is a measure of method accuracy where larger values indicate lower method accuracy. B) The standard deviation of Levenshtein distance is a measure of method precision where larger values correspond to lower precision. C) Call rates. D) Standard deviations of read count intensities.

4. Conclusions

Levenshtein distance is a metric that can be used to calculate accuracy and precision of forensic PCR-MPS methods in compliance with SWGDAM and ENSFI guidelines. The Levenshtein distance has an intuitive interpretation as the edit distance between experimentally determined reads and expected or reference sequences.

Acknowledgements

Supported by Army SBIR W911NF-20-P-0068

Appendix A

Definitions for mean and variance imply the need for a metric that can be used to quantitatively calculate accuracy and precision. A measure that satisfies the axioms of a metric is essential for calculating accuracy and precision in a way analogous to the approach used for PCR-CE methods, including calculation of standard deviations. Metrics are distance functions that must satisfy four axioms [25] p.147. Let $d(x, y)$ be a function measuring the distance between two elements ***x*** and ***y*** in a set of elements S.

1. The distance between two elements in the metric space is non-negative: $d(x, y) \geq 0$
2. The distance between two entities is zero if and only if the two entities are the same: $d(x, x) = 0$
3. The order of comparison does not matter: $d(x, y) = d(y, x)$
4. The distance between two elements is less than or equal to the sum of the distances obtained when an intermediate element is inserted. In other words, the triangle inequality is satisfied: $d(x, z) \leq d(x, y) + d(y, z)$

These properties hold for common metrics such as meters or inches in metric spaces of ordinary experience. These properties also hold when the metric $d(x, y)$ is Levenshtein distance and the elements in the metric space are DNA sequence strings [24].

Appendix B

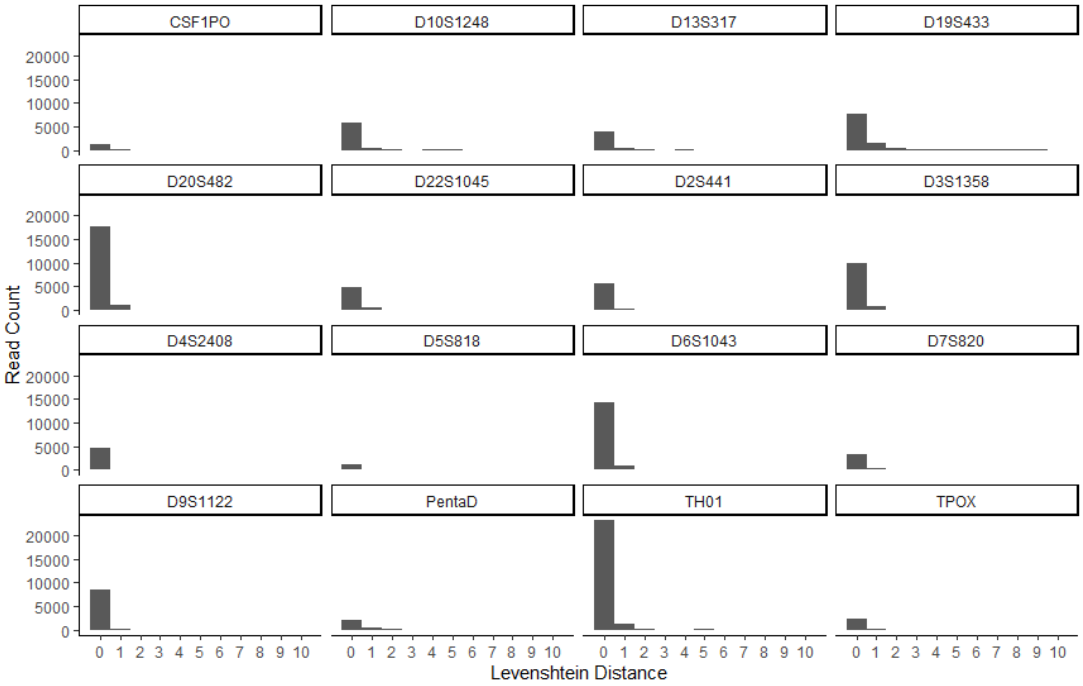

Figure B-1. Histograms for sixteen loci in normal scale.

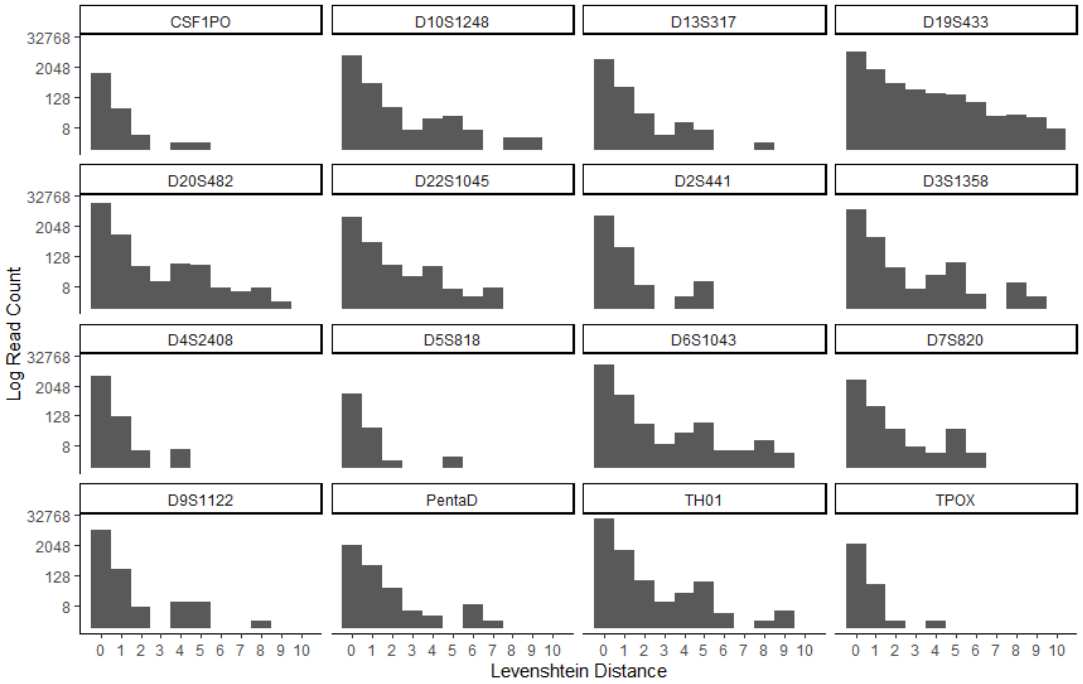

Figure B-2. Histograms for sixteen loci in log scale.

Appendix C

Explanation of Elevated Frequencies of Levenshtein Distances of Four and Five at Loci for Tetrameric Tandem Repeat Motifs

```
D19S433_14_WU      1 AATAAAAATCTTCTCTCTTTCTTCCTCTCTCC----TTCCTTCCTTCCTT     46
                     ||||||||||||||||||||||||||||||||    |||||||||||||||
D19S433_15_AH      1 AATAAAAATCTTCTCTCTTTCTTCCTCTCTCCTTCTTTCCTTCCTTCCTT     50

D19S433_14_WU     47 CCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTACCTTCTTTCCTTCA     96
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
D19S433_15_AH     51 CCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTACCTTCTTTCCTTCA    100

D19S433_14_WU     97 ACAGAATCTTATTCTGTTGCCCAGGCTGGAGTGCAGTGGTACAATTATAG    146
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
D19S433_15_AH    101 ACAGAATCTTATTCTGTTGCCCAGGCTGGAGTGCAGTGGTACAATTATAG    150

D19S433_14_WU    147 CT    148
                     ||
D19S433_15_AH    151 CT    152
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Figure C-1. Alignment of the D19S433 14 WU and 15 AH sequences. The 15 AH sequence is an N+1 stutter artifact of the 14 WU allele sequence with the addition of a sequencing error inside the repeat motif of the stutter artifact. Alignment can be solved using only four edit operations. The bracketed STR sequences are $[CCTT]_{12}[CCTA]_1[CCTT]_1[CTTT]_1[CCTT]_1$ and $[CCTT]_1[CTTT]_1[CCTT]_{11}[CCTA]_1[CCTT]_1[CTTT]_1[CCTT]_1$ respectively. Alignment was performed using EMBOS Needle with default parameter values.

```
D19S433_14_WU      1 AATAAAAATCTTCTCTCTTTCTTCCTCTCTCCTTCCTTCCTTCCTTCCTT     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
D19S433_13_TA      1 AATAAAAATCTTCTCTCTTTCTTCCTCTCTCCTTCCTTCCTTCCTTCCTT     50

D19S433_14_WU     51 CCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTACCTTCTTTCCTTCAACAG    100
                     |||||||||||||||||||| |  || |||||||||||||||||||||||
D19S433_13_TA     51 CCTTCCTTCCTTCCTTCCTT-C--CC-TCCTACCTTCTTTCCTTCAACAG     96

D19S433_14_WU    101 AATCTTATTCTGTTGCCCAGGCTGGAGTGCAGTGGTACAATTATAGCT    148
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
D19S433_13_TA     97 AATCTTATTCTGTTGCCCAGGCTGGAGTGCAGTGGTACAATTATAGCT    144
```

Figure C-2. Alignment of the D19S433 14 WU and 13 TA sequences. The 13 TA sequence is an N-1 stutter artifact of the 14 WU allele sequence with the addition of a sequencing error inside the repeat motif of the stutter artifact. Alignment can be solved using only four edit operations. The bracketed STR sequences are $[CCTT]_{12}[CCTA]_1[CCTT]_1[CTTT]_1[CCTT]_1$ and $[CCTT]_{10}CCCT[CCTA]_1[CCTT]_1[CTTT]_1[CCTT]_1$ respectively. Alignment was performed using EMBOS Needle, with gap open = 5.0 and gap extend = 0.0005.

```
D19S433_14_WU      1 AATAAAAATCTTCTCTCTTTCTTCCTCTCTCCTTCCTTCCTTCCTTCCTT     50
                     ||||||||||||||||||||||||||||||    |||||||||||||||||
D19S433_13_PG      1 AATAAAAATCTTCTCTCTTTCTTCCTCTC----TCCTTCCTTCCTTCCTT     46

D19S433_14_WU     51 CCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTACCTTCTTTCCTTCAACAG    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
D19S433_13_PG     47 CCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTACCTTCTTTCCTTCAACAG     96

D19S433_14_WU    101 AATCTTATTCTGTTGCCCAGGCTGGAGTGCAGTGGTACAATTATAGCT    148
                     ||||||||||||||||||||||||||||||.||||||||||||||||||||
D19S433_13_PG     97 AATCTTATTCTGTTGCCCAGGCTGGAGTACAGTGGTACAATTATAGCT    144
```

Figure C-3. Alignment of the D19S433 14 WU and 13 PG sequences. The 13 PG sequence is an N-1 stutter artifact of the 14 WU allele sequence with the addition of a sequencing error outside the repeat motif of the stutter artifact. Alignment can be solved using five edit operations. The bracketed STR sequences are $[CCTT]_{12}[CCTA]_1[CCTT]_1[CTTT]_1[CCTT]_1$ and $[CCTT]_{11}[CCTA]_1[CCTT]_1[CTTT]_1[CCTT]_1$ respectively. Alignment was performed using EMBOS Needle, with default parameter values.

```
D19S433_14_WU      1 AATAAAAATCTTCTCTCTTTCTTCCTCTC----TCCTTCCTTCCTTCCTT     46
                     ||||||||||||||||||||||||||||||    |||||||||||||||||
D19S433_15_HW      1 AATAAAAATCTTCTCTCTTTCTTCCTCTCTCCTTCCTTCCTTCCTTCCTT     50

D19S433_14_WU     47 CCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTACCTTCTTTCCTTCA     96
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
D19S433_15_HW     51 CCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTACCTTCTTTCCTTCA    100

D19S433_14_WU     97 ACAGAATCTTATTCTGTTGCCCAGGCTGGAGTGCAGTGGTACAATTATAG    146
                     ||||||||||||.||||||||||||||||||||||||||||||||||||||
D19S433_15_HW    101 ACAGAATCTTAATCTGTTGCCCAGGCTGGAGTGCAGTGGTACAATTATAG    150

D19S433_14_WU    147 CT    148
                     ||
D19S433_15_HW    151 CT    152
```

Figure C-4. Alignment of the D19S433 14 WU and 15HW sequences. The 15 HW sequence is an N+1 stutter artifact of the 14 WU allele sequence with the addition of a sequencing error outside the repeat motif of the stutter artifact. Alignment can be solved using five edit operations. The bracketed STR sequences are $[CCTT]_{12}[CCTA]_1[CCTT]_1[CTTT]_1[CCTT]_1$ and $[CCTT]_{13}[CCTA]_1[CCTT]_1[CTTT]_1[CCTT]_1$ respectively. Alignment was performed using EMBOS Needle, with default parameter values.

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