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ORIGINAL ARTICLE

Massively parallel sequencing-enabled mixture analysis of mitochondrial DNA samples

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Abstract

The mitochondrial genome has a number of characteristics that provide useful information to forensic investigations. Massively parallel sequencing (MPS) technologies offer improvements to the quantitative analysis of the mitochondrial genome, specifically the interpretation of mixed mitochondrial samples. Two-person mixtures with nuclear DNA ratios of 1:1, 5:1, 10:1, and 20:1 of individuals from different and similar phylogenetic backgrounds and three-person mixtures with nuclear DNA ratios of 1:1:1 and 5:1:1 were prepared using the Precision ID mtDNA Whole Genome Panel and Ion Chef, and sequenced on the Ion PGM or Ion S5 sequencer (Thermo Fisher Scientific, Waltham, MA, USA). These data were used to evaluate whether and to what degree MPS mixtures could be deconvolved. Analysis was effective in identifying the major contributor in each instance, while SNPs from the minor contributor's haplotype only were identified in the 1:1, 5:1, and 10:1 two-person mixtures. While the major contributor was identified from the 5:1:1 mixture, analysis of the three-person mixtures was more complex, and the mixed haplotypes could not be completely parsed. These results indicate that mixed mitochondrial DNA samples may be interpreted with the use of MPS technologies.

Keywords Mitochondrial DNA · Mixtures · Massively parallel sequencing · Ion S5 · Ion PGM

Introduction

Mitochondrial DNA has become a powerful tool for the identification of human remains and in analyses of certain types of forensic evidence from criminal cases, e.g., hair evidence. The mitochondrial genome's higher copy number per cell, compared with the nuclear genome [1], provides a high sensitivity of detection with challenged or degraded remains, where nuclear markers often provide inconclusive or negative results. In addition, maternal inheritance [2] and well-characterized phylogeny [3–15] of the mitochondrial genome offer useful lineage and bioancestry information. Currently, Sanger sequencing technologies are employed to sequence a limited portion of the mitochondrial genome, often focusing only on the hypervariable regions of the non-coding region. Because the assay is time-consuming and labor-intensive, substantial variation residing in the coding region of the mitochondrial genome is not considered. Moreover, Sanger sequencing is not sufficiently quantitative to resolve mixed mitochondrial DNA profiles [16, 17].

Massively parallel sequencing (MPS) technologies now make it feasible for forensic crime laboratories to sequence the entire mitochondrial genome. Large multiplex, small amplicon panels that amplify the entire mitochondrial genome have been designed for challenged and degraded samples [18–20]. Moreover, the technology has become reasonably robust such that the amount of time and labor needed to sequence the entire mitochondrial genome has been reduced substantially. The high throughput concomitantly provides a much larger amount of useful information. Expanding analysis to the entire mitochondrial genome enables analysis of a previously untapped resource of a large number of single

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nucleotide polymorphisms (SNPs) (up to 75% of total mitochondrial DNA variation) when the mitochondrial coding region is evaluated [4, 5, 7, 21–24]. Analysis of only the mitochondrial control region may provide only limited phylogenetic information as two samples with a control region match do not necessarily belong to the same haplogroup [23]. Thus, sequence data from the entire mitochondrial genome is likely to increase phylogenetic resolution [4, 5, 7, 23, 24]. Additionally, since each molecule (or clonal cluster) is sequenced independently, heteroplasmy detection can be enhanced versus the simultaneous sequencing of each amplicon by Sanger sequencing.

Mixtures are one of the more challenging sample types encountered in forensic casework, and mitochondrial DNA mixture interpretation typically is not attempted with current sequencing technologies used in forensic crime labs. In fact, the current recommendation from the DNA Commission of the International Society of Forensic Genetics (ISFG) states that heteroplasmy evaluation depends, in part, on the limitations of the technology [25]. MPS-generated data are more quantitative than Sanger sequencing data. Studies such as Stewart et al. [26] and Davis et al. [27] visually illustrate the lack of quantitative information provided by Sanger sequencing at varying mixture ratios and heteroplasmic sites, respectively. Combining quantitative information and phylogenetic assignment may make it feasible to effect mixture deconvolution in some samples [16, 17, 20, 28–30]. In this study, two-person mixtures and three-person mixtures of individuals from differing and similar phylogenetic backgrounds were prepared in various ratios. A workflow consisting of the Precision ID mtDNA Whole Genome Panel, Ion Chef, and Ion PGM/S5 sequencer (Thermo Fisher Scientific, Waltham, MA, USA) was used to sequence the mixture samples. Finally, a bioinformatic pipeline using quantitative analysis of positions with multiple allele states, phasing information, and phylogenetics was used to parse mixed haplotypes into their individual components.

Materials and methods

Samples

The policies and procedures approved by the Institutional Review Board for the University of North Texas Health Science Center in Fort Worth, TX, were followed for the collection and use of samples. DNA used in this study was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocols [31] and quantified using the Quantifiler Trio DNA Quantification kit (Thermo Fisher Scientific) following the manufacturer's protocols [32]. Single-source reference samples ($n = 6$) of self-identified Asian and Caucasian individuals, mixed samples ($n = 12$), and positive and negative controls were included in the sequencing runs. Two-person mixtures with contributors of different macrohaplogroups were prepared in 1:1, 5:1, 10:1, and 20:1 major to minor contributor nuclear DNA ratios for both individuals. Two-person mixtures with contributors belonging to the same macrohaplogroup were prepared in 1:1, 5:1, and 1:5 nuclear DNA ratios. Three-person mixtures with contributors of different macrohaplogroups were prepared in 1:1:1 and 5:1:1 nuclear DNA ratios.

Library preparation and massively parallel sequencing

The mitochondrial genome was amplified in each single-source and mixed sample with the Precision ID mtDNA Whole Genome Panel (Thermo Fisher Scientific), a multiplexed panel which generates amplicons of 175 base pairs or less that cover the entire mitochondrial genome in a tiled, overlapping manner [33]. Each amplification was performed with one nanogram of total input nuclear DNA following the manufacturer's protocols. Sequencing libraries were prepared manually using the Precision ID Library Kit (Thermo Fisher Scientific) and the manufacturer's recommended protocols for the "2-in-1 method."

Template preparation was completed on the Ion Chef (Thermo Fisher Scientific) following the manufacturer's recommended protocols [33, 34] for both sequencing runs. Templated Ion Sphere Particles (ISPs) were loaded into an Ion 318 Chip v2 (Thermo Fisher Scientific) for the Personal Genome Machine (PGM; Thermo Fisher Scientific) sequencing run and an Ion 530 Chip (Thermo Fisher Scientific) for the Ion S5 (Thermo Fisher Scientific) sequencing run. The Ion Chips were loaded onto their respective sequencers using the Ion PGM Hi-Q Sequencing Kit for the PGM run and the Ion S5 Sequencing Kit for the S5 run and the manufacturer's respective recommended protocols [33, 34].

Concordance data

An orthogonal methodology was used to generate concordance data for the single-source reference samples included in this study. The concordance data were generated via long-PCR on the Ion PGM and MiSeq (Illumina, San Diego, CA, USA) sequencers as described in Churchill et al. [35] and King et al. [5].

Data analysis

Primary data analyses were completed with the Torrent Suite software v5.2.1. Data were aligned to an "rCRS+80" reference genome to account for the Precision ID mtDNA Whole Genome Panel's tiled, overlapping design [33, 36]. Variant

calls were obtained from the variant call format (VCF) output files generated by the Variant Caller plugin v5.2.1 and were imported into mitoSAVE [37] to generate haplotype calls in standard forensic nomenclature [25, 38]. A minimum of 10 reads (X) and allele ratio of 0.10 were used as thresholds for generating haplotype calls in mitoSAVE. Length heteroplasmies were not included in the final haplotype calls. Additionally, for the mixed samples, the ratio of the reference allele and alternate allele compared to the total read depth for each SNP was obtained from mitoSAVE to use as a quantitative assessment of each contributor's proportion of the mixture. Binary alignment map (BAM) files were viewed in Integrative Genomic Viewer (IGV) for a manual verification of the haplotype calls and to identify any relevant phasing information [39, 40]. A phylogenetic check of the final haplotype calls was performed in HaploGrep v2.1.1 and EMPOP v3 [8, 41, 42]. Finally, performance metrics, including read depth, relative locus performance (RLP), strand balance, and noise, were used to evaluate the quality of the sequencing results. The read depth was used to calculate normalized RLP at each nucleotide position of the mitochondrial genome (i.e., read depth of one nucleotide position divided by the total read depth across the mitochondrial genome for that sample each multiplied by the length of the rCRS (i.e., 16,569)). Strand balance ratios were calculated by dividing the read depth of one strand by the total read depth of that nucleotide position. Noise was calculated by dividing the number of reads not attributed to nominal allele calls at a nucleotide position by the total coverage at that nucleotide position.

Results and discussion

Controls

A positive and negative control was included in each sequencing run to help evaluate the success and performance of each run. The read depth of the negative controls was compared to the read depth of the single-source reference samples by calculating the ratio of the negative controls' average read depth to the single-source samples' average read depth across the mitochondrial genome. The average read depth for both negative controls ranged from 0.04 to 2.55% of the single-source samples' average read depth across the mitochondrial genome. These results are well-below and are in-line with the use of a 0.10 point heteroplasmy threshold for making variant calls. The haplotypes generated for the positive controls in this study were concordant with the NIST standard data described in Riman et al. [43] except for the 1393G/A sequence variant call. The heteroplasmic 1393G/A sequence variant, described by Riman et al. [43], did not reach the 0.10 point heteroplasmy threshold set for this study. The "A" allele was seen at 3% (Ion PGM run) and 4% (Ion S5 run) of the total read depth at that nucleotide position. Differences may be due to sequencing chemistry or variation that can occur with different lots of a cell line.

Single-source reference samples

Haplotype calls for the single-source reference samples were compared to complete mitochondrial genome sequence data generated by long-PCR and sequenced on the MiSeq or PGM. The haplotype calls were completely concordant and then used as references for assessing mixture deconvolution.

Performance metrics of the six single-source samples and two positive controls were evaluated. Average read depth for the eight samples ranged from 368X to 22,188X across the mitochondrial genome. Samples sequenced on the Ion PGM had a slightly lower average read depth, which ranged from 270X to 18,836X, than those samples sequenced on the Ion S5, which ranged from 366X to 24,224X. This difference in average read depth is attributed to the difference in throughput capacities of the Ion Chips used on the two instruments. Thus, an average RLP was calculated to normalize the two sequencing runs and to visualize relative sequencing performance across the mitochondrial genome. Average RLP for the single-source samples ranged from 5.93E-05 to 3.50E-04 (Supplementary Fig. 1). Additionally, read depth across the mitochondrial genome was analyzed on a per strand basis to evaluate balance. Average ratios of read depth for the positive strand ranged from 0.02 to 0.75 with 84% of the nucleotide positions at or above 0.40 (Supplementary Fig. 2). Finally, the level of noise across the mitochondrial genome for the single-source samples was evaluated. Any reads not attributed to nominal allele calls were considered noise. These noise reads potentially could be the result of sequencing errors, PCR errors, alignment errors, NUMTs, or contamination. Average noise for the single-source genomes ranged from 0.0% of the total read depth to 4.86% of the total read depth across the mitochondrial genome, with only eight nucleotide positions above 3% (Supplementary Fig. 3). The nucleotide positions where noise was the highest were scrutinized further. These nucleotide positions (e.g., nucleotide position 13057) generally were associated with homopolymeric regions in the genome. Ion Torrent platforms' difficulty in sequencing through homopolymeric regions has been well-characterized [9, 18, 35, 44, 45], and Supplementary Fig. 4 illustrates the variation in reads that is generated and aligned to a homopolymer. Bioinformatic improvements (e.g., improvements to the alignment of homopolymers) may allow for better characterization of noise or off-target reads potentially allowing the thresholds for which point heteroplasmies and mixtures are called to be lowered.

Mixed samples

Performance metrics

For the mixed samples, average read depth ranged from 401X to 17,466X across the mitochondrial genome. Average RLP for the mixed samples ranged from 7.86E-06 to 3.47E-04 (Supplementary Fig. 5). When evaluating read depth on a per strand basis, average ratios of read depth for the positive strand ranged from 0.02 to 0.71 with 82% of the nucleotide positions at or above 0.40 (Supplementary Fig. 6). The level of reads not attributed to nominal allele calls (i.e., noise) ranged from 0.0 to 4.54% of the total read depth across the mitochondrial genome, with only seven nucleotide positions above 3.0% (Supplementary Fig. 7). The nucleotide positions with the highest level of noise were generally associated with homopolymeric regions in the genome (e.g., nucleotide position 13057). These performance metrics for mixtures were similar to those for single-source samples.

Mixture interpretation

The quantitative MPS data, phasing, and phylogenetics were used to deconvolve mixtures. Mixture data were analyzed quantitatively by calculating the ratio of each allele's read depth to total read depth. These ratios were used to group the sequence variants in each mixed haplotype into three groups: (1) alternate allele present in both contributors' haplotypes, (2) alternate allele present in the major contributor's haplotype, and (3) alternate allele present in minor contributor's haplotype. The "major" versus "minor" designation was decided by assigning the sequence variants with the higher ratio of alternate allele read depth to total read depth as the "major contributor." No specific ratio was selected a priori as no criteria were available to set mixture ratios. Phasing and phylogenetic information were used when sequence variants did not fall into one of the three categories. During manual verification of haplotype calls in IGV, phasing information was collected from amplicons in which two or more nucleotide positions showed evidence of a mixture. Phylogenetic information was acquired from HaploGrep and EMPOP [8, 41, 42].

Two-person mixtures

Two-person mixtures of individuals from differing phylogenetic backgrounds (i.e., haplogroups HV and F1a1a) were analyzed first to assess the bioinformatic processes' ability to parse mixtures of mitochondrial haplotypes with a relatively large amount of genetic differences between the contributors. The quantitative analysis results for each mixture (1:1, 5:1, 10:1, and 20:1) are shown in Table 1. Note that a 1:1 mixture can result as a major:minor because input DNA was based on nuclear DNA amounts in this study. The amount of mitochondrial DNA per individual is related to total DNA but does vary among individuals [1, 46]. While the major contributor's haplotype was fully and accurately identified quantitatively for each mixture, sequence variants associated with the minor contributor's haplotype only were identified above the 0.1 point heteroplasmy threshold in the 1:1, 1:5, 5:1, and 1:10 mixtures, with the 5:1 mixture exhibiting only a partial minor contributor haplotype and the personal point heteroplasmy (14386T/C) in the minor contributor of the 1:1 mixture falling below the 0.1 point heteroplasmy threshold.

Personal point heteroplasmies were difficult to assess as they were low or could be attributed to a minor contributor. In instances (i.e., the 1:5 and 1:10 mixtures) when phase and phylogenetics did not identify whether the 14386T/C was a personal point heteroplasmy from the major contributor or a mixed site with the sequence variant belonging to the minor contributor's haplotype, two possible haplotypes for both the major and minor contributor (four haplotypes in total) were generated. However, phasing and phylogenetic information did help resolve some mixtures (Supplementary Fig. 8a–g). For example, the 4086C/T and 4092G/A point mixtures in the 1:1 mixture had an alternate allele frequency of 48%. These mixture sites could not be assigned with confidence to one of the three categories of (1) alternate allele present in both contributors' haplotypes, (2) alternate allele present in the major contributor's haplotype, or (3) alternate allele present in minor contributor's haplotype (Table 1). This lack of success to parse contributors quantitatively for essentially 1:1 mixtures is expected. Manual verification of the mixture positions in IGV offered additional information as the two sites resided within one amplicon, and the two alternate alleles at these sites were not in-phase with each other (i.e., not sequenced in the same read; Fig. 1). Therefore, in this amplicon, more information about each contributor's genetic profile could be obtained, despite similar read depth for both allele states at the mixture sites. Furthermore, both EMPOP and HaploGrep were used to phylogenetically confirm (or refute) the blind phasing assignments [8, 41, 42]. These tools indicated whether or not each sequence variant would be expected to occur in this haplotype. Alignment issues with indels, reads not making it all the way through an amplicon in one direction, and differing amplification efficiencies can increase the variance for the ratio of allele read depths to the total read depth and thus affect the ability to accurately assign sequence variants to one contributor or another. While such assessments were performed manually herein, anticipated bioinformatic developments (as these applications are increasing rapidly) could facilitate interpretation and improve the ability to parse mitochondrial DNA mixtures.

Given the success of deconvolving two-person mixtures with different haplogroups, two-person mixtures of individuals from the same U2e subclade were analyzed to assess the bioinformatic pipeline's ability to parse mixtures of

Table 1 Quantitative results of two-person mixtures with contributors of different haplogroups (and ancestries). The average for the alternate allele read depth as a ratio to total read depth for each category is provided. The standard deviation is in parentheses

	1:1 mixture	1:5 mixture	5:1 mixture	1:10 mixture	10:1 mixture	1:20 mixture	20:1 mixture
Both contributors	99.50% (0.76%)	99.38% (0.92%)	99.63% (0.74%)	98.44% (3.24%)			
Major contributor	62.38% (2.00%)	73.23% (5.14%)	89.44% (1.81%)	85.73% (1.82%)	96.94% (2.99%)	94.00% (3.24%)	98.12% (1.58%)
Minor contributor	35.96% (3.04%)	24.00% (2.00%)	11.27% (0.90%)	12.86% (1.21%)			

mitochondrial haplotypes with less genetic differences between the contributors. The quantitative analysis results for the 1:1 and 5:1 mixtures are shown in Table 2. This quantitative assessment was able to identify full and accurate haplotypes for the major and minor contributor in each mixture, similar to the results above. Additional phasing and phylogenetic information were not needed to resolve this set of mixed samples (Supplementary Fig. 9a–c). Greater major

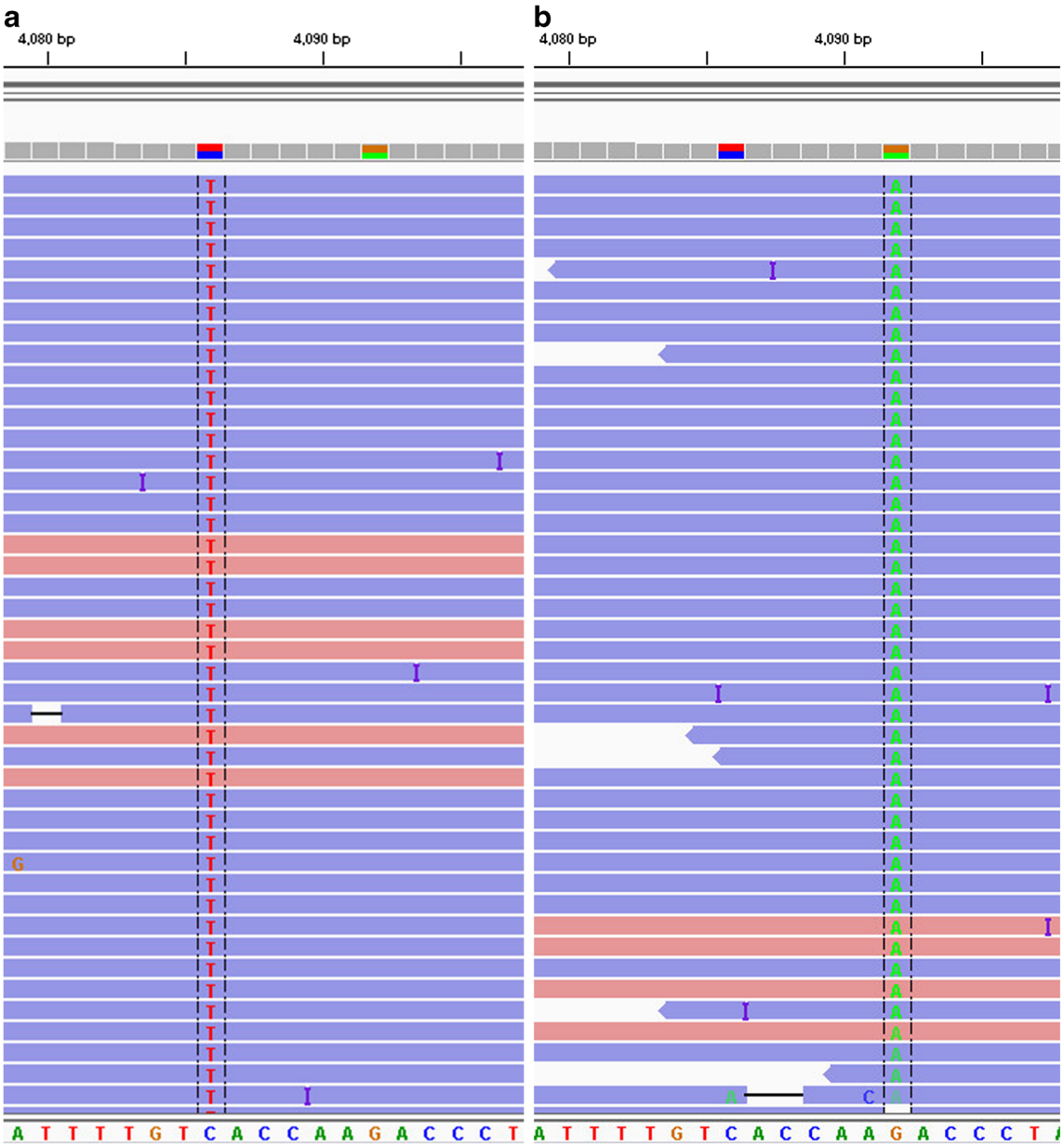

Fig. 1 Viewing the 1:1 mixture's haplotype in IGV. Sorting alignments by base illustrated that the alternate alleles at 4086C/T and 4092G/A were not in-phase with each other (i.e., T and A, respectively were not in the same read)

Table 2 Quantitative results of two-person mixtures with contributors of the same U2e subclade. The average for the alternate allele read depth as a ratio to total read depth for each category is provided. The standard deviation is in parentheses

	1:1 mixture	5:1 mixture	1:5 mixture
Both contributors	99.09% (0.93%)	99.13% (0.94%)	99.06% (1.39%)
Major contributor	59.00% (2.53%)	85.33% (1.75%)	76.50% (3.94%)
Minor contributor	38.80% (1.75%)	12.17% (1.33%)	21.33% (1.21%)

versus minor contributor ratios (i.e., 10:1 and 20:1 ratios) were not attempted based on the mixture findings discussed above.

Three-person mixtures

Mixtures of 1:1:1 and 5:1:1 ratios of individuals from different phylogenetic backgrounds (i.e., haplogroups HV, F1a1a, and U2e2a1) were generated. Phasing information and the presence of tri-allelic nucleotide positions suggested that the number of contributors was greater than two for these mixtures (Fig. 2), which could be more challenging if all three contributors were of the same haplogroup. However, quantitative assessment of the three-person mixtures did not provide a clear delineation of the alternate allele ratios into one of the three groups of (1) alternate allele present in each contributors' haplotypes, (2) alternate allele present in the major contributor's haplotype, or (3) alternate allele present in minor contributor's haplotype where complex mixtures can have more than one minor contributor (Supplementary Fig. 10a, b). While the tri-allelic nucleotide position and amplicons where all three haplotypes were observed (Fig. 2) provided an indication for assessing the quantitative contribution of each individual in the mixture, the amount of shared variants and large number of possible combinations for parsing each sequence variant in the mixture was not feasible with manual deconvolution of the 1:1:1 mixture. Phasing only provided additional information for a small number of amplicons in this mixture (Fig. 2c), but this phasing information should be considered as it could help exclude some individuals from the mixture. With the 5:1:1 mixture, quantitative assessment allowed the shared alleles present in each contributors' haplotype to be identified at a ratio of 99.38% (± 0.74%) and the alternate allele's present in the major contributor's haplotype to be identified at a ratio of 74.78% (±1.92). The range of alternate allele ratios seen for the two minor contributors was too similar to parse manually. The remaining sequence variants that were attributed to the minor contributors were uploaded to EMPOP for a phylogenetic assessment [8]. A haplogroup prediction (F1a1a) for one of the minor contributors was obtained. The sequence variants labeled as "Private Mutations" were deemed "inconclusive" prior to comparison with single-source reference samples as these variants also could have come from the second minor contributor. This phylogenetic assessment provided an accurate haplogroup prediction and an accurate, partial (77.5% complete) haplotype for one of the minor contributors. The remaining seven sequence variants could have been private mutations from minor contributor one or part of minor contributor two's haplotype, but with so many of minor contributor two's sequence variants likely falling below the 0.1 point heteroplasmy threshold, it was difficult to take advantage of any additional phylogenetic assessment. As discussed previously, alignment issues with indels, reads not making it all the way through an amplicon in one direction, and differing amplification efficiencies can increase the variance for the ratio of the alternate allele's read depth to the total read depth and affect the ability to accurately assign sequence variants to one contributor or another. However, nucleotide positions that can be phylogenetically associated to a contributor could be reported with a probability of a profile given certain genotypes. Likely, since MPS data for the most part are quite quantitative, a probabilistic genotyping approach could perform better at parsing contributors [47–50]. Vohr et al. [51] have released a software package for the analysis of mixed mitochondrial DNA samples called mixemt. This software provides a more automated approach to the quantitative and phylogenetic assessment completed manually in this study. However, use of a PCR-based amplification of the mitochondrial genome and current data capacities of Linux-based systems available for this study rendered this software package, in its current state, ineffective for analysis of previously discussed mixtures.

Evaluating number of contributors

The performance metrics, read depth, RLP, strand balance, and noise, of the single-source and mixed samples were compared to search for the presence of trends that potentially could help distinguish between single-source and mixed samples. The results for each of the performance metrics for both groups of samples were found to be comparable. The range of read depth and RLP across the mitochondrial genome was comparable for both the single-source and mixed samples. The topography of the RLP graphs (Supplementary Figs. 1 and 5) displayed similar valleys and peaks and illustrated that the higher and lower performing amplicons were the same for both the single-source and mixed samples. Strand balance and the number of reads attributed to noise also were comparable between the single-source and mixed samples. The nucleotide

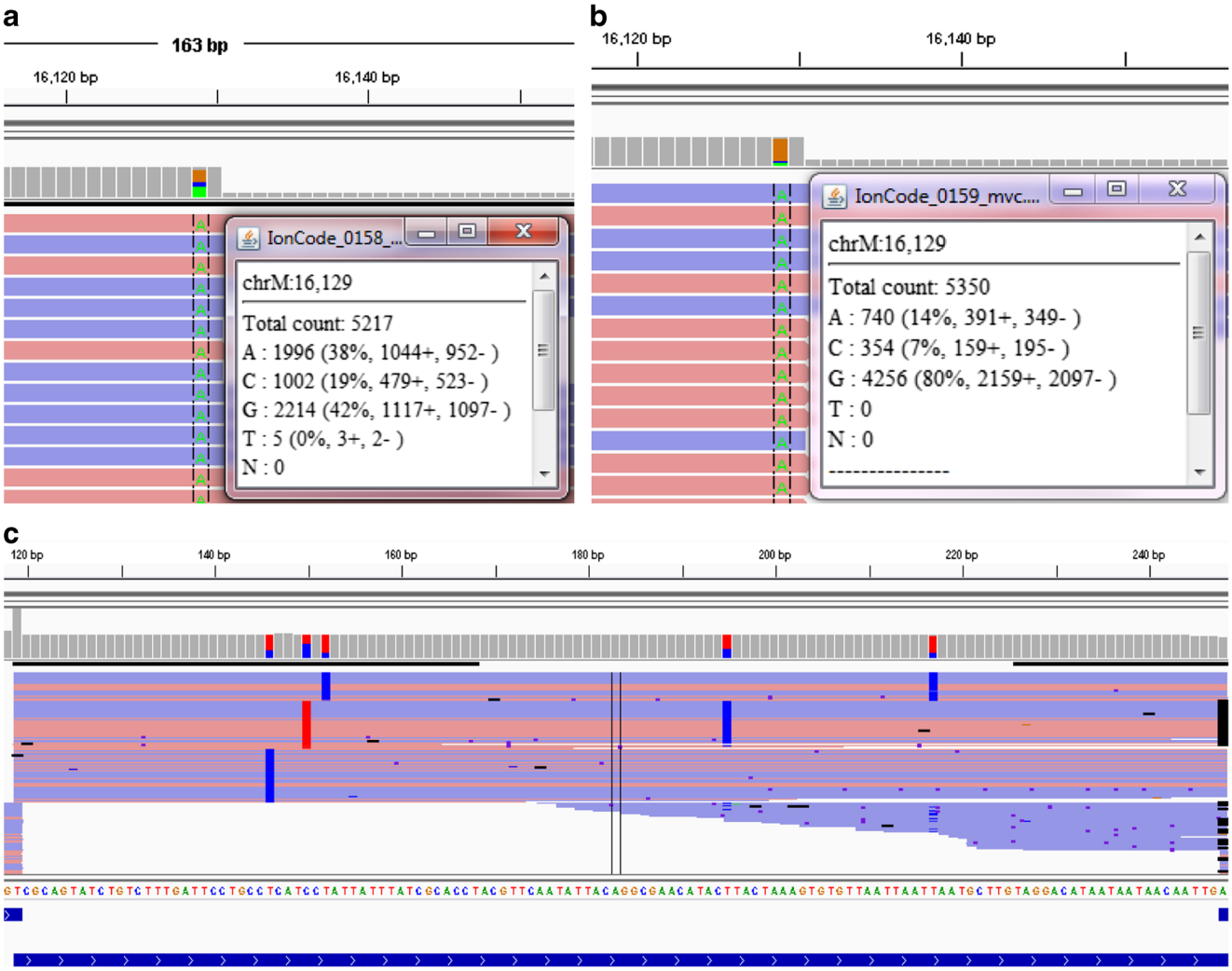

Fig. 2 Images taken from IGV of nucleotide position 16129 in the 1:1:1 (**a**) and 5:1:1 mixtures (**b**) where three different alleles from the three different contributors are present. Figure 2**c** illustrates an amplicon in the 1:1:1 mixture where sorting by base in IGV allows visualization of the three haplotypes of the three-person mixture

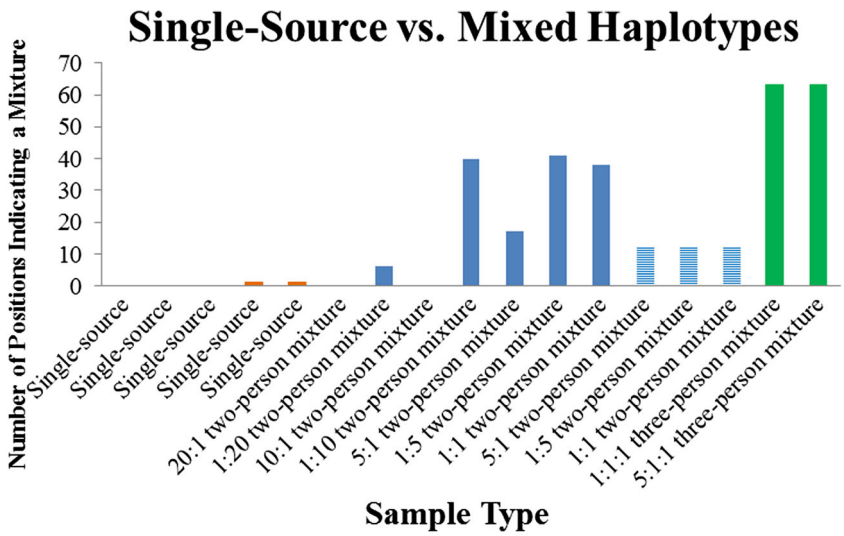

Fig. 3 The number of positions in the sequence data indicating a mixture in each single-source and mixed sample analyzed in this study. A gradual increase in the number of mixture positions is seen from single-source to more complex mixtures. A striped fill pattern is used to indicate mixtures with contributors of similar phylogenetic backgrounds

positions with the highest level of noise across the mitochondrial genome associated with homopolymeric regions in both the single-source and mixed samples.

The final comparison evaluated the number of mixture sites (or point heteroplasmies for single-source samples). King et al. [5] provided the pairwise nucleotide differences between and among haplotypes from three major US population groups. Large population studies such as these provide necessary baseline information on the number of differences that exist between samples of different and similar phylogenetic backgrounds and concomitantly the number of positions that would indicate the presence of a mixture and the potential number of contributors of a mixed sample. Thus, plotting the number of mixture positions provides insight of the potential to predict the number of contributors in a mixed sample (Fig. 3). As expected, an increase in the number of mixture sites (point heteroplasmies for single-source samples) occurs from single-source samples to more complex mixtures. The two-person mixtures exhibited a greater range of the number of mixture positions which can be attributed to two explanations: (1) the 10:1, 1:20, and 20:1 mixtures' minor contributors were not detected above the 0.1 point heteroplasmy threshold, and thus, present more similarly to that of single-source samples and (2) two-person mixtures with contributors from the same haplogroup subclade have fewer differences (on average) between the two individuals than mixtures of individuals from different haplogroups. Despite these confounding factors, the number (i.e., in actuality the distribution of number) of positions indicating a mixture may be a good indicator of the number of contributors (at least up to three) in a mixed sample.

Conclusions

MPS offers the potential for analyzing mixtures using mtDNA sequence data. This study demonstrated, in a similar manner to that of STR typing, that a quantitative approach (i.e., the ratio of alternate alleles to total read depth) can be used to properly assign allele states to major and minor contributors. Qualitatively, phasing information (i.e., multiple SNPs residing within one amplicon) and well-characterized phylogeny of the mitochondrial genome can assist in mixture deconvolution.

Analysis was effective in identifying the major contributor in two-person mixtures with nuclear DNA ratios of 1:1, 5:1, 10:1, and 20:1. SNPs associated with the minor contributor were identified in the 1:1, 5:1, and 10:1 mixtures. For the more complex three-person mixtures, parsing was more difficult but likely can be improved substantially with additional studies and a probabilistic genotyping approach. These results indicate that MPS-based approaches that sequence mitochondrial DNA may be applicable to mixture interpretation compared to analysis with current CE technologies. With continued bioinformatic developments, mitochondrial DNA mixture analysis will become more robust and could become more routine for analysis of challenging samples.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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