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Low-level point heteroplasmy detection in human mitogenomes amplified with different polymerases and sequenced on MiSeq FGx platform

Wykrywanie heteroplazmii punktowej na niskim poziomie w ludzkich genomach mitochondrialnych amplifikowanych różnymi polimerazami i sekwencjonowanych przy użyciu platformy MiSeq FGx

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Abstract

Introduction: Massively parallel sequencing of mitogenomes usually requires prior amplification. The PCR step may influence the quality of the data obtained, especially when low-level heteroplasmy detection is applied.

Aim: The aim of this study was to compare the reliability of two different DNA polymerases in detecting homoplasmic and heteroplasmic substitutions in human mitogenomes.

Materials and Methods: Mitogenomes of five samples were amplified with *Long PCR Enzyme Mix* from Fermentas or *TaKaRa LA Taq DNA Polymerase* from TaKaRa. Then, *NexteraTM XT DNA libraries* were sequenced on MiSeq FGx platform (Illumina). mtDNA substitutions were called for alternative variants above the 1% level.

Results: All homoplasmic substitutions detected in amplicons generated with polymerases studied here and sequenced on MiSeq FGx system were consistently identified as homoplasmies with alternative sequencing methods. *TaKaRa LA Taq DNA Polymerase* was found to be less accurate in low-level heteroplasmy detection than *Long PCR Enzyme Mix* enzyme as more false negative and false positive results were observed for minority variants called above the 1% level. Nevertheless, both PCR systems studied can be successfully used to detect authentic mtDNA substitutions, for which minority variants exceed the 3.61% level assuming at least 10,000x coverage and sequencing *Nextera XT DNA libraries* on MiSeq FGx machine.

Conclusions: The accuracy and sensitivity of point heteroplasmy detection with the MiSeq FGx instrument varies on polymerase used for mtDNA amplification. Therefore, it is recommended to validate the laboratory protocols used for mtDNA substitution detection prior to their implementation for the forensic or medical genetics purposes.

Keywords

mtDNA, mitochondrial genome, heteroplasmy, point heteroplasmy (PHP), massively parallel sequencing (MPS), MiSeq

Streszczenie

Wstęp: Wysokoprzepustowe sekwencjonowanie genomów mitochondrialnych wymaga zazwyczaj wcześniejszej amplifikacji tego materiału genetycznego. Etap PCR może wpływać na jakość uzyskanych danych, szczególnie, gdy stosuje się detekcję heteroplazmii na niskim poziomie.

Cel: Celem niniejszego opracowania było porównanie wiarygodności detekcji homoplazmatycznych i heteroplazmatycznych substytucji w ludzkich genomach mitochondrialnych zamplifikowanych z wykorzystaniem dwóch różnych polimeraz.

Materiał i metody: Pełne genomy mitochondrialne zamplifikowano przy użyciu *Long PCR Enzyme Mix* z Fermentas lub *TaKaRa LA Taq DNA Polymerase* z TaKaRa. Biblioteki przygotowano z wykorzystaniem *Nextera XT DNA libraries* i zsekwencjonowano przy użyciu platformy MiSeq FGx (Illumina). Substytucje w mtDNA identyfikowano dla wariantów mniejszościowych na poziomie powyżej 1%.

Wyniki: Wszystkie substytucje homoplazmatyczne wykryte w produktach PCR zamplifikowanych przy użyciu badanych polimeraz i zsekwencjonowanych na MiSeq FGx były identyfikowane jako homoplazmie za pomocą alternatywnych metod sekwencjonowania. Zaobserwowano, że *TaKaRa LA Taq DNA Polymerase* jest mniej precyzyjna w detekcji heteroplazmii na niskim poziomie niż enzym *Long PCR Enzyme Mix*, ponieważ wprowadza więcej fałszywie negatywnych i fałszywie pozytywnych wyników dla wariantów mniejszościowych powyżej poziomu 1%. Niemniej jednak, obie badane polimerazy są tak samo skuteczne w wykrywaniu autentycznych substytucji w mtDNA, dla których warianty mniejszościowe przekraczają poziom 3,61%, zakładając co najmniej 10 000 – krotne pokrycie i sekwencjonowanie bibliotek *Nextera XT DNA libraries* przy użyciu MiSeq FGx.

Wnioski: Wiarygodność i czułość detekcji heteroplazmii punktowych przy użyciu MiSeq FGx zależy od polimerazy używanej do amplifikacji mtDNA. W związku z tym, zalecane jest zweryfikowanie protokołów laboratoryjnych używanych do detekcji substytucji w mtDNA przed ich zastosowaniem w celach medycznych lub sądowych.

Słowa kluczowe

mtDNA, genom mitochondrialny, heteroplazmia, heteroplazmia punktowa (PHP), masowe sekwencjonowanie równoległe (MPS), MiSeq

Conflicts of interest statement

The authors declare no conflict of interest

Introduction

MPS (Massively Parallel Sequencing) platforms enable sensitive detection of heteroplasmic variants, which is of substantial importance in forensic and medical genetics practice [1, 2]. The threshold for detecting a minority variant using MPS technology depends on several factors that impact the quality of sequencing data and subsequent data analysis. Key factors include coverage depth, sequencing error rate characteristic for the sequencing machine, base calling quality and/or the choice of data analysis algorithms and filtration strategies [3]. Therefore, the detection threshold may vary across experiments and technologies, highlighting the importance of tailoring the analysis to specific experimental contexts and research goals. However, it should be noted that the lower the detection threshold of the minority variant, the greater the risk of calling artifacts resulting from earlier laboratory steps. In fact, the vast majority of MPS-based mitogenome sequencing procedures requires a prior mtDNA amplification. The PCR step may also impact the quality of the data obtained, including the reliability of the minority variants observed. Diverse strategies, such as amplification of long fragments (to avoid NUMTs) [4] and selection of a high-fidelity polymerase (to diminish PCR errors) [3], contribute to enhancing the accuracy and robustness of mitochondrial amplicon sequencing. First reports assuming three different polymerases: *Clontech LA Advantage* (TaKaRa Bio Inc., Kusatsu, Japan), *Long Amp* (New England Biolabs, Ipswich, MA, USA) and *Herculase II Fusion* (Agilent Technologies, Santa Clara, CA, USA) indicate substantial differences in sensitivity, specificity and precision of those enzymes [3]. Here, we analyzed the reliability of alternative polymerases, which we scrutinized across a set of five samples sequenced on the MiSeq FGx platform (Illumina).

Aim

The aim of this study was to assess the reliability of the *TaKaRa LA Taq DNA Polymerase* (Takara Bio Inc., Japan) and the *Long PCR Enzyme Mix* (Fermentas) in detecting substitutions in human mitochondrial genomes.

Materials and methods

Ethics approval and biological material

The protocols used in the present study were approved by the Bioethics Committee of the Nicolaus Copernicus University, Collegium Medicum in Bydgoszcz, Poland (statement no. KB 432/2008). The leftover, DNA isolates that had previously been sequenced with 454 and dideoxy methods as described by Skonieczna et al. [5] were used in this study. Those DNA extracts were previously acquired using a *GeneMATRIX™ Bio-Trace DNA Purification Kit* (EURX, Gdańsk, Poland) from normal colon tissues obtained from Polish colorectal cancer patients. Samples investigated in this study were selected from the set of reliable haplotypes that had previously been determined using the 454 method, validated for the detection of at least 1% heteroplasmy. Sample inclusion criteria were assumed, the availability of DNA extract for further mtDNA amplification and the presence of at least one heteroplasmic position in the haplotype.

Laboratory methods

In this study we have selected two polymerases, which were not previously compared in terms of accuracy of mitogenome amplification for the purposes of massively parallel sequencing. *TaKaRa LA Taq DNA Polymerase* (Takara Bio Inc., Kusatsu, Japan) was subjected to analysis as this enzyme is a part of the *Nextera XT DNA Library Preparation Kit* protocol dedicated by the manufacturer for mitogenome library preparation for sequencing on Illumina platforms. *Long PCR Enzyme Mix* (Fermentas) was chosen as this enzyme has a lower overall error rate than TaKaRa**.**

PCR reactions were performed according to Fendt et al. protocol [6] using *TaKaRa LA Taq DNA Polymerase* (Takara Bio Inc., Kusatsu, Japan) or *Long PCR Enzyme Mix* (Fermentas). Amplification reaction was conducted on a thermal cycler (GeneAmp PCR System 9700 form Applied Biosystems, Foster City, California) in a total volume of 50 µl assuming 2.5 mM $MgCl_2$, 0.4 mM each dNTP, 10 pmol of each primer, 2.5 – 5U *TaKaRa LA Taq DNA Polymerase* (Takara Bio Inc., Kusatsu, Japan) or *Long PCR Enzyme Mix* (Fermentas). The thermal profile for mitogenome amplification described by Fendt et al. [6] was used without any changes. NGS libraries were prepared with the *Nextera™ XT DNA Library Preparation Kit* (Illumina, United States) and *Nextera™ XT Index Kit* (Illumina, United States). Pooled NGS libraries were sequenced using the *MiSeq™ Reagent Kit v3 (600-cycle) Kit* (Illumina, United States) in the same run of the MiSeq FGx System (Illumina, United States) to generate paired-end reads, 2 x 300 bp in length.

Bioinformatic analysis

Bioinformatic analysis was performed as previously described in Skonieczna and Grzybowski [7] by using FastQC software version 0.11.9 [8] and mtDNA-Server [9]. PHP (point heteroplasmy) was identified when at least 40 reads represented minority variant assuming per base quality PHRED score above 30 and similar forward to reverse reads (F/R ratio) as that observed for the majority variant. Statistical significance of the difference between the F/R ratio between minority and majority variants were calculated with Chi-squared test with Yates' correction. BAM files were manually checked using IGV software to confirm the mtDNA substitutions reported. Homopolymer tracts between 303 – 315 and 16180 – 16193 nucleotides were not considered during the analysis. Substitution error rates for MiSeq FGx data was calculated according to the formula described by McElhoe and Holland [10]. False negatives were defined when expected variants (determined with dideoxy and/or 454 method) could not be found in MiSeq FGx sequencing results. False positives were determined when mtDNA variant called with MiSeq FGx platform was not observed in dideoxy and/or 454sequencing data.

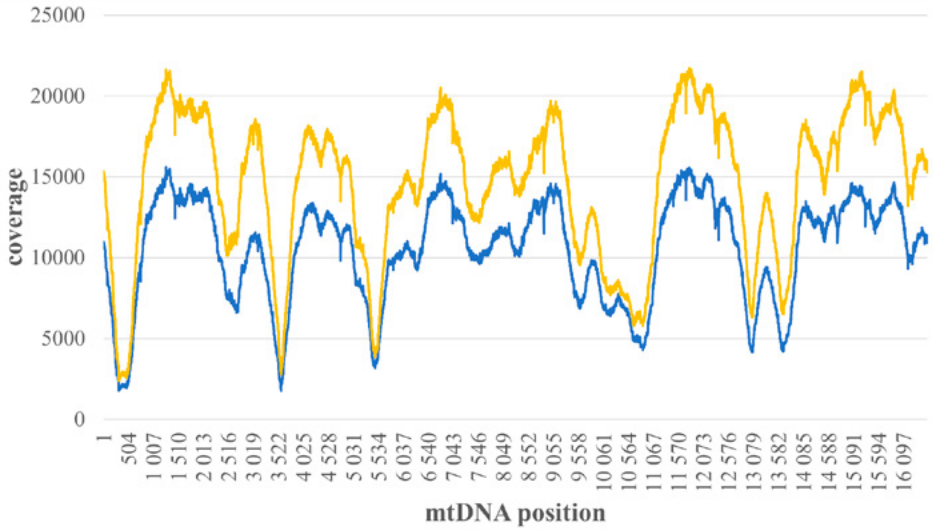

Figure 1. Mean coverage of mtDNA sequences amplified with Long PCR Enzyme Mix from Fermentas (blue plot) or TaKaRa LA Taq DNA Polymerase from TaKaRa (yellow plot)

Rycina 1. Średnie pokrycie sekwencji mtDNA zamplifikowanych z wykorzystaniem Long PCR Enzyme Mix z Fermentas (linia niebieska) lub TaKaRa LA Taq DNA Polymerase z TaKaRa (linia zółta)

Results

Average substitution error rates for MiSeq FGx reads obtained in this study was 0.06% (± 0.01%). Mean coverage (Fig. 1) was about 10,000x for mtDNA amplified with *Long PCR Enzyme MiX* (Fermentas) and about 14,000 for *TaKaRa LA Taq DNA Polymerase* (TaKaRa). The coverage dropped twice as much in positions 208 – 588; 3446 – 3675; 5380 – 5550; 10648 – 10905; 12993 – 13079 and 13608 – 13740 (Fig. 1). The lowest regions covered, for which sequencing depth reaches about 2000 were within the range from 290 to 501 and from 3547 to 3596 nucleotide positions in mitogenomes (Fig. 1). Thus, assuming the obtained coverage depth and bioinformatics criteria for calling minority variants, the level of PHP detection was set here to 1%.

All homoplasmic variants in the five analyzed samples were correctly detected in Fermentas as well as in TaKaRa amplicons sequenced with the MiSeq FGx platform. Indeed, alternative methods (dideoxy and 454 sequencing) gave the same set of homoplasmic substitutions for all samples reanalyzed here with the SBS (sequencing by synthesis) method on the MiSeq FGx platform. Thus, haplotypes obtained here after amplification with Fermentas or TaKaRa enzymes were correctly assigned to the known mtDNA haplogroups (Tab. S1).

Detection of heteroplasmic substitution in PCR products amplified with Fermentas polymerase

Sequencing of Fermentas PCR products with MiSeq FGx platform revealed seven heteroplasmic substitutions at the level from about 1% to about 40% in five analyzed samples (Tab. S1). All of the PHPs above the 2% level were also previously observed after 454 sequencing on GS FLX platform (Roche Diagnostics). Two out of seven (~29%) heteroplasmic substitutions, for which MiSeq FGx sequencing of Fermentas PCR products showed minority variants at the ~1% level were left undetected using alternative MPS method (Fig. 2, Tab. S1).

The frequency difference of the minority variants (excluding potential false positives) between the MiSeq FGx and GS FLX runs ranged from about 1% to 5.87%. One false negative result for Fermentas amplicons (Fig. 2) was observed for PHP that localized at mitochondrial position 16526, for which 454 sequencing showed guanine at the 8% level (Tab. S1).

PHP detection in PCR amplicons generated with TaKaRa enzyme

Sequencing mitogenomes amplified with TaKaRa enzyme revealed eleven point heteroplasmies in four out of five analyzed samples (Tab. S1). Minority variant level ranged from about 1% to 38%. Only four of these PHPs (~36%) were observed in Fermentas amplicons sequenced on MiSeq FGx or GS FLX platforms (Tab. S1). Seven false positive mutations (Fig. 2), for which minority variant did not exceed 2% level were observed in 40% of samples (no. N033 and N110) amplified with TaKaRa polymerase (Tab. S1). Moreover, two false negatives (Fig. 2) with minority variants at the level of 3.6 – 5% (according to MiSeq and GS FLX results, respectively) and at the 8% level (according to GS FLX data) were observed in the D-loop region of samples no. N034 and N098 (Tab. S1), respectively. The frequency difference of the minority variants (excluding potential false positives and false negatives) between the MiSeq FGx and GS FLX runs ranged from about 1% to 14.27%.

Discussion and conclusions

DNA polymerases used for mtDNA amplification manifest diverse error rates, consequently impacting the accuracy of *in vitro* replication. Notably, the exact replication fidelity is crucial in preserving the authenticity of the original mtDNA template. Hence, careful selection of a high-fidelity DNA polymerase tailored for mtDNA amplification emerges as a critical determinant in upholding sequence reliability. Simultaneous-

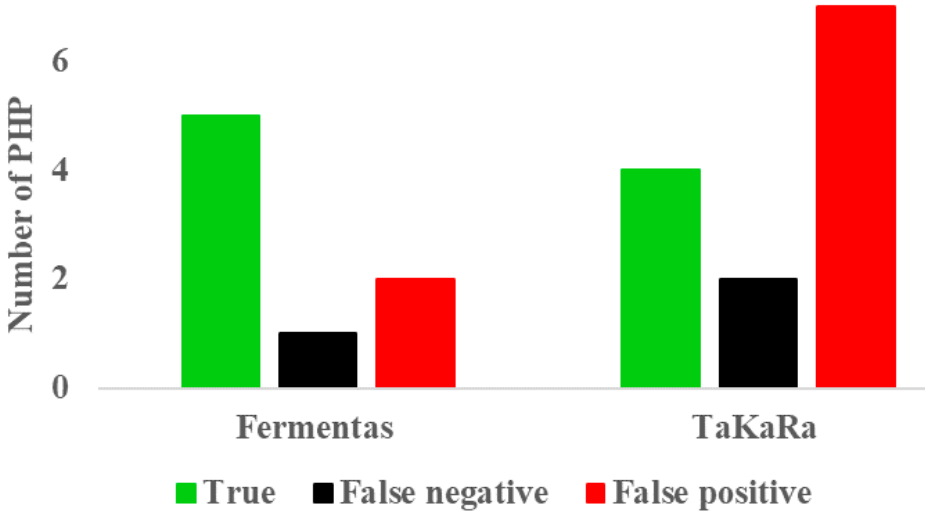

Figure 2. PHPs detected with MiSeq FGx in amplicons generated with Long PCR Enzyme Mix (indicated as Fermentas) or TaKaRa LA Taq DNA Polymerase (indicated as TaKaRa)

Rycina 2. Heteroplazmatyczne substytucje wykryte z wykorzystaniem MiSeq FGx w amplikonach uzyskanych przy użyciu Long PCR Enzyme Mix (oznaczono jako Fermentas) lub TaKaRa LA Taq DNA Polymerase (oznaczono jako TaKaRa)

ly, the analysis of mitochondrial sequence variation becomes more precise and attains heightened accuracy through harnessing MPS technologies. Indeed, MPS enables exact determination of the minority variant percentage contribution at a specific mtDNA site. Moreover, it is currently possible to identify a minority variant at a much lower level than that achievable with the traditional dideoxy method. Certainly, assuming background noise level of the sequencing technology used along with exact coverage depth, it is now possible to detect PHPs below the 10% level. For instance, by employing SBS on the MiSeq platform (Illumina), Holland et al. [11] detected heteroplasmy at the level as low as 2%, while Fazzini et al. [3] or Kloss-Brandstätter et al. [12] reached even lower levels accounting to 1%. Thus, selection of the appropriate high-fidelity enzyme for mtDNA amplification is of a pivotal importance as determining authentic mtDNA profile in forensic genetics is critical for persons identification or investigation of maternal relationships.

Here we compared the accuracy of two PCR enzymes: *TaKaRa LA Taq DNA Polymerase* from TaKaRa and *Long PCR Enzyme Mix* from Fermentas that were used for the entire mitogenome amplification. The strength of this study lies in the usage of the same DNA extracts and the same laboratory procedures to generate MPS libraries, which were sequenced in the same MiSeq FGx run. Also, the same bioinformatic pipeline was used for raw data analysis, thus the whole process of mtDNA haplotypes determination differed only in the PCR step for which TaKaRa or Fermentas DNA polymerase was used. The limitations of this study relate both to the small number of samples analyzed (five) and to the fact that not all polymerases known so far have been subjected to scrutiny. The focus was on the most commonly used one (*TaKaRa LA Taq DNA Polymerase* form TaKaRa) and the one that has a lower overall error rate (*Long PCR Enzyme Mix* from Fermentas). It should be noted, however, that no empirical comparison of these two sets of reagents has been published so far. Despite the fact that the results of the present study require further verification on a larger number of samples, the data presented here and that reported by Fazzini et al. [6] clearly shows that the selection of polymerase for mitogenome amplification may have a significant impact on determining the mtDNA variability at the lowest level, and must be taken into account in analyses performed for the purposes of forensic or medical genetics.

Irrespective of the limitations of this study, we were able to show that TaKaRa enzyme gives two times more false negative and 3.5 times more false positive PHPs than polymerase mix from Fermentas. The observation that points to the higher fidelity of Fermentas enzymes and corresponds well with the overall polymerase error rate, which is about seven times lower for Fermentas in comparison to TaKaRa reagents (about 1×10^{-5} and 7×10^{-5} for those polymerases, respectively, is based on manufacturers' specifications and reports of Hestand et al. [13] and McInerney et al., [4]). The largest differences in the level of detected minority variant reached approx. 13%, for the positions located in the control region and about 5.5% for the sites in the coding region (Table SI). However, both polymerases consistently uncovered the same panel of alternative alleles, for which minority variant exceeds the 3.61% level and none of them gave phantom mutations in the dataset of the present study. (Table SI). Moreover, these haplotypes (with minority variant above 3.61% level) are coherent with those determined with alternative 454 technology and previously published by Skonieczna at el. [5] beside one heteroplasmy that hit position 16526. Indeed, sequencing sample no. N098 on GS FLX platform has shown guanine at the level of 8% at 16526 position, whereas MiSeq FGx run allowed to identify only homoplasmic adenine in both Fermentas and TaKaRa amplicons (Table SI). It seems that the potential to detect minority variants in certain D-loop positions is diminished with MiSeq FGx sequencing runs of *Nextera XT DNA libraries*. Confirmation of this observation can be found in previous reports, which showed that control region heteroplasmies that were identified with Illumina platform had lower levels of minority variants compared to the results of alternative sequencing methods [15, 16]. Moreover, even those D-loop minority substitutions that were identified with a traditional dideoxy sequencing method could not be detected by diverse library preparation methods and Illumina sequencing protocols [15, 16]. Accordingly, the lower sensitivity to detect D-loop minority variants in MiSeq FGx data in comparison to GS FLX data may result from different library preparation methods (tagmentation versus emPCR), which may suggest some stochastic variation in this particular region.

The efficiency and fidelity of different polymerases used for mtDNA amplification was previously investigated by Fazzini et al. [3]. The authors compared three different polymerases: *Clontech LA Advantage, Long Amp and Herculase II Fusion*, which were used to detect minority variants above 1% level. Fazzini et al. [3] study showed that each of those polymerases has different sensitivity, specificity, and precision. In fact, *Herculase II Fusion* gave the best results revealing a much lower number (about 30x) of false positives than the other two being compared [3]. Together, the results described here and those published by Fazzini et al. [3] highlight the need for conducting validations to establish the reliability of protocols (including fidelity of polymerase used for mtDNA amplification) employed to determine mtDNA haplotypes. The laboratory analysis conditions and criteria for reporting reliable minority variants should be used as safeguards to uphold the authenticity of the determined mtDNA profiles. Notably, heteroplasmy has been increasingly considered when evaluating the discrimination potential of mtDNA for forensic purposes [1]. Furthermore, the mtDNA heteroplasmy analysis is more frequently used in medical and clinical genetics [2], often carrying significant implications for patients whose treatment protocols depend on

Supplementary Table I. Heteroplasmic substitutions detected in mitochondrial genomes amplified with Fermentas and TaKaRa polymerases. NS – not significant

Tabela uzupełniająca I. Heteroplazmatyczne substytucje wykryte w genomach mitochondrialnych zamplifikowanych przy użyciu polimeraz wyprudukowanych przez Fermentas i TaKaRa. NS – wynik nieistotny statystycznie

Sample	Haplogroup	Position	Localization	Majority variant	Minority variant	Coverage	% of the minority variant	Major variant	Minor variant	p-value	Coverage	% of the minority variant	Major variant	Minor variant	p-value	454 sequencing [% minority variant]	Dideoxy sequencing
								F/R ratio	F/R ratio				F/R ratio	F/R ratio			
N033	K1a1a	71	D-loop	G	T	12189	Undetected				7730	1.06	0.49	0.52	NS	NO	NO
		72	D-loop	T	G	11991	Undetected				7595	1.22	0.50	0.45	NS	NO	NO
		114	D-loop	T	C	10304	6.30	0.55	0.54	NS	6491	19.27	0.63	0.58	NS	YES [5%]	NO
		16390	D-loop	G	A	15547	1.11	1.12	1.06	NS	10136	Undetected				NO	NO
		16519	D-loop	C	T	13519	Undetected				8858	1.81	0.71	0.66	NS	NO	NO
N034	U5b2c2b	214	D-loop	A	G	5595	3.61	0.54	0.50	NS	7583	Undetected				YES [5%]	NO
		5935	MT-CO1	A	G	12466	24.13	0.93	0.80	NS	15911	29.48	0.99	0.87	NS	YES [30%]	YES
		14666	MT-ND6	T	C	15846	1.29	1.36	1.47	NS	19025	Undetected				NO	NO
N081	H1g1	13635	MT-ND5	C	T	4444	39.64	1.02	1.22	NS	6969	38.48	1.11	1.06	NS	YES [41%]	YES
N098	U5a2a1b	16526	D-loop	A	G	9426	Undetected				28230	Undetected				YES [8%]	NO
N110	J1b1a1	267	D-loop	T	C	1479	Undetected				4345	1.19	0.56	0.53	NS	NO	NO
		2083	MT-RNR2	T	C	8100					0.93	1.44	0.89	0.93	NS	NO	NO
		5462	MT-ND2	C	T	927					0.97	1.41	1.12	0.97	NS	NO	NO
		12829	MT-ND5	A	G	6368					0.67	1.01	0.65	0.67	NS	NO	NO
		16092	D-loop	C	T	5970	22.09	0.80	0.74	NS	15549	17.08	0.82	0.78	NS	YES [18%]	YES

the presence and/or load of specific pathogenic mtDNA mutations. Hence, the standardization of procedures employed in determining mtDNA profiles holds considerable significance.

Concluding, the results obtained in this study suggest that the sensitivity of low-level substitution detection with the MiSeq platform (Illumina) varies on polymerase used for mtDNA amplification. Therefore, selecting a high-fidelity enzyme tailored for amplifying mtDNA is critical to obtain authentic haplotype. We further recommend performing necessary validation of MPS protocols used to determine mitochondrial haplotypes prior to their application for the forensic or medical genetics purposes.

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