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Full Length Research Paper

Sequence analysis of mitochondrial DNA hypervariable region III of 400 Iraqi volunteers

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The aims of this research were to study mitochondrial DNA hypervariable region III and establish the degree of variation characteristic of a fragment. The mitochondrial DNA (mtDNA) is a small circular genome located within the mitochondria in the cytoplasm of the cell and a smaller 1.2 kb pair fragment, called the control region (D-loop). DNA was extracted from blood following a standard phenol-chloroform method. Polymerase chain reaction (PCR) products were purified by EZ-10 spin column then sequenced and detected by using the ABI 3130*xL* DNA analyzer. New polymorphic positions detected as T460A, C426G, T471A, T482A, A493T and C518G may in future be suitable sources for identification purpose.

Key words: Frequency, HVIII, mitochondrial DNA, polymorphic positions.

INTRODUCTION

Unlike the double-helix form of the nuclear DNA, the mtDNA is a circular molecule with only 16,569 base pairs, and is present in the cytoplasm of unfertilized ovum during the reproduction cycle. Mitochondrial DNA does not recombine and thus there is no change between parent and child, unlike nuclear DNA. mtDNA Is only passed on from mother to child and this is an important fact (Ingman and Gyllensten, 2003; Ukhee et al., 2005). There is more sequence divergence in mitochondrial than in nuclear DNA (Brown et al., 1993; Giulietta et al., 2000). Mitochondrial DNA is a useful genetic marker for answering evolutionary questions due to its high copy number, maternal mode of inheritance, and its high rate of evolution (Stoneking and Soodyall, 1996). In modern population genetics research, studies based on mitochondrial DNA (mtDNA) and Y-chromosome DNA are an excellent way of illustrating population structure while tracing uni-parental inheritance and ancestry-mtDNA is maternally inherited while the Y-chromosome is paternally inherited.

Reasons for using mitochondrial DNA rather than DNA within the nucleus includes; first, multiple copies: each mitochondrion contains its own DNA, with many copies of the circular mitochondrial DNA in every cell. It is thought

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Table 1. Primer sequence, Region amplified, Fragment size (bp) and PCR Product length for HVIII.

Primer name	Primer sequence (5' - 3') (Forward; F, Reverse; R)	Region amplified	Fragment size (bp)	PCR Product length
HVIII-1	F: 5'- TCATCAATACAACCCCCGCC -3'	482-501	20	74
	R: 5'- TTGGTTGGTTCGGGGTATGG -3'	555-536	20	
HVIII-2	F: 5'- TCTCATCAATACAACCCCCGC -3'	480-500	21	70
	R: 5'- GGTTCGGGGTATGGGGTTAG-3'	549-530	20	
HVIII-3	F: 5'- TATTTTCCCCTCCCACTCCCA -3'	450-470	21	98
	R: 5'- TTCGGGGTATGGGGTTAGCA -3'	547-528	20	
HVIII-4	F: 5'- CAATACAACCCCCGCCCAT -3'	48-504	19	78
	R: 5'- TTTGGGGTTTGGTTGGTTCG -3'	563-544	20	
HVIII-5	F: 5'- CAATACAACCCCCGCCCATC -3'	486-505	20	77
	R: 5'- TTGGGGTTTGGTTGGTTCGG -3'	562-543	20	

that each mitochondrion contains between 1 and 15, with an average of 4 to 5, copies of the DNA (Reynolds, 2000) and there are hundreds, sometimes thousands, of mitochondria per cell. The result is that there are many thousands of copies of the mitochondrial DNA in every cell. This compares with only two copies of nuclear DNA; second, better protection: the mitochondrion also has a strong protein coat that protects the mitochondrial DNA from degradation by bacterial enzymes. This compares to the nuclear envelope that is relatively weak and liable to degradation; third, higher rate of evolution: DNA alterations (mutations) occur in a number of ways. One of the most common ways by which mutations occur is during DNA replication. An incorrect DNA base may be added; for example, a C is added instead of a G. This creates a single base change, or polymorphism, resulting in a new form. These single base mutations are rare but occur once every 1,200 bases in the human genome. The result is that the rate of change, or evolutionary rate, of mitochondrial DNA is about five times greater than nuclear DNA (Bar, 2000). This is important in species testing, as even species thought to be closely related may in time accumulate differences in the mitochondrial DNA but show little difference in the nuclear DNA and finally, maternal inheritance: A further reason for the use of mitochondrial DNA in species testing, and in forensic science, is its mode of inheritance. Mitochondria exist within the cytoplasm of cells, including the egg cells. Spermatozoa do not normally pass on mitochondria and only pass on their nuclear DNA. The resulting embryo inherits all its mitochondria from its mother (Brown, 2000; Brown, 2002; Tully, 2004).

This polymorphism allows scientists to compare mtDNA from crime scenes to mtDNA from given individuals to ascertain whether the tested individuals are within the maternal line (or another coincidentally matching maternal line) of people who could have been the source of the trace evidence.

MATERIALS AND METHODS

Sample collection

Blood samples were collected from 400 healthy unrelated volunteer donors, recruited from Iraq.

Mitochonderial DNA extraction and amplification

DNA was extracted from blood following a standard phenol-chloroform method. Amplification of HVIII region was carried out using five sets of primers encompassing the three hypervariable regions, respectively (Table 1). A portion of a noncoding region encompasses positions from 438 to 574 for HVIII amplified in accordance with the Anderson reference sequence (Anderson et al., 1981). 20 µL of Master Mix was added to a PCR tube. The pipette tip was changed and 20 µL of Primer Mix added to the PCR tube. 10 µL of extracting DNA was added to the PCR tube after changing the pipette tip again. The whole liquid was allow to settle at the bottom of the tube, and not elsewhere. The volume in the PCR tube was checked using the PCR tube with 50 µL in it; the location of the tube on the grid after putting the mixture in the thermal cycler. 95°C hold for 10 min, 30 cycles of: 94°C for 30 s, 52.5°C for 30 s, 65°C for 1 min, 72°C hold for 10 min, 4°C hold, ∞ infinity is the cycling protocol for amplification of mtDNA PCR.

Purification, cycle sequencing and sequence analysis of mitochonderial DNA

Purification of mitochonderial DNA was by EZ10-spin column DNA cleanup kit 100 prep. The DNA Sequencing of the PCR products was done using the BigDye TM Terminator (Applied Biosystems). The separation of the cycle sequencing products was carried out. Detection was by using the ABI 3730xL DNA Analyzer, cap array size 96 and cap array length 50. The reference sequence described by Anderson et al. (1981) was compared to the data observed. Within the coding region Mitochondrial DNA, sequencing results are studied from a consensus sequence derived from multiple sequence results. Data were analysed by Sequencher™ (SEQUENCER™ 4.7 User Manual for Windows © 1991-2007) and aligned with the Anderson sequence (Anderson et al., 1981) using sequence Navigator software. They were accepted by stating the nucleotide position followed by the code for the polymorphic base (for example, 263G).

Statistical analysis

Genetic diversity for the analyzed DNA fragment was calculated according to the formula:

$$h=(1-\sum x_i^2)n/(n-1)$$

Where n is sample size and xi is the frequency of i-th mtDNA type (Gu, 2001).

The probability of two randomly selected individuals from a population having identical mtDNA types is:

$$(P=\sum x_i^2)$$

Where, p is the frequencies of the observed haplotypes (Jones, 1972).

RESULTS AND DISCUSSION

Haplotypes and variable positions detected in mitochonderial DNA HVIII noncoding region

The study enabled identification of 87 different haplotypes and 29 polymorphic nucleotide positions in HVIII (Table 1). The most frequent variant (H1) was consistent with the Anderson sequence. Substitutions determined during the study are transitions and transversion. This fact is consistent with abundant literature data revealing significant domination of transitions over transversions (Brown et al., 1982; Yang and Yoder, 1999; Mohammed and Imad, 2013., Imad et al., 2014a). Eleven (11) polymorphic positions, T453A, T460A, C462G, T471A, T482A, A493T, G513C, C518G, T523A, G526C and C527G have transverse substitution (Table 2). All the other substitutions determined during the analysis are transitions. The most frequent variant differed in the single position A464G, according to the CRS sequence. The number of analyzed markers has been increased to compensate for the increasing number of profiles in the databases in order to minimize accidental matches between unrelated individuals. Progression of new technology is therefore very slow and the use of SNPs has sometimes met a reluctant reception.

Mitochondrial DNA genetic diversity

Genetic diversity for the analysed DNA fragment was calculated according to the formula: D= 1-∑ *p²* and recorded 0.94%. The calculated value of the genetic diversity should be understood as high in the context of noncoding function of the analysed DNA fragment. The relatively high gene diversity and a relatively low random match probability were observed in this study.

Walsh et al. (1991) and Tang (2002) show that the polymorphism of mtDNA coding area is less than that of mtDNA control region. Therefore, more efficient polymorphic sites should be used to provide an improved discrimination power for forensic mtDNA testing (Homer et al., 2008; Holland et al., 2011; Imad et al., 2014a). As forensic markers, they should be phenotypic neutral to avoid landing investigators into serious situations of medical genetic privacy and ethnics, especially for mtDNA coding area whose mutation often correlated with an increased risk of some diseases. With the whole mtGenome sequences being researched, we are optimistic that the polymorphism sites within mtDNA coding area will be useful in combination with control region SNPs so as to increase the discrimination power of mtDNA.

There is a simple program called Mito Analyzer attached to the database which enables convenient access to information concerning polymorphic positions. In cases where there is an abundance in the sample, for example mass graves in mass disasters, there are newly discovered forensically validated methods such as ESI-MS (David et al., 2013).

Significant assistance for the research was provided by Mitomap computer database, which contains information concerning human mtDNA (Mitomap: A Human Mitochondrial Genome Database, http://www.mitomap.org) (Table 3). This database includes data about currently known variable positions, their possible association with genetic diseases, and references to the literature. There is also a simple program called Mito Analyzer attached to the database which enables convenient access to information concerning polymorphic positions.

Conclusion

Sequence analysis of the noncoding region of mtDNA (HVIII) conducted on a population of 400 unrelated individuals enabled identification of 87 different haplotypes in HVIII. New polymorphic position detected as T460A, C426G, T471A, T482A, A493T and C518G described, may be in future suitable sources for identification purpose.

Conflict of interests

The authors did not declare any conflict of interest.

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Table 2. Variable positions and haplotypes for HVIII.

Anderson	453	456	460	462	464	471	477	482	485	489	493	497	499	504	507	508	511	513	514	516	518	523	526	527	533	538	545	545	553	No. of Individual
	T	C	T	C	A	T	T	T	T	T	A	C	G	T	T	A	C	G	C	C	C	A	G	C	A	A	G	C	C	
H1*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	253
H2	-	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	T	-	1
H3	-	-	C	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	3
H4	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
H5	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	G	G	-	-	-	1
H6	-	-	-	-	-	-	-	C	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	1
H7	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	T	-	-	T	-	-	-	-	-	1
H8	C	-	-	-	-	-	-	-	-	-	-	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H9	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	1
H10	-	-	-	-	G	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H11	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-	3
H12	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	2
H13	-	-	-	-	-	C	-	-	-	-	G	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	T	2
H14	A	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-	T	-	-	-	T	-	2
H15	-	-	-	-	-	-	C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H16	-	-	-	G	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
H17	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	1
H18	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	2
H19	-	-	-	-	-	-	-	-	-	C	-	T	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H20	-	-	C	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H21	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H22	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H23	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	T	1
H24	-	-	-	T	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H25	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	T	-	-	-	-	-	-	-	-	-	-	1
H26	-	-	A	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H27	-	-	-	-	-	-	-	C	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H28	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	T	-	-	-	-	-	1
H29	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	1
H30	-	-	-	-	-	C	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H31	-	-	-	-	-	-	-	-	-	-	G	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H32	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	1
H33	-	-	-	-	G	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H34	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	1
H35	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	A	-	-	-	-	-	-	1

Table 2. Contd.

H36	-	-	-	-	-	A	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	G	-	-	-	-	1
H37	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	4
H38	-	-	-	-	-	-	-	-	-	-	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H39	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H40	-	-	-	-	-	-	-	C	-	-	-	T	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H41	-	-	-	T	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H42	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H43	C	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H44	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	1
H45	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H46	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
H47	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	1
H48	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	6
H49	-	-	-	-	-	-	C	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
H50	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	1
H51	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
H52	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H53	-	-	-	-	-	-	-	-	-	-	-	T	A	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	4
H54	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	G	-	A	-	-	-	-	-	-	-	-	-	-	-	1
H55	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	1
H56	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	2
H57	-	-	-	-	-	-	-	A	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H58	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	G	-	-	-	1
H59	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H60	-	-	-	-	G	-	-	-	C	-	-	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	-	T	-	1
H61	-	T	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H62	-	-	C	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
H63	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	T	-	-	-	-	-	1
H64	-	-	-	-	-	C	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	1
H65	-	-	-	-	-	-	-	-	C	-	-	-	-	-	C	-	-	-	-	-	-	-	-	T	-	-	-	-	-	1
H66	C	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	2
H67	-	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	1
H68	-	-	-	-	-	-	C	-	-	-	-	-	A	-	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	1
H69	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	3
H70	C	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	2
H71	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	T	2
H72	-	-	-	-	-	-	C	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	2

Table 2. Contd.

H73	-	T	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	G	-	-	-	-	-	-	-	1
H74	-	T	-	-	-	-	-	C	-	-	G	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	4
H75	-	-	C	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	1
H76	-	-	-	-	-	-	C	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	2
H77	-	-	-	-	-	-	-	-	C	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	2
H78	C	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H79	-	-	-	T	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	1
H80	-	-	-	-	G	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	A	-	-	2
H81	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	T	1
H82	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	2
H83	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	T	-	-	-	-	-	-	-	-	-	-	1
H84	-	-	C	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H85	-	-	-	-	-	-	-	-	-	C	G	-	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
H86	-	-	-	-	-	-	-	C	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-	-	1
H87	-	-	-	T	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	T	-	C	-	-	-	-	A	T	-	1
Total	-	T	-	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	400

H*, Haplotype; G, guanine; T, thiamine; C, cytosine; A, adenine.

Table 3. Types of mutations in variable positions for HVIII.

Position	Mutation	Type of mutation	Presence in Mitomap	Frequency	Frequency (%)
453	Transition	T-C	Presence	0.035	3.5
453	Transversion	T-A	Presence		
456	Transition	C-T	Presence	0.0378	3.78
460	Transition	T-C	Presence	0.040	4
460	Transversion	T-A	New*		
462	Transition	C-T	Presence	0.040	4
462	Transversion	C-G	New		
464	Transition	A-G	Presence	0.060	6
471	Transition	T-C	Presence	0.020	2
471	Transversion	T-A	New		
477	Transition	T-C	Presence	0.040	4
482	Transition	T-C	Presence	0.040	4
482	Transversion	T-A	New		
485	Transition	T-C	Presence	0.030	3

Table 3. Contd.

489	Transition	T-C	Presence	0.048	4.8
493	Transition	A-G	Presence	0.0375	3.75
493	Transversion	A-T	New		
497	Transition	C-T	Presence	0.0525	5.25
499	Transition	G-A	Presence	0.035	3.5
504	Transition	T-C	Presence	0.035	3.5
507	Transition	T-C	Presence	0.040	4
508	Transition	A-G	Presence	0.0175	1.75
511	Transition	C-T	Presence	0.0175	1.75
513	Transition	G-A	Presence	0.0325	3.25
513	Transversion	G-C	Presence		
514	Transition	C-T	Presence	0.030	3
516	Transition	C-T	Presence	0.015	1.5
518	Transition	C-T	Presence	0.0175	1.75
518	Transversion	C-G	New		
523	Transition	A-G	Presence	0.0125	1.25
523	Transversion	A-T	Presence		
526	Transition	G-A	Presence	0.0225	2.25
526	Transversion	G-C	Presence		
527	Transition	C-T	Presence	0.035	3.5
527	Transversion	C-G	Presence		
533	Transition	A-G	Presence	0.0225	2.25
538	Transition	A-G	Presence	0.015	1.5
545	Transition	G-A	Presence	0.0175	1.75
548	Transition	C-T	Presence	0.0175	1.75
553	Transition	C-T	Presence	0.020	2

New*, new polymorphic positions ; genetic diversity* , genetic diversity for the analysed DNA fragment was calculated according to the formula: $D = 1 - \sum p^2$. Genetic diversity* $D = 1 - \sum p^2 = 0.94 = 94\%$.

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