Amanda Ramos
Cristina Santos
Luis Alvarez
Ramon Nogués
Maria Pilar Aluja

Unitat d'Antropologia Biològica, Departament BABVE, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Barcelona, Spain

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Research Article

Human mitochondrial DNA complete amplification and sequencing: A new validated primer set that prevents nuclear DNA sequences of mitochondrial origin co-amplification

To date, there are no published primers to amplify the entire mitochondrial DNA (mtDNA) that completely prevent the amplification of nuclear DNA (nDNA) sequences of mitochondrial origin. The main goal of this work was to design, validate and describe a set of primers, to specifically amplify and sequence the complete human mtDNA, allowing the correct interpretation of mtDNA heteroplasmy in healthy and pathological samples. Validation was performed using two different approaches: (i) Basic Local Alignment Search Tool and (ii) amplification using isolated nDNA obtained from sperm cells by differential lyses. During the validation process, two mtDNA regions, with high similarity with nDNA, represent the major problematic areas for primer design. One of these could represent a non-published nuclear DNA sequence of mitochondrial origin. For two of the initially designed fragments, the amplification results reveal PCR artifacts that can be attributed to the poor quality of the DNA. After the validation, nine overlapping primer pairs to perform mtDNA amplification and 22 additional internal primers for mtDNA sequencing were obtained. These primers could be a useful tool in future projects that deal with mtDNA complete sequencing and heteroplasmy detection, since they represent a set of primers that have been tested for the non-amplification of nDNA.

Keywords:
Mitochondrial DNA / Nuclear DNA sequences of mitochondrial origin / Primer design
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1 Introduction

It is now accepted that mitochondria descend from prokaryotic endosymbionts and that inter-organelle DNA transfer has been rearranging genetic material between the nucleus and mitochondria. Moreover, it seems that the transfer of DNA from mitochondria to the nucleus has contributed significantly to the evolution and function of eukaryotic genomes (for a review see Leister [1]). Almost all the present-day mitochondrial DNA (mtDNA) transfers to the nucleus give rise to non-coding sequences, the so-called nuclear DNA sequences of mitochondrial origin (NUMTs). Human NUMT loci are evenly distributed within and among chromosomes [2–4], and up to 9857 bp of the human mtDNA can be found at a single nuclear locus [5]. However, only about a third of all NUMTs present in the human nuclear genome are due to insertions of mitochondrial sequences, the rest being originated as duplications of pre-existing NUMTs [6]. Moreover, a subset of human NUMTs is highly rearranged, comprising sequences derived from different regions of the organellar chromosome that have undergone inversions, deletions and duplications [3–5, 7].

A comparison of published compilations of NUMTs clearly shows significant discrepancies among data [8]. For example, in relatively recent studies, Ricchetti *et al.* [9], Mishmar *et al.* [10] and Hazkani-Covo and Dan Graur [11] identify 211, 247 and 452 NUMTs, respectively. According to Lascaro *et al.* [8] these discrepancies are due both to an unwise

Correspondence: Amanda Ramos, Unitat d'Antropologia Biològica, Departament Biologia Animal, Biologia Vegetal i Ecologia, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Barcelona, Spain
E-mail: amanda.ramosr@campus.uab.cat
Fax: +34-935811321

Abbreviations: ABC, ammonium bicarbonate; **BLAST,** Basic Local Alignment Search Tool; **mtDNA,** mitochondrial DNA; **nDNA,** nuclear DNA; **NUMT,** nuclear DNA sequences of mitochondrial origin

application of Bioinformatic methods and to the use of a not yet correctly assembled nuclear genome. Thus, to optimize the quantification and location of NUMTs, the authors produced a consensus compilation of Human NUMTs – Reference Human Numt Sequences (RHNumtS)– by applying various bioinformatics and experimental approaches. This allows the identification of 190 human NUMTs that could constitute a valuable tool in the implementation of experimental designs of mtDNA amplification and sequencing.

The human mtDNA has been widely used in population genetics, phylogeographic and phylogenetic studies, because it presents a high copy number *per* cell, a compact organization, is maternally transmitted and provides an easy access to an orthologous set of genes with little or no recombination, with rapid evolution and that are selectively neutral [12, 13]. However, this traditional view has been challenged. Many works have been published pointing out: (i) the possibility of recombination of the mtDNA [14, 15]; (ii) the hypothesis that mtDNA frequency variation is due to natural selection [16–18] and (iii) that mitochondrial DNA heteroplasmy is not an exceptional condition related to mitochondrial disease [19–27].

The majority of population studies based on the mtDNA sequencing have focused on the control region, which constitutes less than 7% of the mitochondrial genome. However, the routinary study of the coding region has been performed for a long time to study the implication of mtDNA in diseases such as cancer [28]. Moreover, in the last years, the rapid development of automated DNA sequencing technology has permitted to study the complete mtDNA genome in a large number of healthy individuals [29–35]. Many of these studies, particularly those that deal with the study of mtDNA in pathological samples, have been severely criticized and a large number of manuscripts addressing errors related to the interpretation of mtDNA results (particularly mtDNA heteroplasmy) have been published [21, 36–42]. One of the critiques reported is that NUMTs are a potential source of contamination when PCR is used to study mtDNA [42]. Although this problem has previously been considered to be muted because of the high copy number of mtDNA over the corresponding nuclear loci, caution is warranted [42–44] since amplification of overlapping NUMTs paralogous to the mitochondrial genome indicates that co-amplification of nuclear mitochondrial pseudogenes is a real problem for accurate sequence interpretation, and particularly for the interpretation of mtDNA heteroplasmy [42, 44]. Parr *et al.* [44], in a study performed with ρ0 cells, suggested that among the factors that determine whether an NUMT will or not co-amplify with mtDNA, there are the region of the mtDNA targeted by the PCR and the number of copies of the NUMT. Moreover, samples of ancient DNA or a tissue with a reduced quantity of mtDNA copy number, in both physiological (sperm) and pathological states, also seem to be important factors that could determine the amplification of NUMTs [44, 45]. Although, Goios and co-workers [44, 45] stated that, in standard sequencing of samples used in a population characterization [29, 30], the amplification of NUMTs does not constitute a real problem (since the mtDNA content of samples is much higher than the content of nuclear DNA (nDNA) and the detection of mtDNA heteroplasmy is not a priority), Yao *et al.* demonstrate that it can occur even when standard samples are used [42].

To date, primers to amplify the entire mtDNA have already been published [31, 34, 46], however, none of the published sets completely prevents the amplification of NUMTs. The main goal of this work was to design, validate and describe a set of primers to specifically amplify and sequence the complete human mtDNA, allowing the correct interpretation of mtDNA heteroplasmy in healthy and pathological samples.

2 Materials and methods

2.1 Primer design and validation

In order to analyze the whole mitochondrial genome, primers previously published [34] were tested and new ones were designed. The primer design was performed using the Lasergene 7.2 software, from the DNASTAR package, following the general criteria of primers design [47]. Particular attention was devoted to adjust, as much as possible, the melting temperatures (T_m) of all the primer pairs.

To discard co-amplifications of nDNA and mtDNA, PCR primers were submitted to the Basic Local Alignment Search Tool (BLAST) available in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) [48]. BLAST finds regions of local similarity between sequences comparing nucleotide sequences with sequence databases and calculating the statistical significance of matches. In this work, the specific tool Basic BLAST, optimized for highly similar sequences (Megablast), was performed using the *Reference genomic sequence database* for *Homo sapiens* (refseq_genomic: Genomic sequences from National Center for Biotechnology Information Reference Sequence Project). A primer pair was discarded if both primers showed homology (expected number of chance matches in a random model – E-value – lower than 1) inside the same chromosome region.

After BLAST validation, primers were further tested in PCR experiments employing total DNA standard samples (see Section 2.2) and nDNA obtained from sperm cells. The nDNA isolation was performed by sperm differential lyses using the QIAmp DNA Investigator Kit (Qiagen) according to the manufacturer's specifications. To guarantee the complete mtDNA elimination, the initial washing step mentioned in QIAmp DNA Investigator Kit manual was repeated at least four times. Moreover, to ensure the integrity of nDNA, Y-chromosome STRs were amplified using AmpFlSTR® Y-filer™ PCR kit (Applied Biosystems), under conditions recommended by the manufacturer and all the markers were correctly amplified.

Table 1. Validated primers to amplify the complete mtDNA in nine overlapping fragments. Melting temperatures (T_m) and annealing temperature (T_a) for each pair of primers are also presented

Fragment	Fragment length	Name	Sequence (5′–3′)	Primers pair length (bp)	T_a	T_m
1	1822	14898for	tagccatgcactactcaccaga	22	60	60.3
		151rev	ggatgaggcaggaatcaaagac			
2	1758	16488for[a)]	ctgtatccgacatctggttcct	22	60	60.3
		1677rev[a)]	gtttagctcagagcggtcaagt			
3	2543	1404for[a)]	acttaagggtcgaaggtggatt	22	57	58.4
		3947rev	tcgatgttgaagcctgagacta			
4	3005	3734for	aagtcaccctagccatcattcta	23	61	58.9
		6739rev	gatatcatagctcagaccatacc			
5	2709	6511for	ctgctggcatcactatactacta	23	58	58.9
		9220rev	gattggtgggtcattatgtgttg			
6	1738	8910for[a)]	cttaccacaaggcacacctaca	22	61	60.3
		10648rev	ggcacaatattggctaagaggg			
7	1866	10360for	gtctggcctatgagtgactaca	22	61	60.3
		12226rev	cagttcttgtgagctttctcgg			
8	1853	11977for	ctccctctacatatttaccacaac	24	63	59.3
		13830rev	aagtcctaggaaagtgacagcga	23		60.6
9	1872	13477for[a)]	gcaggaatacctttcctcacag	22	63	60.3
		15349rev[a)]	gtgcaagaataggaggtggagt			

a) Oligonucleotides are from Torroni *et al.*, [34].

2.2 Total DNA extraction, amplification and sequencing of the entire mtDNA

Total DNA from blood samples was extracted using JETQUICK Blood DNA Spin Kit (Genomed) and isoamilic phenol-chloroform [49].

Optimized primers and PCR conditions for the mtDNA amplification are reported in Table 1. The PCR mix for each sample consisted of 50 pmol of each primer, 200 μM of each dNTP, 2 mM of $MgCl_2$, 10 × NH_4-based reaction buffer, 1 U of *Taq* DNA polymerase, and 30 ng of DNA in a final volume of 50 μL. The PCRs were performed in a G-Storm GS1 thermocycler and the amplification program consisted of an initial denaturation step of 5 min, followed by 35 cycles of PCR (1 min at 94°C, 40 s at annealing temperature (T_a) and 2.5 min at 72°C), and a final extension step of 5 min at 72°C. The PCR amplification results were visualized by electrophoresis in agarose gels (2%).

PCR products were purified using the JETQUICK PCR Purification Spin Kit (Genomed) and the mtDNA was fully sequenced in both strands using 62 primers (Table 2). Sequence reactions were carried out using the sequencing kit BigDye Terminator v.3 (Applied Biosystems) according to the manufacturer's specifications and were run in an ABI 3130XL sequencer (Servei de Genòmica, Universitat Autònoma de Barcelona).

2.3 Cloning

PCR products that, in the optimization process, show consistently more than a one size product, were cloned into the pCR®4-TOPO® vector, using the TOPO TA Cloning® Kit for Sequencing (Invitrogen). Ten clones were sequenced using the previously explained methodology.

3 Results

3.1 Primer design and validation

From the primers reported in the literature for the complete mtDNA amplification, we evaluated the 11 primer pairs published by Torroni *et al.* [34] since, compared with other works, the authors define a small number of fragments (see Table 2 in Torroni *et al.* [34]). These primers were tested using BLAST. After this analysis, four pairs of primers that show high similarity with nuclear regions were rejected.

The BLAST analysis allows detecting two major problematic regions for primer design. The first one, located between 3914 and 9074 mtDNA positions, presents a 98% similarity with a region within chromosome 1 (see BLAST results as online Supporting Information data); the second one encompasses the region located between 9582 and 14479 mtDNA positions and it shows a 88% similarity with a region within chromosome 5 (see BLAST results as online Supporting Information data). For these regions new primers were designed and verified until the selective amplification of mtDNA was obtained. The 3914–9074 region (Fig. 1) was split into two fragments (fragments 4 and 5 in Fig. 1); For each primer pair, one of the primers was designed outside the 3914–9074 region, preventing the amplification of the homologue region of chromosome 1. For the 9582–14479 region (Fig. 1), minimum of four fragments with ~2000 bp were necessary to completely amplify it. Thus, the previous strategy could only be applied

Table 2. Primers designed to sequencing the whole mtDNA after PCR amplification in nine overlapping fragments

Fragment	Name/sequence (5′–3′)				
1	14898for	15416for	15966for		
	tagccatgcactactcaccaga	tacacaatcaaagacgccctc	agtctttaactccaccattag		
	151rev	16281rev	15825rev		
	ggatgaggcaggaatcaaagac	gttggtatcctagtgggtgag	gtgaagtatagtacggatgct		
2	16488for	411for	909for		
	ctgtatccgacatctggttcct	cggtatgcacttttaacagtc	gattaacccaagtcaatagaa		
	1677rev	1159rev	638rev		
	gtttagctcagagcggtcaagt	taagctgtggctcgtagtgt	ggtgatgtgagcccgtctaaa		
3	1404for	2028for	2646for	3239for	
	acttaagggtcgaaggtggatt	gatagaatcttagttcaactt	ggttcagctgtctcttacttt	gcagagcccggtaatcgcata	
	3947rev	3382rev	2801rev	2263rev	
	tcgatgttgaagcctgagacta	ttcgttcggtaagcattagga	taatgcaggtttggtagttta	gggtgtgaggagttcagttat	
4	3734for	4346for	4896for	5468for	5995for
	aagtcaccctagccatcattcta	gaacccatccctgagaatcca	taccaaatctctccctcacta	cacgctactcctacctatctc	cagctctaagcctccttattc
	6739rev	6154rev	5571rev	5017rev	4421rev
	gatatcatagctcagaccatacc	ggaactagtcagttgccaaag	aagtattgcaacttactgagg	gaggagtatgctaagattttg	cttatttagctgaccttactt
5	6511for	7111for	7713for	8311for	
	ctgctggcatcactatactacta	acaccctagaccaaacctacg	tcctaacactcacaacaaaac	tagcattaacctttaagtta	
	9220rev	8600rev	8000rev	7359rev	
	gattggtgggtcattatgtgttg	agaatgatcagtactgcggcg	caacgtcaaggagtcgcaggt	ctactattaggacttttcgct	
6	8910for	9393for	9874for		
	cttaccacaaggcacacctaca	cgagaaagcacataccaaggc	taatatttcactttacatcca		
	10648rev	10154rev	9647rev		
	ggcacaatattggctaagaggg	ttctatgtagccgttgagttg	agctcaggtgattgatactcc		
7	10360for	10892for	11461for		
	gtctggcctatgagtgactaca	atcaacaacaacctatttagc	actcttaaaactaggcggcta		
	12226rev	11673rev	11163rev		
	cagttcttgtgagctttctcgg	gtttggatgagaatggctgtt	cgggtgatgatagccaaggtg		
8	11977for	12500for	12988for		
	ctccctctacatatttaccacaac	tgtgcctagaccaagaagtta	ctagcagcagcaggcaaatca		
	13830rev	13297rev	12763rev		
	aagtcctaggaaagtgacagcga	ggttgatgccgattgtaacta	cgatgaacagttggaataggt		
9	13477for	13950for	14440for		
	gcaggaatacctttcctcacag	ctatctaggccttcttacgag	atactcctcaatagccatcgc		
	15349rev	14838rev	14325rev		
	gtgcaagaataggaggtggagt	catcatgcggagatgttggat	aactttaatagtgtaggaagc		

for flanking fragments (fragments 6 and 9 in Fig. 1), and the primers used to amplify the two fragments that were placed completely within the region (fragments 7 and 8 in Fig. 1) were designed in low-sequence similarity regions.

After BLAST validation, PCR optimization was performed using three samples of total DNA extracted using JETQUICK Blood DNA Spin Kit (Genomed). Surprisingly, the outcome of the amplifications of fragments between positions 12012–13828 and 3798–6739 resulted in more than one PCR product. However, a new amplification with a different sample isolated with isoamilic phenol-chloroform [49] was carried out and only one PCR fragment (with the expected size) was obtained (Fig. 2). These results allow to hypothesize that: (i) the observed additional bands correspond to deletions in mtDNA; (ii) the amplification of an NUMT not detected by BLAST analysis; (iii) the extraction method influences the DNA quality and by consequence the PCR performance and specificity, resulting in unspecific amplification of nuclear or mtDNA. To test these hypotheses the fragment 12012–13828 was cloned into the pCR®4-TOPO® vector, using the TOPO TA Cloning® Kit for Sequencing (Invitrogen) and ten clones were sequenced. The resulting sequences were tested with BLAST showing a complete homology with mtDNA. A careful analysis reveals that the PCR forward primer hybridized with two close regions of mtDNA: the target region and another one that present several differences with the primer. Therefore, the amplification result is an artifact that can be attributed to the poor quality of DNA. For this reason, the forward primer was redesigned and tested again as previously described.

Finally, to discard co-amplifications of nDNA and mtDNA, which could not be deduced by BLAST analysis, PCR amplifications were performed using isolated nDNA obtained from sperm cells. The PCR primers were tested and no positive amplifications were observed.

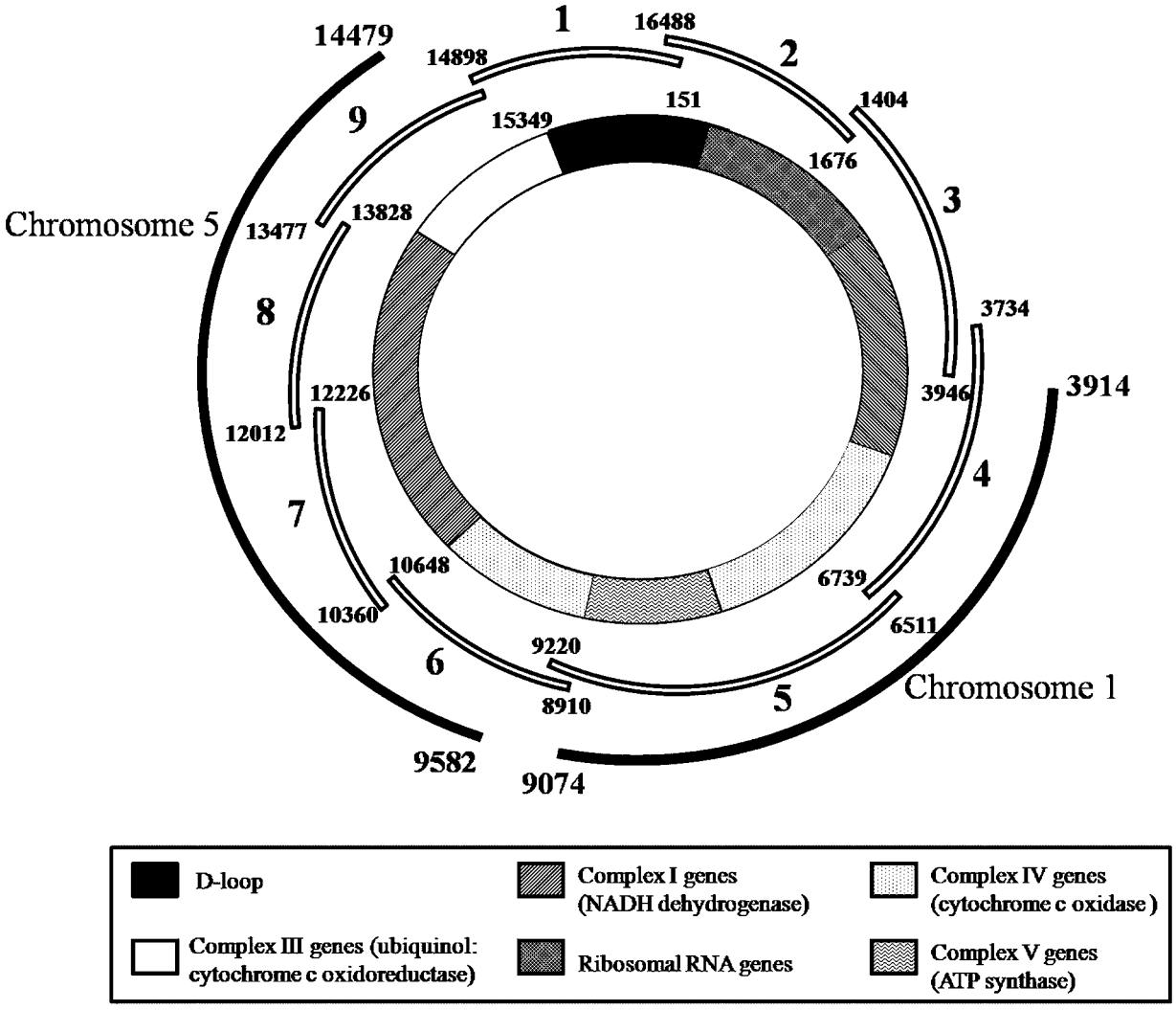

Figure 1. Schematic representation of mtDNA. The nine overlapping fragments defined to PCR amplify the complete mtDNA genome are represented as well as the two nuclear regions with high homology with mtDNA.

3.2 PCR and sequencing primers to mtDNA analysis

For these primers, validated as previously explained, melting temperatures were adjusted as much as possible and the annealing temperatures were obtained by a PCR gradient being selected the highest temperature that shows positive amplification. In Table 1 PCR primers and annealing and melting temperatures to amplify the nine overlapping fragments that cover the entire mtDNA (Fig. 1) are reported. Some of the primers were previously published by Torroni *et al.* [34] and the remaining ones were designed for this study.

Since the size of fragments is of ~2000 bp, internal primers were designed to fully sequence both strands of each fragment. In Table 2 the sequence of the primers to sequence each fragment in both stands are shown. All the primers were tested to sequence three samples and a good efficiency of sequencing was obtained.

4 Discussion

The PCR amplification and sequencing of mtDNA is routinely used in many research fields; however, the interpretation of results, particularly of mtDNA heteroplasmy, could be complicated by the co-amplification of NUMTs [42, 44]. Therefore, an effort to design PCR primers that selectively amplify mtDNA must be performed. In this work we report a set of primers to amplify the whole mtDNA that were validated using the BLAST analysis and by the performance of PCR amplifications using nDNA isolated from sperm cells.

The BLAST search allowed the identification of two problematic regions in primer design (region 3914–9074 of the mtDNA present in chromosome 1 and region 9582–14479 present in chromosome 5). Region 3914–9074 was previously reported [8] as presenting a high sequence similarity with a region within chromosome 1, a result

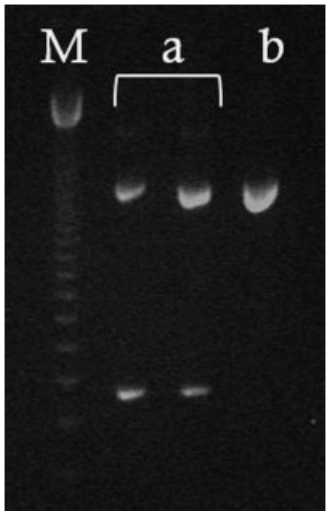

Figure 2. Gel electrophoresis showing amplification results between positions 12012 and 13828 using blood samples extracted with different methods. (a) JETQUICK Blood DNA Spin Kit (Genomed); (b) isoamilic phenol-chloroform.

that is in accordance with our results. However, the presence of an NUMT in chromosome 5, which shows a high identity with the 9582–14479 region of the mtDNA, was not reported in previous studies; accordingly, this region could represent a non-reported NUMT and this deserves further investigation.

According to Goios *et al.* [45] the amplification of NUMTs can only be obtained when mtDNA is almost completely removed from the samples, such as those resulting from preferential semen lyses. Thus, to test the proposed set of primers for the selective amplification of mtDNA, we used a sperm sample that was submitted to DNA extraction using four steps of preferential lyses and for which Y-STRs were successfully amplified and no signs of amplification with the designed primers were detected. This result indicates that the proposed primers specifically amplify mtDNA and we can ensure that no NUMTs will be amplified if this set of nine pair primers is used.

The primers proposed amplification regions of about 2000 bp of mtDNA. As a consequence, in some samples (such as ancient and forensic ones) it would be almost impossible to obtain PCR amplifications with this set of primers. To overcome this problem, we suggest that additional primers within each fragment could be designed using a similar strategy to that applied for fragment 9582–14479, that is, locating primers in regions that show low homology with nDNA.

5 Concluding remarks

In conclusion, we report a set of primers that permit the selective amplification and sequencing of the whole mtDNA in nine overlapping fragments. These primers could be a useful tool in future projects that deal with mtDNA complete sequencing since they represent the first published set of primers tested for the non-amplification of nDNA and therefore suitable for the screening of mtDNA heteroplasmy in both standard and pathological samples.

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