Primer designing strategy for amplification and sequencing of the complete mitochondrial genome of *Semnopithecus hypoleucos*

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Abstract

The mitochondrial genome is highly informative for evolutionary analysis of organism lineages and phylogenetic studies. The availability of robust primers for amplifying complete mitochondrial genomes is a crucial step in current mitogenome studies. However, organism specific characteristics such as variable transition to transversion substitution ratios seen in some groups pose challenges for the development of universal, or at least broadly applicable, primer pairs for this purpose. This study reports on a strategy of primer design and optimization (PDO) where regions of known mtDNA genes can be used for choosing primers for amplification, sequencing and assembly of entire mitochondrial genomes of several closely-related species. In brief, taking advantage of the circular organization of mtDNA, primers are first designed for amplification of “long” products using the 5’ region of one conserved gene and a 3’ region from another conserved gene. Additional primers are then used to amplify “short” regions to fill in gaps to allow for complete assembly of the genome. We show how we were able to use this approach to successfully amplify entire mitochondrial genomes from a non-human primate species (*Semnopithecus hypoleucos*), and also how this provided data useful for annotation of the assembled genome data.

Background

A thorough understanding of genetic diversity is an important step for developing appropriate conservation plans for any group of organisms (Oakenfull et al. 2000). Mitochondrial (mt) DNA has become popular for these studies since it provides rich sets of information relevant to evolutionary biology, population genetics and phylogenetics through its maternal inheritance and relatively high mutational rates (Avise and Saunders 1984; Avise 1986; Dasmahapatra et al. 2010; Nabholz et al. 2019). Moreover, the high copy number and circular nature of mtDNA tends to make it less prone to degradation and therefore may provide material for complete analysis compared, for example, to nuclear sequences. These qualities have shown mtDNA to an important genetic tool in tracking large scale comparative studies of evolutionary relationships among individuals, populations and species.

Construction of phylogenetic trees is a useful tool for analyzing evolutionary relationships of genes between species. Many of these studies rely exclusively on a small part of the mtDNA, such as cytochrome oxidase subunit I (COI) (Webb and Moore 2000; Kerr et al. 2009; Khedkar et al. 2016a); cytochrome b (cyt b) (Chang et al. 2010; Khedkar et al. 2016b), or others. Such approaches are known, however, to underestimate the influence of variation seen in the complete mitochondrial genome on evolutionary processes (Springer et al. 2012; Pozzi et al. 2014). For example, comparative studies of protein coding genes tend show high levels of similarity compared to non-coding regions which can be more highly variable. It is also well known that certain parts of the mitochondrial genome, such as the D-loop region evolve faster than the highly conserved 16S rRNA and 12S genes (Gerber et al. 2001). This implies that phylogenetic relationships among species are better inferred from the use of the complete mitochondrial genome sequences.

Although several complete mitochondrial genome sequences have been published (Matsui et al. 2009; Li et al. 2009; Kim et al. 2009; Ma et al. 2010; Kurabayashi et al. 2010; Finstermeier et al. 2013; Pozzi et al. 2014; Zhang et al. 2017), data for several species and/or species groups is still incomplete due to technical problems related to the availability of robust primers (Ramos et al. 2011; de Freitas et al. 2018). This is especially true for closely-related species such as some of those belonging to primate clades. In some groups such as humans, for example, high mutation rates in the mtDNA can lead to a high degree of variability between individuals (Howell et al. 1996; Wilson et al. 1985). In other primates, the transition to transversion substitution ratio was found to be high in mtDNA (Brown et al. 1982).

Generally three strategies (described below) are in use for obtaining complete mitochondrial genome sequences, but each of them still include procedural challenges (Rizzi et al. 2012),

- i. Direct isolation of mitochondria from the tissue following the nucleic acid purification and direct sequencing through an NGS platform. This method requires large quantities of tissue, and even commercial kits available may not be adequate when dealing with non-invasive methods as well as old, museum samples.
- ii. Obtain total genomic DNA, and then sequence the whole genome and extract the mitochondrial genome sequences through bioinformatics procedures. A challenge here is that the bioinformatics analysis demands infrastructure and expertise that may be hard to come by.
- iii. Obtain genomic DNA from specific tissues followed by enrichment of mitochondrial DNA through PCR and primer walking. This approach requires robust primer design capable of covering the entire mitochondrial genome, or at least fragments of the genome, which can be combined to cover entire region of interest. However, for some primate species, many primers do not show applicability for cross amplification of mitochondrial DNA from related species. This may be a reason that very few primate mitochondrial genomes have been published to date as compare to other organisms (Roos et al. 2011).

Our study reports a method for designing primers that can be effectively applied in amplification of entire mitochondrial genomes of *S. hypoleucos* an endangered primate species in India and may this strategy can be applied to closely related primate species. Primer pairs are specifically designed for covering both large and small segments of the mitochondrial genome which demonstrate amplification challenges.

Materials And Methods

Ethical Statement

We did not perform experimentation directly on any animals; therefore ethical permission was nonobligatory for this study. The authors do not have conflict of interest to declare.

Experimental outline

The flowchart of the primer design and optimization (PDO) protocol is provided (Fig. 1). Some of the important steps of the PDO method are discussed in the following section.

Downloaded reference mitochondrial sequences

For the initial design of robust primer pairs, 25 whole mitochondrial genome sequences were downloaded from NCBI Genbank and other reference sequence databases (Table 1). Among 25 species studied here, 16 belonged to Colobinae family, two are from the Ponginae, two are from the Homininae, two are from Cercopithecinae, and one each from the Cebinae, Gorillinae and Hylobatidae.

Alignment of Sequences

The mt DNA sequences of these primate species were aligned using CodonCode aligner. Aligned regions longer than typical primer sequences were selected to represent conserved sequences, and forward and reverse primers were designed from them.

Primer design and testing its applicability in primate clade

Primer design is a critical part of any PCR based study. Considerations for primer design include: (i) primer melting temperature, (ii) length and GC content of the primer, (iii) resultant PCR amplified product length, (iv) formations of hair pin loops or other secondary structures, (v) primer specificity. In this study, twenty four primers (12 pairs) were designed using the software program Primer3 ver. 0.4.0 (Unterssaaar et al. 2012) and confirmed for their quality criteria as described above using the online tool Oligocalc (Kibbe 2007) (Table 2).

Test data

This study used pre-collected and catalogued material from *S. hypoleucos* from the DNA repository of Paul Hebert Centre for DNA Barcoding and Biodiversity Studies, Aurangabad for testing the efficiency of newly designed primers.

Results

Using the multiple sequence alignment, primers for amplifying mitochondrial DNA from the primate species studied here were designed (Fig. 1; Table 2). As shown in Table 2, these primers have similar length, GC content and annealing temperature requirements.

Primers designed for covering small fragments of mitochondrial region

Some primers were designed to cover shorter segments of the mitochondrial genome. Primers for the ND1, ND2, COI, COII, 16S rRNA genes along with trnaL, trnaI, trnaQ, trnaM, trnaW, trnaA, trnaN, trnaC, trnaY, trnaS2, trnaD, trnaK are shown in Table 2, part B. For PCR amplification, template DNAs (30 ng/μL) were added to the PCR reaction mixture (23.7 μL) containing 2.5 μL of 10x PCR buffer (KAPA Biosystems, Inc. Wilmington, Massachusetts, United States), 0.5 μL of 50 mM MgCl₂, 2.0 μL of 2.5 mM dNTPs, 0.2 μL of

Taq polymerase enzyme (5 units/ μL), 0.5 μL of each primer (10 mM) and 16.5 μL of nuclease free water. The thermal cycling program used was set as follows: 95 $^{\circ}\text{C}$ (3 min, 1 cycle) followed by 35 cycles of 95 $^{\circ}\text{C}$ (30 s), 48 $^{\circ}\text{C}$ (40 s), 72 $^{\circ}\text{C}$ (1 min) and a final extension at 72 $^{\circ}\text{C}$ (10 min). Figure 2 shows the products generated using these primer pairs.

Primers for longer regions of the mitochondrial genome

Two primer pairs designated as PHCDBS 1F, 1R and PHCDBS 2F, 2R were designed to amplify larger portions of the mitochondrial DNA. A combination involving other primer sets such as PHCDBS 3F and PHCDBS 14R were also used to cover a region of 10kb (Table 2). Another primer combination (PHCDBS 14F + PHCDBS3R) was used to cover the remaining mitochondrial region of 7kb (Table 2). For PCR amplification, template DNA samples (30 ng/ μL) were added to the PCR reaction mixture (27.5 μL) containing 12.5 μL of Q5 high fidelity 2X master mix (New England Biolabs, Ipswich, Massachusetts, United States), 5.0 μL of template DNA and 7.5 μL nuclease free water, 1.25 μL of Forward and reverse Primer each. The PCR thermal cycling program set as follows: 98 $^{\circ}\text{C}$ (30 s); 35 cycles of 98 $^{\circ}\text{C}$ (10 s), 50 $^{\circ}\text{C}$ (30 s), 72 $^{\circ}\text{C}$ (6 min) followed by final extension at 72 $^{\circ}\text{C}$ (2 min). As shown in the Fig. 3, the designed primer sets were successfully amplified by PCR.

Tests of sequence coverage

To test the sequence coverage of different primer pairs, the larger fragments (I and II) were sequenced using a next generation sequencer (Illumina HiSeq 2500). Small fragments were sequenced bidirectionally on a Sanger Sequencing Platform (Genetic Analyzer ABI 3730 xl) using standard operating protocols. Sequences obtained were analyzed using bioinformatic curation methods, and mitochondrial assemblies were obtained. A graphical representation of actual primer positions and the regions covered are depicted in Fig. 4.

This genome assembly was also compared to reference genomes and found to be fully aligned in respect to gene order and genome coverage.

Discussion

Studies have shown that datasets derived from complete mitochondrial genome sequences appear to offer more consistent information about evolutionary relationships among species of higher taxa such as primates, and that these can be used effectively to establish the timescale of their evolution (Finstermeier et al. 2013; Kurabayashi and Sumida 2013). In contrast, studies using single or small numbers of genes to analyze evolutionary relationships have often reported rapid radiations or unresolved relationships, largely because the conclusions are based on the use of relatively small numbers of informative sites (Matsui et al. 2009). Phylogenies generated using complete mitochondrial genomes have also been shown to have considerably higher levels of statistical support when compared to analyses based on single genes (Liedigk et al. 2014). Therefore, the use of these larger datasets also has the potential to raise even a weak phylogenetic signal to a level above that of random noise (Hillis and Bull 1993).

However, owing to factors such as differing transition to transversion substitution ratios between even closely related species, it is often challenging to find primers suitable for comparative studies of complete mitochondrial genomes. More specifically, for many primate species, even for closely related species, attempts to use the same pair of primers for cross species amplification often fails.

The present study was planned with the goal of studying evolutionary questions related to primate phylogeny that are yet to be resolved in general for several species (Pozzi et al. 2014), and in particular for resolution of relationships among several primate species found in India. For this goal, a new approach was developed to obtain complete mitochondrial genome sequences from a collection of closely related primate species. The approach we have used is novel compared to methods used and proposed by others (Wu et al. 2004; Chuang et al. 2006; Chen et al. 2009; Yang et al. 2009; Chang et al. 2010; Yang et al. 2010).

The protocol shown in Fig. 1 describes the method that relies first on the use of conserved regions identified from alignments of published primate mitochondrial genomes. These alignments reveal several conserved regions where primer design algorithms are then used to identify primers for amplification beginning at the 5' end of one region (such as PHCDBS 3F) and the 3' end primer of another region (such as PHCDBS14R). This single primer pair can amplify approximately half (6726 bp) of the entire mitochondrial genome. In a similar manner, another primer pair using PHCDBS 14F as the 5' prime end primer and PHCDBS 3R as a 3' prime end primer was used to cover another large segment (9837 bp) of the mitochondrial genome (Fig. 5).

One of the potential challenges of using this method is the possibility of poor coverage in certain regions (Fig. 4, Table 2). This may be due to uncertain rates of substitutions or the possible existence of pseudogenes inserted into the nuclear genome, as suggested by various authors (Thalmann et al. 2004; Raaum et al. 2005; Pozzi et al. 2014; Finstermeier et al. 2013). To address this, apart from the primers used above to amplify large portions of mitochondrial genome, twelve other primer pairs were also designed for the amplification of fragments covering smaller segments of the genome. Most of these smaller amplification products represent the conserved regions of individual genes. These smaller products can also be used to detect amplification of any pseudogene copies of mitochondrial genes that may have inserted into the nuclear genome (Chiou et al. 2011). These primers were also optimized for annealing temperatures to minimize the possibility of non-specific amplification (Figs. 1 & 2). Even at annealing temperatures 6⁰C lower, non-specific amplification was not observed (Schoenbrunner et al. 2017). The primers used to successfully amplify the primate mitochondrial genome of *S. hypoleucos* along with their resultant sequence analysis are shown in the supporting data (Supplementary Fig. S1; Supplementary Table S1).

Overall, this strategy may help in minimizing sequencing costs using Sanger sequencing platforms (Ughade et al. 2019) and for validation of NGS based data in genome assemblies. The primer design also ensures that there is sufficient overlap of the different amplified fragments in order to obtain the complete genome sequences, including the primer sites and flanking nucleotides (Fig. 3).

Applying the strategy mentioned in Fig. 1 of designing primers for amplification of both long and short segments of the mitochondrial genome can be applied to characterization of the entire mitochondrial genome of many different closely-related species to *S. hypoleucos*. Beginning with a download of the entire mitochondrial genomic sequences of a species within a given family (from Genbank or other sources) our algorithm to design appropriate primers (Fig. 4) can easily be implemented. Subsequently, the designed primer sets are used to validate successful PCR amplification and build the genome assembly representing the entire mitochondrial genome from species with mitochondrial genomes that have not yet been adequately characterized and analyzed.

Conclusion

Mitochondrial DNA represents one of the most informative molecules for evolutionary studies. Amplification of the entire mitochondrial genome requires the use of robust primers. This study suggests a method of primer design and optimization (PDO) where first long amplification products are produced using 5' primers from the conserved region of one gene and 3' primers from conserved region of another gene. Additional primer sets representing shorter segments of the genome are also used to fill in gaps in order to complete the mitogenome sequencing. Using this strategy, the mitochondrial genome of *S. hypoleucos* was successfully amplified and sequenced. Applying this strategy of designing primers using conserved regions of known mtDNA sequences may be utilized for amplification and characterization of the entire mitochondrial genome sequences from many other species where groups of closely related species are known to exist.

Declarations

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Authors Contribution:

Vipin Hiremath: Conceiving research idea; sample collection, conduction of experiments;

Chandrakant Jadhav: Data analysis;

GD Khedkar: Conceiving research idea; writing manuscript

Competing interests: The authors do not have conflict of interest to declare.

Consent for publication: Not applicable.

Ethics approval consent to participate: Not applicable.

Abbreviations

MtDNA

Mitochondrial DNA; PDO:Primer design and optimization; COI:cytochrome oxidase subunit I

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Tables

Table 1. List of reference sequences of primate species used for primer designing

Sr. No.	Species Name	Accession number	Sub family	Family
1	<i>T. vetulus</i>	NC_019582.1	Colobinae	Cercopithecidae
2	<i>T. shortridgei</i>	KP834334.1	Colobinae	Cercopithecidae
3	<i>T. pileatus</i>	NC_024529.1	Colobinae	Cercopithecidae
4	<i>T. obscurus</i>	AY863425.1	Colobinae	Cercopithecidae
5	<i>T. johnii</i>	NC_019583.1	Colobinae	Cercopithecidae
6	<i>T. hatinhensis</i>	NC_019579.1	Colobinae	Cercopithecidae
7	<i>T. germaini</i>	NC_019580.1	Colobinae	Cercopithecidae
8	<i>T. francoisi</i>	NC_023970.1	Colobinae	Cercopithecidae
9	<i>T. cristatus</i>	NC_023971.1	Colobinae	Cercopithecidae
10	<i>S. entellus</i>	DQ355297.1	Colobinae	Cercopithecidae
11	<i>P. roxellana</i>	DQ355300.1	Colobinae	Cercopithecidae
12	<i>P. nemaus</i>	DQ355302.1	Colobinae	Cercopithecidae
13	<i>P. badius</i>	DQ355301.1	Colobinae	Cercopithecidae
14	<i>P. melalophos</i>	DQ355299.1	Colobinae	Cercopithecidae
15	<i>P. pygmaeus</i>	NC_001646.1	Ponginae	Hominidae
16	<i>P. abelii</i>	NC_002083.1	Ponginae	Hominidae
17	<i>P. hamadryas</i>	NC_001992.1	Cercopithecinae	Cercopithecidae
18	<i>P. troglodytes</i>	NC_001643.1	Homininae	Hominidae
19	<i>P. paniscus</i>	NC_001644.1	Homininae	Hominidae
20	<i>N. larvatus</i>	DQ355298.1	Colobinae	Cercopithecidae
21	<i>M. sylvanus</i>	NC_002764.1	Cercopithecinae	Cercopithecidae
22	<i>H. lar</i>	NC_002082.1	–	Halobatidae
23	<i>G. gorilla</i>	NC_001645.1	Gorillinae	Hominidae
24	<i>C. guereza</i>	AY863427.1	Colobinae	Cercopithecidae
25	<i>C. albifrons</i>	NC_002763.1	Cebinae	Cebidae

Table 2. Details of primers designed to amplify mitochondrial genome of *S. hypoleucos*

Figures

Primer name	Forward Primer Sequence		Products	T _m (°C)	GC%	Annealing temp. (°C)	Predicted product size	Primer position	
								Start	End
A. Primers designed for amplification of large fragments									
PHCDBS 1F	ATAC CAA	TAGC ATCC	Left half region between 16S rRNA and COII	46	45	55.3	6726	1092	7818
	CAAC								
PHCDBS 1R	TTTA AGGC	GCTG ATTT				40	53.2		
	CACT								
PHCDBS 2F	CCCG TATT	CAGT TTAG	Right half region between COII and 16S rRNA	46	40	53.2	9837	7819	1091*
	TCTT								
PHCDBS 2R	CCAG ATT	GAGA ATTC				40	53.2		
	ATGT								
B. Primers designed for amplification of small fragments									
PHCDBS 3F	ATAC CAA	TAGC ATCC	16SrRNA	44	45	55.3	659	1092	1750
	CAAC					53.2			
PHCDBS 3R	CCAG ATT	GAGA ATTC				40			
	ATGT								
PHCDBS 4F	ACCT AAAT	AGAA CCCA	16SrRNA	48	40	53.2	651	1640	2290
	GACA					55.3			
PHCDBS 4R	TGAC GTGG	TTGT TCTT				45			
	AGCA								
PHCDBS 5F	TAAA CGGA	TCCA CCTA	16SrRNA, tRNA-L, ND1	48	45	55.3	656	2182	2837
	ACAC					55.3			
PHCDBS 5R	TGGG TTAC	TCCT GTAG				45			
	TTGT								
PHCDBS 6F	TTAC TCCT	TTTA AGCC	ND1, tRNA-I, tRNA-Q	48	45	55.3	650	2751	3400
	AGCC					51.2			
PHCDBS 6R	TATG AAAG	AAGA GGCA				35			
	AATG								
PHCDBS 7F	CCCT TAGC	TTTC TGAG	tRNA-Q, tRNA-M, ND2	48	45	55.3	576	3387	3962
	TGAG					57.3			
PHCDBS 7R	GTGG TAAG	GAGC TGAG				50			
	GTAA								
PHCDBS 8F	TTGG TTCC	TTAT CATA	ND2, tRNA-W, tRNA-A	48	36.36	54.7	685	3865	4549
	CT					57.3			
PHCDBS 8R	AGGC AGCT	TTAG AGGA				50			
	ATGC								
PHCDBS 9F	TCCT TACT	AGCA CTTC	tRNA-N, tRNA-C, tRNAY, COI	46	38.10	54.0	689	4420	5108
	AATCA					51.2			
PHCDBS 9R	AGGT GTGG	TTTT GTTT				35			
	GAAT								
PHCDBS 10F	TACT ATCA	CTGC ACTG	COI	48	45	55.3	632	5023	5654
						55.3			

	AACG									
PHCDBS	GTAG	AAAT				45				
10R	GATG	GTGG								
	GAGA									
PHCDBS	ATTT	CCCC	GTCT	COI		48	35	51.2	581	5602
11F	AAAC	AATA						53.2		6182
PHCDBS	CAAT	AAAG								
11R	CCTA	GGAA				40				
	TCCA									
PHCDBS	TGGA	TTCC		COI, tRNA-S2, tRNA-D		48	40	53.2	610	6163
12F	TAGG	CTTT						51.2		6772
	ATTG									
PHCDBS	TAGA	ACTT					35			
12R	TGCG	TTTT								
	GAAG									
PHCDBS	GGCT	CCTT	TATT	COII		48	45	55.3	606	6692
13F	TCCC	TAGT						55.3		7297
PHCDBS	GATG	GTAA					45			
13R	AGGA	GGGG								
	TTAT									
PHCDBS	CCCG	CAGT		COII, tRNA-K		48	40	53.2	608	7211
14F	TATT	TTAG	TCTT							7818
PHCDBS	TTTA	GCTG					40	53.2		
14R	AGGC	ATTT								
	CACT

Figure 1

Figure 2

Gel images showing the amplification success of the newly designed set of primers for shorter segments of the mitochondrial genome.

Figure 3

Gel images showing the amplification success of the newly designed setof primers. L- 10KB ladder; A- amplified product 10 kb and B-amplified product 7kb)

Figure 5

Circular mitochondrial diagram showing the position of primers and regions covered (Larger fragments).

Supplementary Files

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- [SupportingFigureS1.jpg](#)
- [SupplementarytableS1.docx](#)