The Journal of Animal & Plant Sciences, 27(3): 2017, Page: 1015-1023
ISSN: 1018-7081

A NONINVASIVE MOLECULAR APPROACH: EXPLOITING SPECIES-LOCUS-SPECIFIC PCR PRIMERS IN DEFEATING NUMTS AND DNA CROSS-CONTAMINATION OF CERCOPITHECIDAE

M. A. B. Abdul-Latiff[1,2], N. R. Aifat[1], S. Yaakop[1] and B. M. Md-Zain[1*]

[1]School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600, Bangi, Selangor, Malaysia; [2]Centre of Research for Sustainable Uses of Natural Resources (CoR-SUNR), Faculty of Science Technology and Human Development, Universiti Tun Hussein Onn Malaysia, 86400, Parit Raja, Batu Pahat, Johor, Malaysia
Corresponding author's email address: abgbadd@ukm.edu.my

ABSTRACT

The lack of a standardized, noninvasive molecular approach to studying genetic aspects of primates has made it hard for primatologists to decode the evolutionary history of these species. Researchers must optimize their own techniques to fully exploit the available samples. Lack of species-locus-specific primers also contributes to difficulties in using noninvasive genetic samples. Thus, the objectives of this study were to develop a standardized technique to collecting samples noninvasively, propose newly designed species-locus-specific primers, and optimize conditions for polymerase chain reaction (PCR) for *Macaca fascicularis*, *M. nemestrina*, *Trachypithecus cristatus*, and *T. obscurus*. Nine new species-locus-specific primers for three different loci of mitochondrial DNA, namely D-loop, cytochrome oxidase subunit I (COI), and cytochrome *b*, were successfully designed. These primers proved to be efficient in amplifying larger datasets (up to ~1,000 bp) of the targeted species in the optimized PCR conditions. The species-locus-specific primers are able to anneal to host DNA alone in highly contaminated feces of highlighted species. They can also offer alternatives measures in avoiding contamination related to nuclear insertion of mitochondrial pseudogenes (numts).

Key words: Noninvasive, species-locus-specific primers, numts, *Macaca*, *Trachypithecus*.

INTRODUCTION

Over the last two decades, extensive behavioral and ecological studies have been conducted on the Southeast Asian primates (Md-Zain *et al.*, 2014); however, little is known about their genetic information (Vun *et al.*, 2011). Conservation efforts related to these Southeast Asian primates must focus on genetics aspects, as many researchers have emphasized the importance of genetic factors in influencing extinction. Lynch *et al.* (1995) showed that small populations might decline in fitness due to the accumulation of detrimental mutations. This has occurred in the case of the endangered gray-headed lemur (*Eulemur cinereiceps*) of Madagascar; these species have apparently undergone significant genetic bottlenecks, and their effective population sizes are well below total population estimates (Mittermeier *et al.*, 2009). The advancement in genetics has greatly affected the perspective on Southeast Asian primate conservation, especially in terms of molecular systematics, speciation, biogeography, and differences between individuals and populations (Roos *et al.*, 2003).

The noninvasive molecular approach has marked advancement in genetics research primates. The subject does not need to be captured, restrained, or even observed, as DNA can be extracted from feces, shed hairs, shed feathers, sloughed or shed skins, or saliva (Morin *et al.*, 1993; Morin and Woodruff, 1996). Morin *et al.* (1993) were among the earliest to use a noninvasive molecular approach in studying primates, *Pan troglodytes*, to analyze the gene flow patterns of the species. This was followed by extensive study on primate genetics using noninvasive DNA samples, which provide valuable information on population genetics, evolutionary history, and genetic structures of wild populations without requiring direct contact (Valderrama *et al.*, 1999; Jalil *et al.*, 2008; Abdul-Latiff *et al.*, 2014 a, b).

The development of novel molecular techniques that allow amplification of a single or a few copies of DNA to a million copies of DNA for a particular DNA sequence, such as polymerase chain reaction (PCR), have made it possible for primatologists to investigate and study primates in greater detail. In fact, this technique has been complemented with a growing number of field observational studies in recent years. However, with all the commercially available starting materials such as *Taq polymerase* and PCR buffer, PCR may still fail. Its efficacy may be hugely affected by the selection of oligonucleotide primers (Dieffenbach *et al.*, 1993). Oligonucleotide primers are crucial in determining the efficacy and sensitivity of PCR: Without a functional primer set, there will be no PCR product (He *et al.*, 1994).

In this study, we focused on four distinct species of Cercopithecidae, silvered leaf monkey (*Trachypithecus cristatus*), dusky leaf monkey (*T. obscurus*), long-tailed macaque (*Macaca fascicularis*) and pig-tailed macaque (*M. nemestrina*). Thus, we intend to provide sample collection methods, species-specific primers, and optimized PCR profiles for noninvasive DNA samples from all four species in order to facilitate more research on them.

MATERIALS AND METHODS

Feces Collection and DNA Extraction: Fecal samples were collected from wild populations of *M. fascicularis*, *M. nemestrina*, *T. cristatus*, and *T. obscurus* in their original habitats. The initial step in collecting fecal samples is identifying samples from targeted species. *M. fascicularis* and *M. nemestrina* conventionally have brown-colored feces, in contrast to leaf monkeys which have greenish-colored feces as a result of their frugivorous omnivore and folivorous herbivore diets, respectively (Figure 1) (Md-Zain and Ch'ng, 2011). Samples were collected, labeled, and subsequently stored in absolute ethanol (99%) in order to preserve the traces of DNA in the feces. DNA was then extracted from 0.5–1.0 g of feces samples using the innuPREP Stool DNA Kit (Analytik Jena) following the manufacturer's protocol.

Primer Design and PCR Optimization: A total of seven sequences were retrieved from GenBank (Table 1) and used as templates in designing primer. The Primer-BLAST (Ye *et al.*, 2012) program was used to generate candidate primer pairs, as well as minimum T_m differences between primer pairs. The Needleman-Wunsch global alignment algorithm (Needleman and Wunsch, 1970) is used by Primer-BLAST to check the specificity of the primer pairs to the template sequences. The primers obtained were analyzed for their physical characteristics using OligoAnalyzer 3.1 (Owczarzy *et al.*, 2006).

Two sets of PCR conditions were optimized in this study, employing Thermo Scientific Phusion Flash-High-Fidelity PCR Master Mix and Promega 5X Green GoTaq® Flexi Buffer. Both PCR cycles were run following the manufacturers' protocol to determine the annealing temperature before further optimization stages using a MyGeneMG96G Thermalcycler (Longgene Scientific Instruments Co. Ltd.) and Eppendorf Mastercycler Nexus (Eppendorf North America, Inc.). The thermal gradient cycler was utilized for the same PCR mixtures and cycle conditions for both PCR reagents. We also exploited the Wallace rule (T_m=64.9+41*(G+C-16.4)/(A+T+C+G) (Wallace *et al.*, 1979) in predicting the best annealing temperature, and subsequently we performed a thorough optimization procedure through increments and decrements of volumes and concentrations of PCR reagents and DNA templates. Negative controls were employed in both PCRs and results were observed on 1.5% agarose gel in a 1X TAE buffer. PCR conditions and cycles for both PCR reagents were shown in Table 2 and Table 3.

DNA Sequencing and Analysis: PCR products with successful amplifications were sent to 1st Base SdnBhd, Malaysia, for sequencing purposes, after purification using the innuPREPDOUBLEpure Kit (Analytik Jena). The results (chromatograms in ABI file format) were analyzed using BioEdit software v7.2.3 (Hall, 1999), MEGA6 (Tamura *et al.*, 2013), and the GenBank BLASTn application to evaluate the quality and authenticity of DNA sequences obtained for both primer reactions.

RESULTS

Nine primer pairs were successfully designed for the three designated loci with respect to all four selected species (Table 4). The primer pairs were able to amplify the targeted loci in the range of ~500–1,000 bp after combining the results of both forward and reverse reactions. Physical characteristics of the primers, such as melting temperature (T_m), primer length, GC content (%), molecular weight (g/mole), and the extinction coefficient (L/mole*cm, nmole/$^{OD_{260}}$ and µg/$^{OD_{260}}$) were presented in Table 5. The lengths of primers were 20–21 mer, which directly correlated with the molecular weight range of 5947.9–6431.2 g/mole. The GC content of the primer pairs was carefully analyzed in the selection of primer pairs; this was determined to be 35–50% of GC, except in the cases of LATIFF1784_F and LATIFF1779_R, which contained slightly higher GC contents of 57.1 % and 60%, respectively. The T_m of each primer was estimated, as this would be a key factor in assessing the annealing temperature of the primer to the respective DNA templates. Altogether, the T_m of primer pairs was in the range of 49–55ºC, except for LATIFF1784_F and LATIFF1779_R, whose temperatures were 56.6ºC and 59.5ºC, respectively. Finally, the primer pairs were analyzed for their complementary and dimer sequences to increase the efficiency of DNA template amplifications (Table 6).

Annealing temperatures were the first parameters assessed in the PCR optimizations in this study using a gradient PCR thermocycler. We used the T_m of each primer as a point of reference to deduce the annealing temperature by ranging the annealing temperature ± 5ºC from the T_m of the primer itself (Rolfs *et al.*, 1992). The best annealing temperature obtained was observed 50–52ºC. This resulted in higher annealing temperatures (Table 1). Once the annealing temperatures

had been changed to constant variables, the remaining parameters such as volumes and concentrations of the PCR reagents used were carefully optimized using experimental increment-decrement methods. PCR products using the Phusion Flash-High-Fidelity PCR Master Mix for all nine primer pairs showed excellent results without the need to optimize the PCR reagents and cycles (Figure 2). Promega 5X Green GoTaq® Flexi Buffer PCR reagents, on the other hand, were optimized to increase the sharpness of the bands and avoid the formation of primer-dimer or amplification of unspecific byproducts. The optimized concentrations and volumes of Promega 5X Green GoTaq® Flexi Buffer were as follows, 5 µl of 1X GoTaq® Green Flexi Buffer, 2.5 µl of 25 mM $MgCl_2$, 0.7 µl of 10 mM dNTP, 0.3 µl of 10 µM for both forward and reverse primers, 0.2 µl of 1.25 U GoTaq® DNA polymerase, 1 µl of <0.5 µg/50 µl DNA templates, and 15 µl of ddH_2O (Figure 3).

PCR products with the best visible bands on agarose gel electrophoresis were sequenced to analyze the effectiveness of the PCR optimization. The chromatogram results for all PCR products were analyzed using BioEdit software v7.2.3 (Hall, 1999) to determine the quality and success of DNA amplification. Several vital characteristics were taken into account in this analysis to further support the premise that we were amplifying the targeted DNA using the best primer pairs, optimum PCR conditions, and excellent purity of DNA templates. The chromatogram needed to have well formed, distinctive, single-color peaks that were evenly separated; in addition, the absence of noisy background signals was required. A total of 18 chromatograms corresponding to nine primer pairs exhibited excellent results concerning the efficiency of the proposed primers and optimized PCR conditions (Figure 4).

Sequences from both reactions of forward and reverse primers were extracted and analyzed through GenBank BLASTn to ensure that targeted loci of the selected species were amplified and not unwanted byproducts. The total alignment scores from all alignment segments, the percentage of queries covered by the alignment to the database sequence, the best expected value (E value) of all alignments from the database sequence, and the highest percent identity (Max ident) of all query-subject alignments were the key parameters in ensuring the specificity of the DNA sequences. Each sequence has been verified to have a minimum of 98% specificity with respect to Max ident and an E-value ≤ 0.

Table 1. List of source sequence in primer design.

Species	GenBank ref no	mtDNA locus	Sites
M. nemestrina	DQ002004	D-loop	1-467
M. nemestrina	AY972643	COI	1-641
M. nemestrina	HM071135	Cytochrome *b*	1-1112
M. fascicularis	JX113358	D-loop	1-1093
M. fascicularis	KF305937	COI	5864-7432
M. fascicularis	KF305937	Cytochrome *b*	14747-15887
T. obscurus	AY863425	D-loop	15468-16560
T. cristatus	KJ174503	D-loop	15466-16557
T. cristatus	KJ174503	Cytochrome *b*	14190-15330

Table 2. Initial concentration and volumes for each PCR reagents.

Promega	Phusion Master Mix
1X GoTaq® Green Flexi Buffer	1X Phusion Flash PCR Master Mix
0.1–1.0 µM of primer pairs	0.5 µM of primer pairs
<0.5 µg/50 µl of template DNA	1–10 ng/20 µl of template DNA
1–4 mM of $MgCl_2$	
0.2 mM of dNTP	
.25 U of GoTaq® DNA polymerase	
ddH_2O	
Total = 25 µL	Total = 20 µL

Table 3. PCR cycles for Promega and Phusion Master Mix reagents

Stage	Promega		Phusion Master Mix	
Initial denaturation	94 ºC – 3min		98 ºC – 10s	
Denaturation	94 ºC – 1min		98 ºC – 1s	
Annealing	51-56.8 ºC – 30s	35 cycles	51-56.8 ºC – 30s	30 cycles
Elongation	72 ºC – 90s		72 ºC – 15s	
Final Elongation	72 ºC – 7 min		72 ºC – 1 min	

Table 4. Nine designed primer pairs for designated species and loci.

No	Primer name	Sequence (5’ -3’)	Locus	ºC	Species	Product size (bp)
1	LATIFF138_F	5'- TTCTTATGTTGTACGACCTCT -3'	D-loop	51.0	*M. nemestrina*	500
2	LATIFF139_R	5'- CTCAAGTATAGCTACCCCCAC -3'				
3	LATIFF1784_F	5'- CTGGAGTCATAGGTACAGCCC -3'	COI	56.8	*M. nemestrina*	650
4	LATIFF1779_R	5'- CCTCCTCCAACGGGGTCAAA -3'				
5	LATIFF1528_F	5'- CTCCAATACGCAAATCCAAC -3'	Cyt *b*	51.0	*M. nemestrina*	750
6	LATIFF1599_R	5'- CAGCTGGAATGTAGTTGTCT -3'				
7	LATIFF1638_F	5'- ACAGTCCTAGTATTAACCTGC -3'	D-loop	52.0	*M. fascicularis*	1000
8	LATIFF1689_R	5'- CAAGGGGTGTTTAGTGAAGT -3'				
9	LATIFF148_F	5’- GACATCGGAACCCTGTATTT -3’	COI	52.2	*M. fascicularis*	1100
10	LATIFF149_R	5’- CTCCCACGGTAAATAGGAAG - 3’				
11	LATIFF1018_F	5’- CAATACACTACTCACCAGAC-3’	Cyt *b*	50.0	*M. fascicularis*	1000
12	LATIFF1069_R	5’-TAGGTTGTTTTCGATTAGGG - 3’				
13	LATIFF1978_F	5'- AAGTGCTTAACCGTCCATAG -3'	D-loop	52.0	*T. obscurus*	800
14	LATIFF1909_R	5'- AAGGCAAATTATGTGGGAGT -3'				
15	LATIFF2568_F	5'- CACTCCCTCATTGACTTACC -3'	Cyt *b*	52.0	*T. cristatus*	1000
16	LATIFF2509_R	5'- GGAGGCTAGTGGTATTAGGA -3'				
17	LATIFF2598_F	5'- GTCCAATTTTATGTGCCCTA -3'	D-loop	50.0	*T. cristatus*	1000
18	LATIFF2512_R	5'- TAGGCAGTAAGCTTTTGTTT -3'				

Table 5. Physical characteristics of designated primer pairs.

No	Primer name	T_m (ºC)	Length	GC Content (%)	Molecular Weight (g/mole)	Extinction Coefficient		
						L/mole*cm	nmole/OD_{260}	µg/OD_{260}
1	LATIFF138_F	50.7	21	38.1	6353.2	188900	5.29	33.63
2	LATIFF139_R	54.2	21	52.4	6295.1	195800	5.11	32.15
3	LATIFF1784_F	56.6	21	57.1	6431.2	203400	4.92	31.62
4	LATIFF1779_R	59.5	20	60.0	6047.0	185900	5.38	32.53
5	LATIFF1528_F	51.8	20	45.0	5999.0	191800	5.21	31.28
6	LATIFF1599_R	52.1	20	45.0	6163.0	192000	5.21	32.10
7	LATIFF1638_F	51.4	21	42.9	6365.2	202500	4.94	31.43
8	LATIFF1689_R	52.3	20	45.0	6252.1	203500	4.91	30.72
9	LATIFF148_F	52.2	20	45.0	6092.0	191200	5.23	31.86
10	LATIFF149_R	52.2	20	50.0	6135.0	201700	4.96	30.42
11	LATIFF1018_F	49.8	20	45.0	5999.0	194000	5.15	30.92
12	LATIFF1069_R	49.6	20	40.0	6209.1	194600	5.14	31.91
13	LATIFF1978_F	52.2	20	45.0	6101.0	196800	5.08	31.00
14	LATIFF1909_R	52.0	20	40.0	6245.1	209700	4.77	29.78
15	LATIFF2568_F	52.5	20	50.0	5947.9	174700	5.72	34.05
16	LATIFF2509_R	52.7	20	50.0	6277.1	208900	4.79	30.05
17	LATIFF2598_F	50.2	20	40.0	6058.0	186400	5.36	32.50
18	LATIFF2512_R	49.7	20	35.0	6153.0	190600	5.25	32.28

Table 6. The recognized complementary and dimer sequences of each primer.

No.	Primer Name	Complementary Sequence	Dimer sequence
1	LATIFF138_F	5'-AGAGGTCGTACAACATAAGAA-3'	5'-TTCTTATGTTGTACGACCTCT-3'
2	LATIFF139_R	5'-GTGGGGGTAGCTATACTTGAG-3'	5'-CTCAAGTATAGCTACCCCCAC-3'
3	LATIFF1784_F	5'-GGGCTGTACCTATGACTCCAG-3'	5'-CTGGAGTCATAGGTACAGCCC-3'
4	LATIFF1779_R	5'-TTTGACCCCGTTGGAGGAGG-3'	5'-CCTCCTCCAACGGGGTCAAA-3'
5	LATIFF1528_F	5'-GTTGGATTTGCGTATTGGAG-3'	5'-CTCCAATACGCAAATCCAAC-3'
6	LATIFF1599_R	5'-AGACAACTACATTCCAGCTG-3'	5'-CAGCTGGAATGTAGTTGTCT-3'
7	LATIFF1638_F	5'-GCAGGTTAATACTAGGACTGT-3'	5'-ACAGTCCTAGTATTAACCTGC-3'
8	LATIFF1689_R	5'-ACTTCACTAAACACCCCTTG-3'	5'-CAAGGGGTGTTTAGTGAAGT-3'
9	LATIFF148_F	5'-AAATACAGGGTTCCGATGTC-3'	5'-GACATCGGAACCCTGTATTT-3'
10	LATIFF149_R	5'-CTTCCTATTTACCGTGGGAG-3'	5'-CTCCCACGGTAAATAGGAAG-3'
11	LATIFF1018_F	5'-GTCTGGTGAGTAGTGTATTG-3'	5'-CAATACACTACTCACCAGAC-3'
12	LATIFF1069_R	5'-CCCTAATCGAAAACAACCTA-3'	5'-TAGGTTGTTTTCGATTAGGG-3'
13	LATIFF1978_F	5'-CTATGGACGGTTAAGCAC-3'	5'-AAGTGCTTAACCGTCCATAG-3'
14	LATIFF1909_R	5'-ACTCCCACATAATTTGCCTT-3'	5'-AAGGCAAATTATGTGGGAGT-3'
15	LATIFF2568_F	5'-GGTAAGTCAATGAGGGAGTG-3'	5'-CACTCCCTCATTGACTTACC-3'
16	LATIFF2509_R	5'-TCCTAATACCACTAGCCTCC-3'	5'-GGAGGCTAGTGGTTTAGGA-3'
17	LATIFF2598_F	5'-TAGGGCACATAAAATTGGAC-3'	5'-GTCCAATTTTATGTGCCCTA-3'
18	LATIFF2512_R	5'-AAACAAAAGCTTACTGCCTA-3'	5'-TAGGCAGTAAGCTTTTGTTT-3'

Figure 1. Fecal samples of 1) *M. fascicularis* from UKM Permanent Forest Reserve, Bangi, Selangor; 2) *M. nemestrina* from Paloh, Kluang, Johor; 3) *T. cristatus* from Kuala Selangor, Selangor; and 4) *T. obscurus* from Larut Hill, Taiping, Perak.

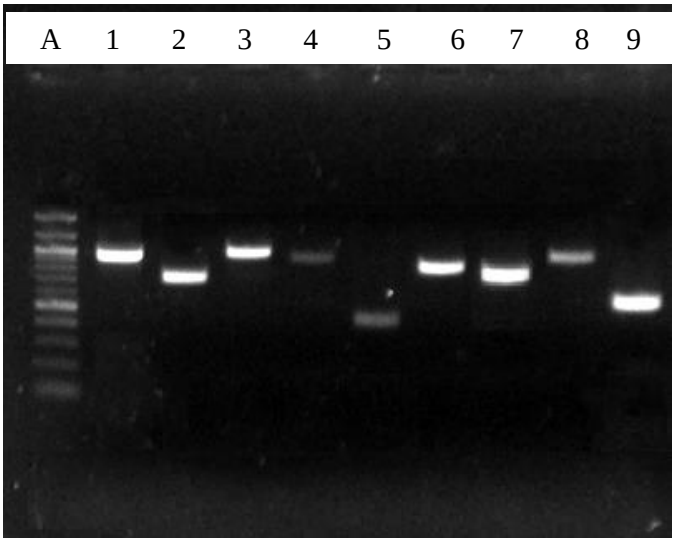

Figure 2. Result of PCR process using Finzymes. A= DNA Ladder 100bp, Cyt *b* [1= *M. fascicularis*, 2= *M. nemestrina*, 3= *T. cristatus*], D-loop [4= *M. fascicularis*, 5= *M. nemestrina*, 6= *T. cristatus*, 7= *T. obscurus*], COI [8= *M. fascicularis*, 9= *M. nemestrina*]

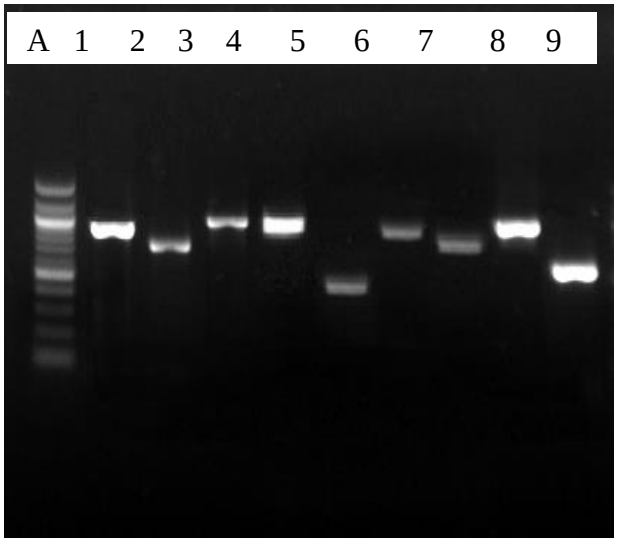

Figure 3. Result of PCR process using Promega GoTaq®. A= DNA Ladder 100bp, Cyt *b* [1= *M. fascicularis*, 2= *M. nemestrina*, 3= *T. cristatus*], D-loop [4= *M. fascicularis*, 5= *M. nemestrina*, 6= *T. cristatus*, 7= *T. obscurus*], COI [8= *M. fascicularis*, 9= *M. nemestrina*]

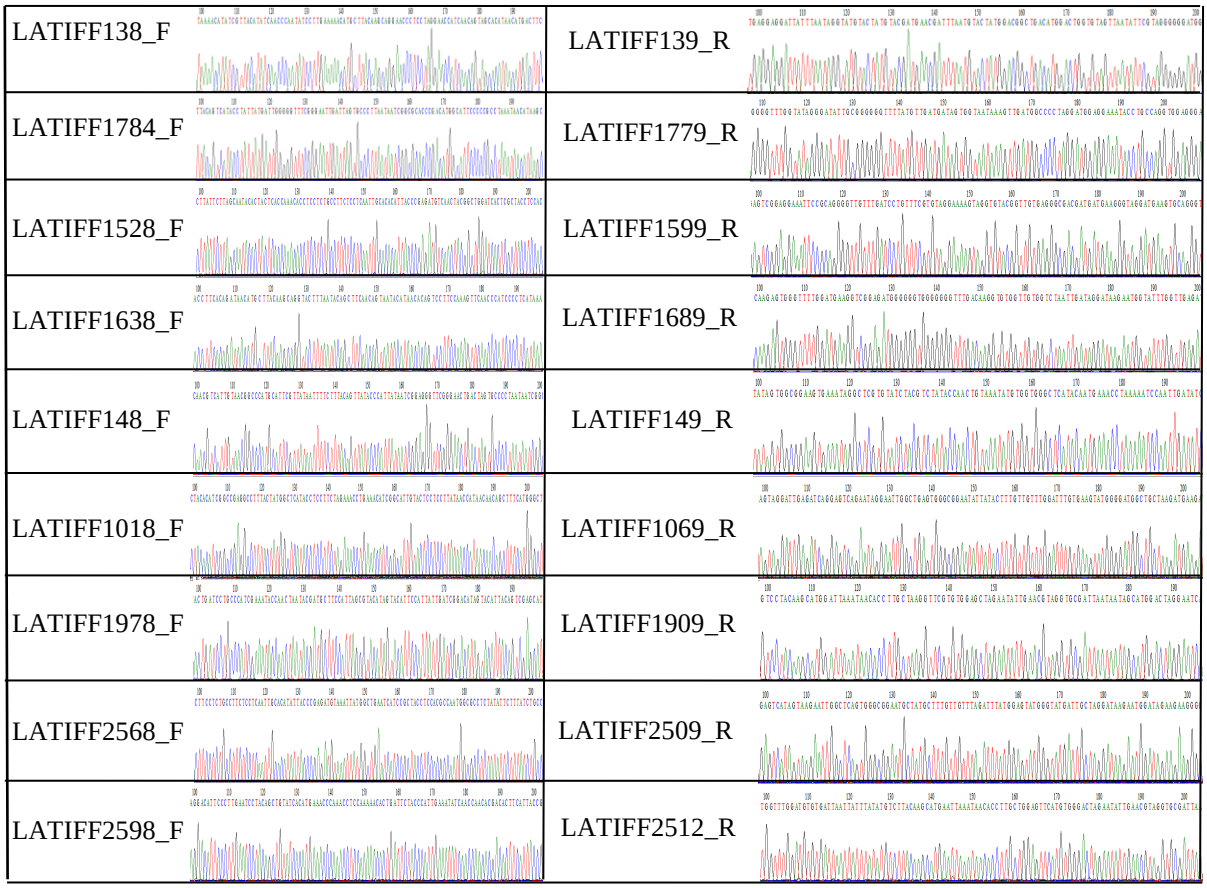

Figure 4. DNA chromatograms for all PCR products obtained with optimized PCR conditions using the respective primers. All the peaks are well formed with distinctive evenly separated single colored peaks, with total absence of noisy signal in the background.

DISCUSSION

Numerous factors should be taken into account when designing PCR primers, as these plays a key role in the efficacy of the approach, preferably with minimal length (Wu *et al.*, 1991). Minimal length will dictates the denaturation temperature in the region of 55–56ºC as this will greatly increase the specificity and efficacy of PCR (Singh and Kumar, 2001). The general agreement is the primer should be in the length of 18-27 as it will minimize the chances of a secondary hybridization site (Sharma and Katoch, 2006). Dieffenbach *et al.*, (1993) suggested primers with 18–24 mer are best for specific sequences, if the annealing temperature in the PCR reactions is within 5ºC of the primer's T_m (dissociation temperature of the primer). The optimal GC% [GC% = (G + C) / length of sequence] content in primer oligonucleotides is 50–60%, as it is directly proportional to the melting and annealing temperatures of primers due to the triple hydrogen bonds between the pairs.

The most well-known and largely used universal primer are only able to amplify short sequences as it have to anneal across species, genera and orders, thus offering limited evolutionary history of a taxa. However, with the development of our species-locus-specific primer, we are now able to amplify large datasets from mtDNA (up to ~1,000 bp). For the noninvasive molecular approach in this study, the DNA templates were extracted from highly degradable and contaminated feces. Feces is claimed to be the most trusted, noninvasive source of DNA samples for genomic sequencing used by primatologists (Kohn, 2010). While feces is a ready-to-collect DNA source in the field, it also poses tremendous challenges; for instance, microorganisms from an individual's gut microbiota will often be passed along with feces during the waste elimination process, causing a severe contamination hazard (Marrero *et al.*, 2009). Moreover, fecal samples often contain chemicals that can inhibit PCR, thereby greatly reducing the chances of amplifying the targeted DNA locus (Perry *et al.*, 2010). In essence, employing specific-locus-species primers were crucial to greatly increase the chances of primer annealing to targeted loci and altogether avoid amplifying unwanted byproducts.

Nuclear insertion of mitochondrial pseudogenes (numts) represents a cytoplasmic mitochondrial DNA sequence that has naturally been integrated into an organism's nuclear genome over an evolutionary timescale, giving rise to nDNA sequences of mitochondrial origin (Lopez *et al.*, 1994). However, upon the insertion of mtDNA into nDNA, most numts will presumably lose their function (Bensasson *et al.*, 2001); they are even assumed to be "genetic fossils," or the remains of dead or damaged genes (Gaziev and Shaikhaev, 2010), which is why they are called pseudogenes. The "natural contamination" of numts can cause serious flaws, especially in studies involving the exploitation of mtDNA as genetic markers in evolutionary studies, phylogenetics, population genetics, species identifications, medical diagnosis, and forensic medicine. Aside from the discovery of numts in humans and many other species, Karanth (2008) have definitively illustrated the presence numts in primates, specifically colobines. These are known to have structural alterations, deletions, insertions, and nucleotide substitutions (Tourmen *et al.*, 2002) which will ultimately compromise the outcomes of mtDNA-generated study. Thus the species-locus-specific primer that we propose is one way of defeating numts; given that numts undergoes sequence alterations, it will specifically anneal to the mtDNA template and not numts.

Conclusion: The increasing threats faced by primates worldwide should be complemented by increasing efforts to study them genetically, ecologically, and behaviorally to advance conservation efforts. At this critical time, we cannot afford to face difficulties in finding a way to salvage evolutionary information from endangered species of primates. Thus, we have aimed to provide all the necessary information concerning feces collection and identification, as well as the primers needed and the optimized PCR conditions. It is hoped that the methodology proposed here will facilitate research on the highlighted species while avoiding the problems of numts in employing mtDNA as focal datasets.

Acknowledgements: The authors acknowledge Universiti Kebangsaan Malaysia, Universiti Tun Hussein Onn Malaysia and Department of Wildlife and National Parks, Malay Peninsular, for providing necessary funding, facilities and assistance. This research was supported by Grants FRGS/1/2012/STWN10/UKM/02/3, UKM-GUP-2011-168, TD-2014-022, DLP-2013-006, KOMUNITI-2011-023 and ERGS/1/2013/STWN10 /UKM/02/1 and AP-2015-004.

REFERENCES

Abdul-Latiff, M.A.B., F. Ruslin, V.F. Vun, A. Mohd-Hashim, J.J. Rovie-Ryan, P. Abdul-Patah, M. Lakim, C. Roos, S. Yaakop and B.M. Md-Zain (2014a). Phylogenetic relationships of Malaysia's long-tailed macaques, *Macaca fascicularis*, based on cytochrome *b* sequences. ZooKeys, 407, 121–140.

Abdul-Latiff, M.A.B., F. Ruslin, H. Faiq, M.H. Hairul, J.J. Rovie-Ryan, P. Abdul-Patah, S. Yaakop & B.M. Md-Zain (2014b). Continental Monophyly and Molecular Divergence of Peninsular Malaysia's *Macaca fascicularis fascicularis.* BioMed Research International. Article ID 897682.

Bensasson, D., D.X. Zhang, D.L. Hartl, and G.M. Hewitt (2001). Mitochondrial pseudogenes: evolution's misplaced witnesses. Trends in Ecology & Evolution, 16, 314–321.

Dieffenbach, C.W., T.M.J. Lowe and G.S. Dveksler (1993). General concepts for PCR primer design. PCR Methods and Applications. 3, S30-S37.

Gaziev, A.I. and G.O. Shaikhaev (2010). Nuclear mitochondrial pseudogenes. Molecular Biology, 44, 358–368.

Hall, T.A (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl.Acids.Symp., 41, 95–98.

He, Q., M. Marjamaki, H. Soini, J. Mertsola and M.K. Viljanen. (1994). Primers are decisive for sensitivity of PCR. Biotechniques, 17:82–87.

Jalil, M.F., J. Cable, J. Sinyor, M. Ancrenaz, M.W. Bruford and B. Goossens (2008). Riverine effects on mitochondrial structure of Bornean orang-utans (*Pongo pygmaeus*) at two spatial scales. Molecular Ecology, 17, 2898–2909.

Karanth, K.P (2008). Primate numts and reticulate evolution of capped and golden leaf monkeys (Primates: Colobinae). J. Biosciences, 33, 761–770.

Kohn, M.H (2010). Noninvasive genome sampling in chimpanzees. Molecular Ecology, 19, 5328–5331.

Lopez, J.V., N. Yuhki, R. Masuda, W. Modi, and S. J. O'Brien (1994). Numt, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. J. Mol. Evol., 39, 174–190.

Lynch, M., R. Conery and R. Burger (1995). Mutation accumulation and the extinction of small population. The American Naturalist, 146, 489–518.

Marrero, P., R. Fregel, V.M. Cabrera and M. Nogales (2009). Extraction of high-quality host DNA from feces and regurgitated seeds: a useful tool for vertebrate ecological studies. Biological Research, 42, 147–151.

Md-Zain, B.M., and C.E. Ch'ng (2011). The activity patterns of a group of cantor dusky leaf monkeys (*Trachypithecus obscurus halonifer*). International J. Zoological Research, 7, 59 –67.

Md-Zain, B.M., F. Ruslin and W.M.R. Idris (2014). Human-Macaque Conflict at the Main Campus of Universiti Kebangsaan Malaysia. Pertanika J. Tropical Agricultural Science, 37, 73–85.

Mittermeier, R.A., J. Wallis, A.B. Rylands, J.U. Ganzhorn, J.F. Oates, E.A. Williamson, E. Palacios, E.W. Heymann, M.C.M. Kierulff, L. Yongcheng, J. Supriatna, C. Roos, S. Walker, L. Cortés-Ortiz and C. Schwitzer (2009). Primates in peril: The world's 25 most endangered primates 2008-2010. Primate Conservation, 24, 1–57.

Morin, P.A. and D.S. Woodruff (1996). Noninvasive genotyping for vertebrate conservation. In: Wayne RK, Smith TB, eds. Molecular genetic approaches in conservation. Oxford University Press (Oxford), 298–313p.

Morin, P.A., J. Wallis, J.J. Moore, R. Chakraborty and D. Woodruff (1993). Non-invasive sampling and DNA amplification for paternity exclusion, community structure, and phylogeography in wild chimpanzees. Primates, 34, 347–356.

Needleman, S.B. and C.D. Wunsch (1970). A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Molecular Biology, 48, 443–453.

Owczarzy, R., A.V. Tataurov, Y. Wu, J.A. Manthey, K.A. McQuisten, H.G. Almabrazi, K.F. Pedersen, Y. Lin, J. Garretson, N.O. McEntaggart, C.A. Sailor, R.B. Dawson, A.S. Peek (2006). IDT SciTools: a suite for analysis and design of nucleic acid oligomers. Nucleic Acids Res, 36, W163–169.

Perry, G.H., J.C. Marioni, P, Melsted and Y. Gilad (2010). Genomic-scale capture and sequencing of endogenous DNA from feces. Molecular Ecology, 19, 5332–5344.

Rolfs, A., I. Schuller, U. Finckh and I. Weber-Rolfs (1992). PCR: Clinical Diagnostic and Research. Springer-Verlag Berlin, Heidelberg.

Roos, C., T. Ziegler, J.K. Hodges, H. Zischler and C. Abegg (2003). Molecular phylogeny of Mentawai macaques: taxonomic and biogeographic implications. Molecular Phylogenetics and Evolution, 29, 139–150.

Singh, V.K. and A. Kumar (2001). PCR Primer Design. Molecular Biology Today, 2, 27–32.

Sharma, R. and V.M. Katoch (2006). Designing of oligonucleotides: probes and primers for diagnosis, epidemiology and research in medicine. J. Communicable Diseases, 38, 305–316.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution, 30, 2725–-2729.

Tourmen, Y., O. Baris, P. Dessen, C. Jacques, Y. Malthiery and P. Reynier (2002). Structure and chromosomal distribution of human mitochondrial pseudogenes. Genomics, 80, 71– 77.

Vun, V.F., M.C. Mahani, M. Lakim, A. Ampeng and B.M. Md-Zain (2011). Phylogenetic

relationships of leaf monkeys (*Presbytis*; Colobinae) based on cytochrome *b* and 12S rRNA genes. Genetics and Molecular Research, 10, 368–381.

Valderrama, X., W. B. Karesh, D. E. Wildman and D. J. Melnick (1999). Noninvasive methods for collecting fresh hair tissue. Molecular Ecology, 8, 1749–1750.

Wallace, R.B., J. Shaffer, R.F. Murphy, J. Bonner, T. Hirose and K. Itakura (1979). Hybridization of synthetic oligodeoxyribo nucleotides to phi chi 174 DNA: the effect of single base pair mismatch. Nucleic Acids Res., 6, 3543–3557.

Wu, D.Y.W., B.K. Ugozzoli, J. Pal, Qian and R.B. Wallace (1991). The effect of temperature and oligonucleotide primer length on specificity and efficiency of amplification by the polymerase chain reaction. DNA Cell Biology, 10, 233–238.

Ye, J., G. Coulouris, I. Zaretskaya, I. Cutcutache, S. Rozen and T.L. Madden (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics, 13, 134.