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OPTIMIZATION OF PARTIAL Cyt *b* GENE SEQUENCE FROM SELECTED ANCIENT *Presbytis* MUSEUM SKIN SPECIMENS

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Ancient DNA analyses hold great potential to improve our understandings on phylogeny, phylogeography, and the population history of extinct and extant species (Barnett *et al.*, 2007). According to Rohland *et al.* (2004), museum specimens are important sources of biological materials for a large number of ancient DNA studies. Most importantly, museum specimens are the only sample source available from the extinct populations, enables the investigation of past and present diversity and detection of genetic diversity losses that may have incurred over a period of time.

Unlike modern genetic analyses, ancient DNA studies are characterized by low quality DNA and thus render limited potential for satisfactory results obtained from amplification and sequence analyses. Therefore, a suitable extraction procedure is needed to obtain DNA from the museum specimens. In this study, we selected dry skin specimens from the museum collection because not all collections in museums are preserved (frozen or ethanol immersion), and dry skins may be the only source of genetic material with poor molecular characterizations (Nadia & Morgante, 2007).

Seven dry skin specimens of primate *Presbytis* were procured from Sarawak Museum and Sabah Museum. All specimens were dated from 43 to 124 years old (Table 1). The DNA extraction were performed using the QIAamp DNA Investigator Kit (Qiagen). Although there are several well-established protocols, have been used to obtain and amplify DNA segments from museum specimens (Shedlock *et al.*, 1997; Yang *et al.*, 1997; Pichler *et al.*, 2001; Junqueira *et al.*, 2002), nevertheless, these protocols require special reagents, which are costly and time consuming.

Two primer pairs were designed for the *Cyt b* region of the mitochondrial DNA (mtDNA). Both primer pairs were designed for *P. hosei* and *P. frontata* (Table 2). Two sequences of *P. hosei* and *P. frontata* retrieved from GenBank were used as templates for the primer design. The Primer-BLAST (Ye *et al.,* 2012) program was used to generate candidate primer pairs for the template sequences and minimum T_m differences between primer pairs. The Needleman-Wunsch global alignment algorithm was employed to check the specificity of the primer pairs corresponding to the template sequences (Needleman & Wunsch, 1970). Physical characteristics taken into account in designing species-specific primers included the sequence length, percentage of guanine-cytosine (GC) content, molecular weight (g/mole), and complementary sequences.

The polymerase chain reaction (PCR) was carried out in a 25 µl final volume by using MyTaq™ Red Mix from Bioline, (Country). As suggested by the manufacturer, the PCR reaction set up included 12.5 µl of MyTaq™ Red Mix, (Country) 1.0 µl of each 20 µM primer (forward and reverse), 3.0 µl of DNA template, and water added up to the final volume of 25 µl. The PCR cycling conditions were set as followings; 95°C for 3 min, 30 cycles of 95°C for 15 sec, gradient ranged annealing temperature (*P. hosei*: 48.8°C–51.0°C; *P. frontata*: 49.2°C–52.4°C) for 30 sec, and 72°C for 10 sec, followed by incubation at 72°C for 10 min. Negative controls were included in each PCR run. In order to optimize the annealing temperature for each primer pair, the amount of DNA template used was kept constant. The PCR products were subjected to electrophoresis using 1.5% agarose gel in a 1X TAE buffer. In the event of successful bands of amplifications, PCR products were sent to First Base Sdn. Bhd., Malaysia for sequencing. Both forward and reverse primers were conducted in the sequencing process. The quality of amplified DNA was quantified by chromatogram using BioEdit software. The chromatogram was ascertained with the following properties; distinctive, single-color peaks that were evenly separated, well formed and absence of noisy background.

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Table 1. List of museum skin samples

No	Code No.	Species Name	Date of specimen	Locality	Types of sample
1	BMPF 55	*P. frontata*	8 Oct 1962	Nanga Takalit, Sg Mengiong, Balleh Sarawak	Skin
2	BMPF 57	*P. frontata*	23 Sept 1962	Nanga Takalit, Sg Mengiong, Balleh Sarawak	Skin
3	BMPF 59	*P. frontata*	4 Sept 1929	Ulu Majong, Sarawak	Skin
4	BMPH 71	*P. hosei*	29 March 1972	Kg. Pandasan, Kota Belud, Sabah	Skin
5	BMPH 72	*P. hosei*	10 Jan 1969	Kg. Pinawantas, Kudat Sabah	Skin
6	BMPH 77	*P. hosei*	25 Nov 1964	Kuching, Sarawak	Skin
7	BMPH 79	*P. hosei*	Sept 1891	Gunung Dulit, Sarawak	Skin

Table 2. Two designed primer pairs for *Cyt b* locus

Primate Species Primer name	Sequence (5'-3')	Annealing Temperature (ºC)	PCR Product Size (bp)
Presbytishosei			
AIFAT6901_F	5'- GATAATTATACCCCAGCCAA -3'	48.8	150
AIFAT6903_R	5'- GATATGCTTTGTTGTTTGGA -3'		
Presbytisfrontata			
AIFAT6909_F	5'- ACTCTTCACTTTACACTACC -3'	49.2	210
AIFAT6911_R	5'- AGTATTTAGTGGGTTAGCTG -3'		

A temperature of 48°C–53°C was applied using a gradient PCR machine (PTC-100 Thermal Cycler by MJ Research, Inc., Waltham, MA, USA) for both primer pairs (*Cyt b P. hosei* and *P. frontata*). Only one sample (DNA) from each species (*P. hosei* [BMPH72] and *P. frontata* [BMPF59]) was chosen to optimize the annealing temperature of the primers.

In this study, both of the primer pairs had the same average estimated T_m (49°C). The results showed that both primer pairs were able to amplify the targeted loci, approximately 200bp (Figure 1 and Figure 2). However, each T_m tested for this gene produced poor amplification products (fainter bands) than were obtained for a T_m between 51°C and 53°C. The highest T_m that can produce the best visible product on agarose gel was chosen for each of the primer pairs in order to obtain the highest specificity of the annealing of the primer to its perfect matched template (McPherson & Moller, 2006). The sequencing results were aligned to the control sequence retrived from GenBank to ensure that the designed primers annealed to the specific target. Both primer pairs were able to amplify the *Cyt b* gene, sized 100bp to 200bp (four samples from *P. hosei* and three samples from *P. frontata*). Previous ancient DNA studies using degraded DNA did not report positive results for DNA sequences longer than 250bp (Paabo *et al.,* 2004).

In this study, both primers designed specifically to the selected species were able to amplify all the museum skin specimens utilized in this study. By using specific-locus-species primers, amplifying unwanted by-products which could occur with the usage of universal primers can be avoided (Syed-Shabthar *et al.*, 2013). In addition, the analysis of DNA from degraded samples is technically demanding since the availability of DNA is limited. Several factors should be taken into account when designing PCR primers because these factors affect the efficacy of the approach tremendously. For example, when using a primer with minimal length, a denaturation temperature of 55–56°C should generally be used; this will greatly increase the specificity and efficacy of PCR (Singh & Kumar, 2001).

PCR optimization has proved to be an important approach to obtain positive results in research involving ancient DNA samples (Elliza *et al.*, 2015). The successful use of suitable parameters and chemical reactions to examine museum skin specimens showed that ancient specimens may be valuable biological samples to compare the past and present genetic diversity values. of *P. hosei* and *P. frontata* which are nearing to extinct. Museum samples can fulfill this task through ancient DNA analyses, and most Malaysian museums have a good representation of all primate taxa.

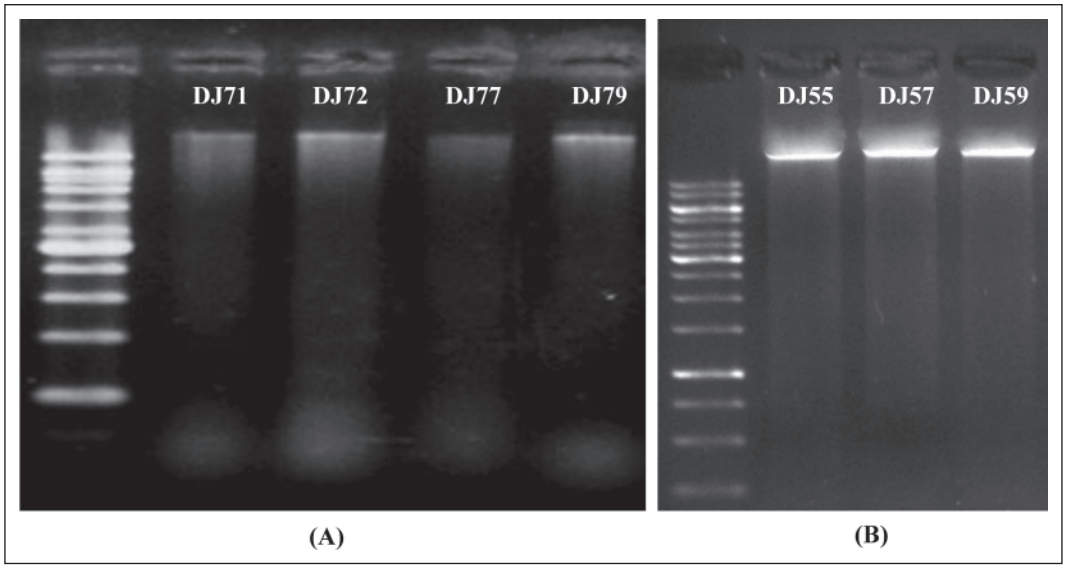

Fig. 1. Results of DNA extraction for samples *P. hosei* (A) and *P. frontata* (B).

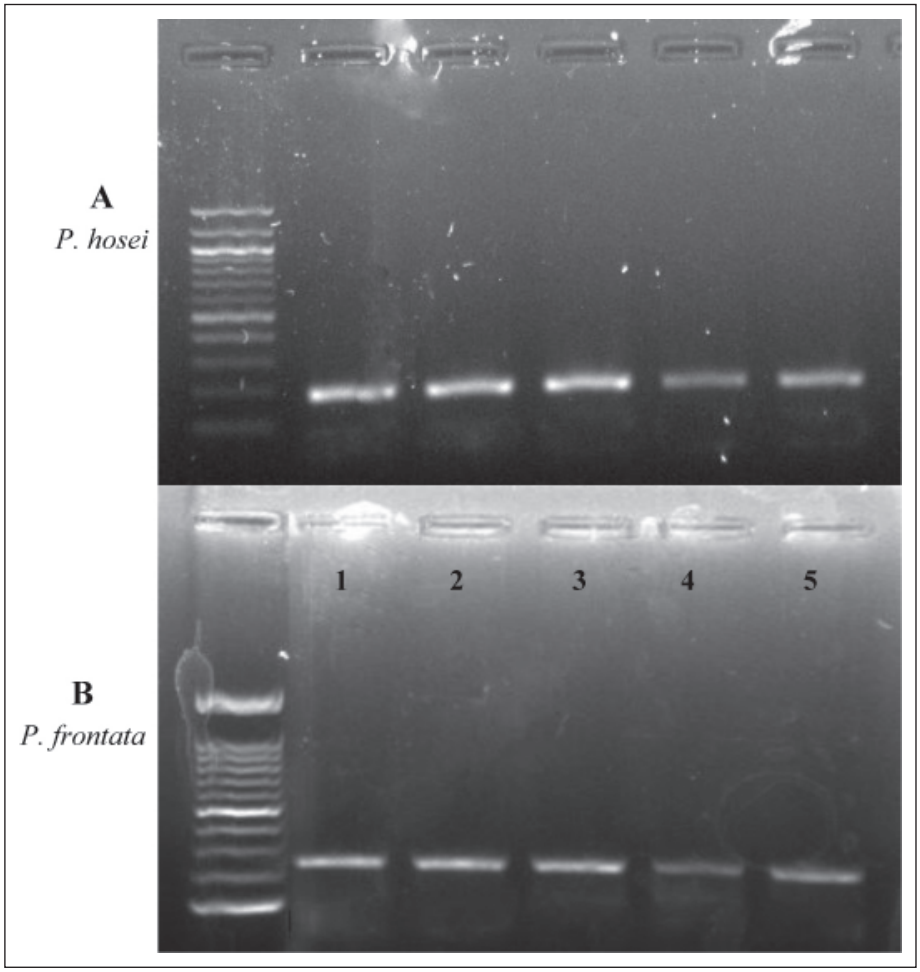

Fig. 2. DNA bands of gradient PCR on 1.5% agarose gel for *Cyt b* gene. The best T_m was 48.8°C for *P. hosei* and 49.2°C for *P. frontata*. Each well on agarose gel indicates T_m for *P. hosei* and *P. frontata*, respectively: Well 1 = 48.8°, 49.2°C; Well 2 = 49.2°, 49.7°C; Well 3 = 49.7°, 50.6°C; Well 4 = 50.2°, 51.5°C, Well 5 = 51.0°, 52.4°C.

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