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DISCRIMINATION OF TWO SPECIES OF ORANGUTANS (*PONGO* SP.): A RAPID PROTOCOL FOR REHABILITATION CENTRES AND ZOOS

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ABSTRACT

Currently orangutans are found in widely fragmented and isolated populations. Sumatran orangutan is primarily found in northern Sumatra, and the Bornean orangutans is distributed in Central, West, and East Kalimantan, Sarawak and Sabah. The determination of intra- and inter-species variation between Bornean and Sumatran orangutans is been stated to be essential for both the management of orangutan reintroduction projects and the planning of conservation strategies to preserve the remaining wild populations. This study aimed to identify two species of Orangutans (*Pongo* sp.) by means of RFLP (Restriction Fragment Length Polymorphisms) analyses of mitochondrial DNA (mtDNA). An approximately 540 bp single fragment of the ND5 gene near the 5'-region was PCR amplified for all samples tested. Digestion pattern for both *Alu*I and *Mse*I were different between two groups of ND5 fragments in this study. Present result showed a rapid protocol to identify these two species by means of RFLP (Restriction Fragment Length Polymorphism) analyses of mtDNA (mitochondrial DNA). This technique can be applied easily to rehabilitation centres and zoos to resolve species discrimination problem.

Key words: Orangutans, *Pongo* sp., Sumatra, Borneo, discrimination

INTRODUCTION

The endangered orangutans (*Pongo* sp.) in the wild are only found on the islands of Borneo and Sumatra. Now, the orangutan is listed as a CITES Appendix 1 Endangered Species (most endangered). Orangutans are taxonomically classified as two distinct subspecies, the Bornean (*Pongo pygmaeus pygmaeus*) and the Sumatran (*Pongo pygmaeus abelii*), based primarily on their distinctive morphological and behavioral

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characteristics (Groves 1971). However, some authors have recently argued that the populations should be promoted to species status (Zhi *et al.* 1996; Xu & Arnason 1996; Muir *et al.* 2000) because the differences between the two subspecies are more identical than those relatively recognized species *e.g.* chimpanzee (*Pan troglodytes*) vs. bonobo (*Pan paniscus*), horse (*Equus caballus*) vs. donkey (*Equus asianus*). These studies have been based on morphology (size, hair color, beard, size of cheek pads) and genetics (allozymes, nuclear RFLPs, mtDNA sequence, and chromosomal inversions) which seem to have correlations with the island of origin.

At present orangutans inhabit in a widely fragmented and isolated populations. While Sumatran orangutan is primarily found in northern Sumatra, and the Bornean is distributed in Central, West, and East Kalimantan, Sarawak and Sabah, they were never found in Brunei and South Kalimantan (Rijksen & Meijaard 1999). The determination of intra- and inter-species variation between Bornean and Sumatran orangutans has been noted to be essential for both the management of orangutan in the reintroduction projects and the planning of conservation strategies for the remaining wild populations (Janczewski, Goldman & O'Brien 1990; Uchida 1996).

This study aimed to identify two species of Orangutans (*Pongo* sp.) by means of RFLP (Restriction Fragment Length Polymorphisms) analyses of mitochondrial DNA (mtDNA).

MATERIALS AND METHOD

Samples

Blood samples are taken from orangutans being rehabilitated at the Rehabilitation Centers. Heparinized blood samples are stored at -20 °C until used. There are 20 blood samples used, 18 of which were originated in Borneo and the two were from Sumatra.

DNA preparation

Blood samples will be subjected to QIAGEN DNA blood extraction kit using the manufacturer's suggested protocol.

General PCR and RFLP methods

Polymerase Chain Reaction (PCR). Whole genomic DNA extracts were used for PCR reactions. PCR reaction mixtures contain 1mM $MgCl_2$, 1x Taq buffer, 0.2 mM primers, 1 U Taq polymerase (PROMEGA). PCR was performed under the conditions of Zhang et al (2001): 94° C 40s, 56 °C 40s, 72 °C 30s, for 35 cycles, with 94 °C 12 min at the beginning and 72 °C, 10 min at the end. ND5 region of mitochondrial DNA were amplified using primers of

ND5f (forward) 5'-TAA-CCG-CCC-TCA-CCT-TAA-CTT-CCC-3' (24 bp)

ND5r (reverse) 5'-GGT-CAG-GAT-GAA-GCC-AAT-GTC-G-3' (22 bp)

Restriction Fragment Length Polymorphisms (RFLP). PCR products were digested overnight by using restriction enzymes (Table 1) at 37°C.

Table 1. Restriction Enzymes used for PCR-RFLP analyses

No.	Restriction Enzymes	Recognition Sites
1.	*Alu*I	AGCT
2.	*Hha*I	GCGC
3.	*Mse*I	TTAA
4.	*Sau*3A	GATC

The digested products were separated on 5-6% PAGE in TBE buffer then stained with silver. Fragment sizes were determined using a 100 bp DNA ladder marker (BioRad).

RESULTS AND DISCUSSION

An approximately 540 bp single fragment of the ND5 gene near the 5'-region was PCR amplified for all samples tested (Fig. 1).

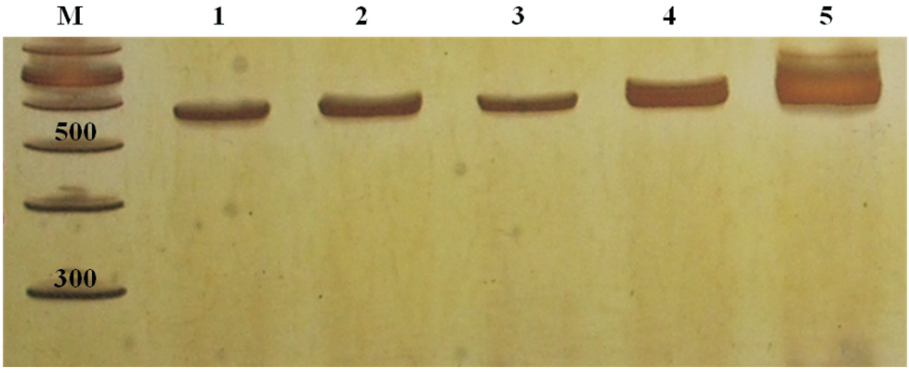

Figure 1. ND5 gene fragments of mtDNA amplified using primers reported by Zhang et al (2001); fragment size ± 540 bp; M: DNA ladder marker; 1-5: representative samples

Using reference sequences from DNA database for Sumatran and Bornean species (Genbank accession numbers are AF255448-449, AF255450-452, AF255454), the most appropriate restriction enzymes that indicated a remarkable differences among squences of two species of Orangutan were determined. Among four restriction enzymes restriction enzymes (*Alu*I, *Hha*I, *Mse*I and *Sau*3A), two restriction enzymes were selected for RFLP (restriction fragment length polymorphisms) analyses, these are *Alu*I and *Mse*I (Table 2). Digestion result using *Alu*I yielded two fragments of approximately 220 and 320 bp respectively for Bornean and three fragments of 50, 220 and 270 bp for Sumatran orangutans. Furthermore *Mse*I digested the PCR product into two fragments of 220 and 320 bp for Bornean and two fragments of 50 and 490 bp for Sumatran orangutans. Hence although Bornean and Sumatran orangutans revealed two fragments, their sizes were different. Observed numbers of DNA fragments after enzymatic digestion is summarized in Table 3.

Table 2. Reference sequences from DNA database are given with Pa (Sumatran species) and Pp (Bornean species) letters. Recognition sites of *Mse*I (TTAA), *Alu*I (AGCT) and Sau3A (GATC) are indicated with underlines; No recognition sites for *Hba*I

```
Pa ND5-A    1
ATCCCCCCCCATTACCGCTACCCTCATTAACCCCAACAAAAAAAACTCATACCCCCACTAT   60
Pa ND5-B    1 ..........__.....................................   60
Pa ND5-C    1 ..........__.....................................   60
Pp ND5-A    1 ..........C.....G.....................   60
Pp ND5-B    1 ..........C.....G.....................   60
Pp ND5-C    1 ..........C.....G.....................   60

Pa ND5-A   61
GTAAAAACGGCCATCGCATCCGCCTTTACTATCAGCCTTATCCCAACAACAATATTCATC   120
Pa ND5-B   61 .......T.....................................T...   120
Pa ND5-C   61 .....................................................T...   120
Pp ND5-A   61 ..........T.....................................T...   120
Pp ND5-B   61 ..........T.....................................T...   120
Pp ND5-C   61 ..........T.....C.....T...   120

Pa ND5-A   121
TGCCTAGGACAAGAAACCATCATCACAAACTGATGCTGAACAACCACCCAGACACTACAA   180
Pa ND5-B   121 .....G.....   180
Pa ND5-C   121 .....   180
Pp ND5-A   121 .....G.....T.....A.....G...   180
Pp ND5-B   121 .....G.....A.....G...   180
Pp ND5-C   121 .....G.....T.....A.....G...   180

Pa ND5-A   181
CTCTCACTAAAGCTTCAAACTTGACTACTTCTCCATAACATTCCTCCCCGTAGCACTACTC   240
Pa ND5-B   181 .....__.....   240
Pa ND5-C   181 .....__.....T..   240
Pp ND5-A   181 .....____.T.....GT..   240
Pp ND5-B   181 .....____.T.....GT..   240
Pp ND5-C   181 .....____.T.....GT..   240

Pa ND5-A   241
ATCACTTGATCCATTATAGAATTTTCACTATGGTATATAGCCTCAGACCCAAACATCAAC   300
Pa ND5-B   241 .....   300
Pp ND5-A   241 G....C.....T.....G.....   300
Pp ND5-B   241 G....C.....T.....G.....   300
Pp ND5-C   241 G....C.....T.....G.....   300

Pa ND5-A   301
CAATTTCTCAAATTCCTCCTCATTTTCCTAATCGCCATAATTATCCTAGTCACTGCCAAC   360
Pa ND5-B   301 .....T.....A.....T   360
Pa ND5-C   301 .....C.....T.....   360
Pp ND5-A   301 .....CT.....A.....C.....TA.....A.....   360
Pp ND5-B   301 .....CT.....A.....C.....TA.....A.....   360
Pp ND5-C   301 .....CT.....A.....C.....TA.T.....A.....   360
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Table 2. Continued

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Pa ND5-A   361
AACCTACTCCAACTCTTCATCGGCTGAGAAGGCGTAGGAATCATATCCTTCCTGCTCATT   420
Pa ND5-B   361 .............................G.......G.....  420
Pa ND5-C   361 .............................G.............  420
Pp ND5-A   361 .............................G.....................A.....  420
Pp ND5-B   361 .............................G.....................A.....  420
Pp ND5-C   361 ...................................................A.....  420
Pa ND5-A   421
AGTTGATGATACGCCCGAACAGACGCTAACACAGCAGCTATTCAAGCAATCCTATACAAT   480
Pa ND5-B   421 .............................C.....G.....  480
Pa ND5-C   421 .............................C.....G.....  480
Pp ND5-A   421 ...........T.............C...........C.....................C   480
Pp ND5-B   421 ...........T.............C...........C.....................C   480
Pp ND5-C   421 ...........T.............C...........C.....................C   480

Pa ND5-A   481 CGT                                    483
Pa ND5-B   481 ...                                    483
Pa ND5-C   481 ...                                    483
Pp ND5-1   481 ...                                    483
Pp ND5-3   481 ...                                    483
Pp ND5-5   481 ...                                    483
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Table 3. Summary of digestion profiles with *Alu*I and *Mse*I

mtDNA type	Number of DNA fragments after digestion	
	Alu I	*Mse* I
Sumatra	3 (ca. 50+220+270 bps)	2 (ca. 50+490 bps)
Borneo	2 (ca. 220+320 bps)	2 (ca. 220+320 bps)

As reported by Xu and Arnason (1996), the molecular differences between the two orangutans *P. abelii* and *P. pygmaeus* are considerably greater than those between species of hominoids (common/pygmy chimpanzee) and some other mammals (harbor/grey seals). Therefore, the two orangutans should be given the rank of separate species, *P. abelii*, Sumatra orangutan, and *P. pygmaeus*, Bornean orangutan. Analysis of molecular variation is commonly used in evaluation of animal populations for purposes of taxonomic. Furthermore the obtained result of Muir *et al.* (2000) based on mitochondrial DNA analyses suggested that mitochondrial lineage of Sumatran and Bornean orangutans has been isolated for an extended period of time. Hence, for practical uses, species discrimination of Orangutans is critical step to be applied to rehabilitation centers and zoos.

But, the new result revealed a rapid protocol to identify these two species by means of RFLP (Restriction Fragment Length Polymorphism) analyses of mtDNA (mitochondrial DNA). This technique can be applied easily to rehabilitation centres and zoos to resolve species discrimination problem there. Nonetheless Warren *et al.* (2001) found that four distinct subpopulations were identified in Borneo, as a result further study are required for assessment of molecular variation within Bornean orangutan using the same method.

CONCLUSION

*Alu*I and *Mse*I could be used for species discrimination of Orangutans by means of PCR-RFLP analyses.

Applicability of the PCR-RFLP protocol reported here should be tested further in future study.

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