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GENETIC IDENTIFICATION OF CRITICALLY ENDANGERED ORANGUTANS IN CAPTIVITY

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Abstract: A large numerals of the orangutans' genetic status in captivity is unknown to its exact, thus complicates the translocation process to their true natural habitat. A study was carried out to identify 37 captive orangutans in Peninsular Malaysia at the species and subspecies level using mitochondrial DNA (mtDNA) displacement loop (D-loop) region DNA sequences. Orangutan genetic samples were provided by the Bukit Merah Orang Utan Island (BMOUI), Zoo Negara, Zoo Taiping and the A' Famosa. Total genomic DNA was extracted from both fecal and blood samples. Polymerase chain reaction (PCR) was performed using specific primers for the orang utan. Data analyses were carried out using distance and character based approaches, namely the neighbor joining (NJ), maximum parsimony (MP), and Bayesian techniques. About 8 individuals were identified as *Pongo pygmaeus pygmaeus,* 20 as *P. p. morio,* 5 as *P. p. wurmbii,* and *4* as *P. abelii.* All samples were successfully identified with high probabilities. The results obtained in this study contributes to improve the understanding of captive orangutans' genetic identification, and indirectly, a guide to the authorities in developing plans for the management in captivity and conservation of primates in Malaysia using molecular data.

Keywords: Orangutan, *Pongo*, mitochondrial DNA, genetic identification, captive

Introduction

Orangutans, genus *Pongo* are the only great apes found in Asia, and they are only found on the Borneo and Sumatra islands (Brandon-Jones *et al.,* 2004). Taxonomically, orangutans on both islands were initially considered to represent two different subspecies, *Pongo pygmaeus pygmaeus* and *P. p. abelii* (Jalil *et al.,* 2008); although the behavioral and morphological features of these groups are almost the same, they are quite distinct in term of molecular genetics (Ryder & Chemnick, 1993). However, recent data have established the orangutan taxonomy as representing three different species, namely the Bornean orangutan, *P. pygmaeus,* the Sumatran orangutan, *Pongo abelii* and the Tapanuli orangutan, *Pongo tapanuliensis* based on morphometric, behavioral and genomic analyses (Nater *et al.,* 2017). Further taxonomic classification of Bornean orangutans had suggests the existence of three subspecies. The first three groups *P. p. pygmaeus* in Sarawak and Northwest Kalimantan, *P. p. morio* in Sabah and east Kalimantan, and *P. p. wurmbii* in southwest and central Kalimantan (Warren *et al.,* 2001).

Orangutans are classified as critically endangered species based on the IUCN Redlist 2017. Over the years, natural populations of orangutans have been declining due to factors such as hunting, habitat loss and illegal trades (Robinson & Bennet, 2000). Several ex situ institutions have been developed to overcome the declining trend of orangutan populations in the wild. In fact, translocation and rehabilitation efforts, such as the establishment of rehabilitation centers at Bohorok in north Sumatra (Indonesia), Sepilok in Sabah (Malaysia), Batang Ai and Semenggoh in Sarawak (Malaysia), and Tanjung Puting in central Kalimantan (Indonesia), are being implemented by various stakeholders

(Rosen & Byers, 2002). Although in situ conservation is one of the best ways to protect endangered animals, not all species are able to be effectively protected and maintained in their natural environment (Kasso & Balakrishnan, 2013). Consequently, focus of establishing zoos and other forms of captivity has changed from entertainment and exhibition to captive breeding centers, which contribute to education and ex situ conservation (Snyder *et al.,* 1996). The *ex-situ* initiative was taken by several institutions in Malaysia, including the Bukit Merah Orang Utan Island, Perak (BMOUI) and some zoos, as a complement to *in-situ* conservation.

Molecular phylogeny studies involving primates in Malaysia are relatively limited due to the small numbers of research publications and the available GenBank sequences (Md-Zain *et al.,* 2008). In fact, most molecular studies on Malaysian primates had focused on Cercopithecidae rather than orangutans (Md-Zain *et al.,* 2010; Vun *et al.,* 2011; Abdul-Latiff *et al.,* 2014a; 2014b, Liedigk *et al.,* 2015; Aifat *et al.,* 2016; Abdul-Latiff *et al.,* 2017). Molecular studies of the orangutan had mainly focused on the population genetic structure of wild population (Muir *et al.,* 2000; Goossens *et al.,* 2005; Jalil *et al.,* 2008).

The mtDNA control region sequences have long been used to analyze population diversity in primates due to their high mutation rates (Abdul-Latiff *et al.,* 2014a). The displacement loop (D-loop) mtDNA region is one of the best loci for assessing orangutan molecular variation, as well as the geographic origin within Borneo, due to the highly informative genetic structure (Ang *et al.,* 2011). Through the knowledge of genetic structure and taxonomic classification, species extinction could be minimized by developing suitable population management programs (Arif & Khan, 2009; Ang *et al.,* 2012). Hence, it is also applicable for species conservation, identification of sample origin, and reintroduction efforts (Zhi *et al.,* 1996; Syed-Shabthar *et al.,* 2013; Rosli *et al.,* 2014). The main objective of this study is to identify orangutans in captivity at the species and subspecies level as well as the geographic origin of the orangutans by using D-loop gene of mtDNA.

Materials and Methods

DNA Extraction and Polymerase Chain Reaction (PCR)

All 37 samples collected in this study are listed in Table 1. Orangutan fecal and blood samples were collected from BMOUI. Blood samples also collected from Zoo Negara, Kuala Lumpur, Malaysia, Zoo Melaka, Melaka, Malaysia and A' Famosa, Melaka, Malaysia on FTA cards that were available in the Department of Wildlife and National Parks, Malaysia collections. Each genetic sample from blood was extracted using the QIAGEN DNeasy Blood and Tissue Kit, following the manufacturer's protocol. The mtDNA genomes from (FTA) of nucleic acids samples, they were extracted using the WHATMAN® GenSolve Recovery Kit, also following the manufacturer's protocol. About 0.5–1.0 g of DNA was extracted from fecal samples using the innuPREP Stool DNA Kit (Analytik Jena) following the manufacturer's protocol.

Table 1: Details on the genetic samples used in this present study from Peninsular Malaysia

No	Code/Accession Number	Species/Name	Sampling Location	Type of Samples
1	OU05	*P. p. morio*	Taiping Zoo	Fta card
2	OU09	*P. p. wurmbii*	Taiping Zoo	Fta card
3	OU16	*P. abelii*	Zoo Negara	Fta card
4	OU21	*P. p. morio*	Zoo Negara	Fta card
5	OU33	*P. p. morio*	Zoo Negara	Fta card
6	OU38	*P. abelii*	A' Famosa	Fta card
7	OU39	*P. abelii*	A' Famosa	Fta card
8	OU41	*P. p. morio*	Zoo Melaka	Fta card
9	OU42	*P. p. wurmbii*	Zoo Melaka	Fta card
10	OU45	*P. p. morio*	Zoo Melaka	Fta card
11	OU46	*P. p. wurmbii*	Zoo Melaka	Fta card
12	OU47	*P. p.morio*	Taiping Zoo	Fta card
13	OU49	*P. abelii*	Zoo Negara	Fta card
14	OU51	*P. p. morio*	Zoo Negara	Fta card
15	OU52	*P. p. morio*	Bukit Merah	Fta card
16	OU54	*P. p. morio*	Bukit Merah	Fta card
17	OU56	*P. p. pygmaeus*	Bukit Merah	Fta card
18	OU57	*P. p.morio*	Bukit Merah	Fta card
19	SKPPA 549	Carlos	Bukit Merah	Fecal
20	SKPPA 552	BJ	Bukit Merah	Fecal
21	SKPPA 553	Harry	Bukit Merah	Fecal
22	SKPPA 554	Nicky	Bukit Merah	Fecal
23	SKPPA 555	Hiroshi	Bukit Merah	Fecal
24	SKPPA 556	June Jr.	Bukit Merah	Fecal
25	SKPPA 558	Baboon	Bukit Merah	Fecal
26	SKPPA 559	Tuah	Bukit Merah	Fecal
27	SKPPA 560	Careena	Bukit Merah	Fecal
28	SKPPA 561	Ah ling	Bukit Merah	Fecal
29	SKPPA 562	Sonia	Bukit Merah	Fecal
30	SKPPA 567	Kate	Bukit Merah	Fecal
31	SKPPA 568	Jidin	Bukit Merah	Fecal
32	SKPPA 569	Fatt Fatt	Bukit Merah	Fecal
33	SKPPA 570	Hang lipo	Bukit Merah	Blood
34	SKPPA 571	Marina	Bukit Merah	Blood
35	SKPPA 572	Cha cha	Bukit Merah	Blood
36	SKPPA 573	April	Bukit Merah	Blood
37	SKPPA 574	Adam	Bukit Merah	Fecal

Polymerase chain reaction (PCR) was employed to amplify the targeted locus in the mtDNA genome, which is a partial fragment of the D-loop gene, using Mastercycler® Nexus (Eppendorf North America, Inc.). PCR was performed using Phusion™ Flash High-Fidelity PCR Master Mix (Finnzymes, OY), which has extreme speed (extension times of 15 s/kb or less), high accuracy (proofreads DNA polymerase with a fidelity of 25 x *Taq polymerase*) and a very high yield in reduced times. The primers used in this study were 5′-CGAAGCTTGATATGAAAAA CCATCGTTG-3′ (Warren_D1F; Warren *et al.*, 2001) and 5′-AAACTGCAGCCCCTCAGA ATGATATTTGTCCTCA-3′ (Warren_D1R; Warren *et al.,* 2001). PCR reactions were carried out under the following parameters: initial denaturation at 98°C for 30 seconds, followed by 35 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds, an extension at 72°C for 30 seconds, and a final extension stage at 72°C for 10 minutes. The PCR product was purified using the Vivantis G-F1 PCR Clean-up Kit, and the purified PCR products were sent to 1st Base Laboratories Sdn Bhd (Malaysia) for sequencing.

Sequence and Phylogenetic Analysis

The sequence results obtained from 1st Base Laboratories Sdn Bhd were proofread and edited using Bioedit Sequence Alignment Editor. Sequence similarity searches were performed using the GenBank BLAST application to validate the DNA sequences obtained. Phylogenetic tree and genetic distance analyses of the dataset were performed and determined from the sequence analysis using PAUP 4.0b10 (Swofford, 2002) and MEGA version 4.0 (Tamura *et al.,* 2011). Three methods of phylogenetic tree reconstructions were carried out, as follows: the distance-based method (*neighbor joining,* NJ) using MEGA version 4.0 (Tamura *et al.,* 2011), the character-based method (*maximum parsimony,* MP) with Phylogenetic Analysis and Using Parsimony (PAUP) version 4.0b10 (Swofford, 2002) and Bayesian inference using MrBayes 3.1 (Huelsenback & Ronquist, 2001). The Kimura two-parameter model was selected for NJ phylogenetic reconstructions (Kimura, 1980). The MP phylogenetic tree was determined using heuristic search methods and 1,000 random stepwise additions with the application of a 50% consensus–majority rule concept (Swofford, 2002). In the MP analysis, average transitions and transversions were calculated on average. The MP phylogenetic tree was constructed using a tree bisection and reconnection (TBR) algorithm, and all the trees constructed underwent 1,000 bootstrap replications to obtain the bootstrap confidence level.

Modeltest version 3.7 software (Posada & Crandall, 1998) was used to select the best substitution model for the partial D-loop sequences using the Akaike information criterion (AIC). The best substitution model was applied in the Bayesian analysis using MrBayes 3.1.2 software. The most suitable model that fit the data was the TPM3 + G model with a gamma shape parameter of 0.1650. We ran Metropolis-coupled Markov chain Monte Carlo (MCMC) method with 400,000 generations, with a split frequencies probability (P) of 0.009214, and the tree was sampled every 10 generations. The first 25% of the trees obtained in the analysis were discarded as burn-in (1,000 trees discarded from 400,000 trees), a majority-rule consensus of remaining trees was constructed and posterior probabilities were summarized for each branch.

Results

Genetic Distance and Nucleotide Diversity

A total of 53 DNA sequences in a 305-bp D-loop locus contained 163 variable sites; among these, 67 sites were parsimony informative characters, and the remaining 32 sites were parsimony uninformative. The nucleotide composition for all 305-bp sequences was as follows: thymine = 18.6%, cytosine = 42.8%, adenosine = 30.4%, and guanine = 8.2%. The genetic distances (Table 2) between subspecies of Bornean orangutans were much closer to each other, at 0.034 from *P. p. pygmaeus* to *P. p. morio*, 0.028 from *P. p. pygmaeus* to *P. p. wurmbii*, and 0.030 from *P. p. morio* to *P. p. wurmbii.* In contrast, all subspecies of captive Bornean orangutan showed high genetic distance from

captive Sumatran orangutan, with the following distances: 0.151 from *P. abelii* to *P. p. morio*, 0.147 from *P. abelii* to *P. p. wurmbii*, and 0.145 from *P. abelii* to *P. p. pygmaeus.*

Table 2: Pairwise distance of orangutan samples based on Kimura-2-Parameter algorithm

	P. p. pygmaeus	***P. p. morio***	***P. p. wurmbii***	***P. abelii***
P. p. pygmaeus				
P. p. morio	0.034			
P. p. wurmbii	0.028	0.03		
P. abelii	0.144	0.151	0.145	

Phylogenetic Relationships

The NJ phylogeny tree (Figure 1) was generated using the Kimura two-parameter model with 1,000 bootstrap replications. The NJ tree showed that Sumatran orangutans remained monophyletic from Bornean orangutans, which was supported with a 99% bootstrap value. Samples of Bornean orangutans were divided into two clades—Clade A and Clade B. Clade A portrays subspecies of *P. p. pygmaeus* originating from Sarawak, with a bootstrap value of 85%. In contrast, Clade B is the assemblage of the subspecies of *P. p. morio* and *P. p. wurmbii,* supported by a 95% bootstrap value. Samples of *P. p. morio* originating from east Kalimantan were further separated into different populations of *P. p. morio* originating from Sabah and *P. p. wurmbii* originated from southwest and central Kalimantan, supported by a 36% bootstrap value.

The MP tree (Figure 2) is consistent with the NJ tree (CI = 0.8540, HI = 0.1460, RI = 0.9111, RC = 0.7780, and tree length = 226). Captive Sumatran orangutans were separated with the captive Bornean orangutan populations into two main clades, namely a Borneo clade and Sumatran Clade. Captive Bornean orangutan subspecies were separated into two clades—Clade A and Clade B. Clade A represents *P. p. wurmbii* orangutans originating from southwest and central Kalimantan, with a 98% bootstrap value, while Clade B represents *P. p. pygmaeus* originating from Sarawak and two populations of *P. p. morio* originating from east Kalimantan and Sabah, supported by a 52% bootstrap value.

The Bayesian inference phylogenetic tree (Figure 3) results are inconsistent with those of the NJ and MP trees. There are no clear separations between captive Sumatran and Bornean orangutans. The population of *P. p. morio* originating from east Kalimantan is supported with 0.99 posterior probabilities. In contrast, the population originating from Sabah is supported with 0.77 posterior probabilities. The subspecies of *P. p. wurmbii* and *P. p. pygmaeus* are grouped together, with 0.85 and 0.69 posterior probabilities, respectively.

Subspecies Identification

We had successfully identified 37 orangutan samples from the BMOUI, Zoo Negara, Zoo Melaka and A' Famosa with high probability. Most of the orangutans in the BMOUI were of unknown origin. Only a few individuals (BJ, Carlos, Nicky, and Baboon) were recorded originating from Sarawak, and they could possibly be *P. p. pygmaeus.* The individual named Nicky was wrongly recorded and grouped as *P. p. morio* based on the analysis. She was grouped with Harry, Ah Ling, Hiroshi Careena, Sonia, Hang Lipo, Marina, April, June Jr., and Cha Cha. In contrast, the individuals named BJ, Carlos, Baboon were correctly recorded and identified as *P. p. pygmaeus*, together with Adam, Kate, and Jidin. The samples coded as Fatt Fatt and Tuah were classified as *P. p. wurmbii.* In another case, Zoo Melaka had incorrectly identified sample code OU45 as *P. p. morio;* the analysis determined that this individual belongs to *P. p. pygmaeus.* The subspecies identification of Zoo Negara and A' Famosa were synchronized with data recorded by the management.

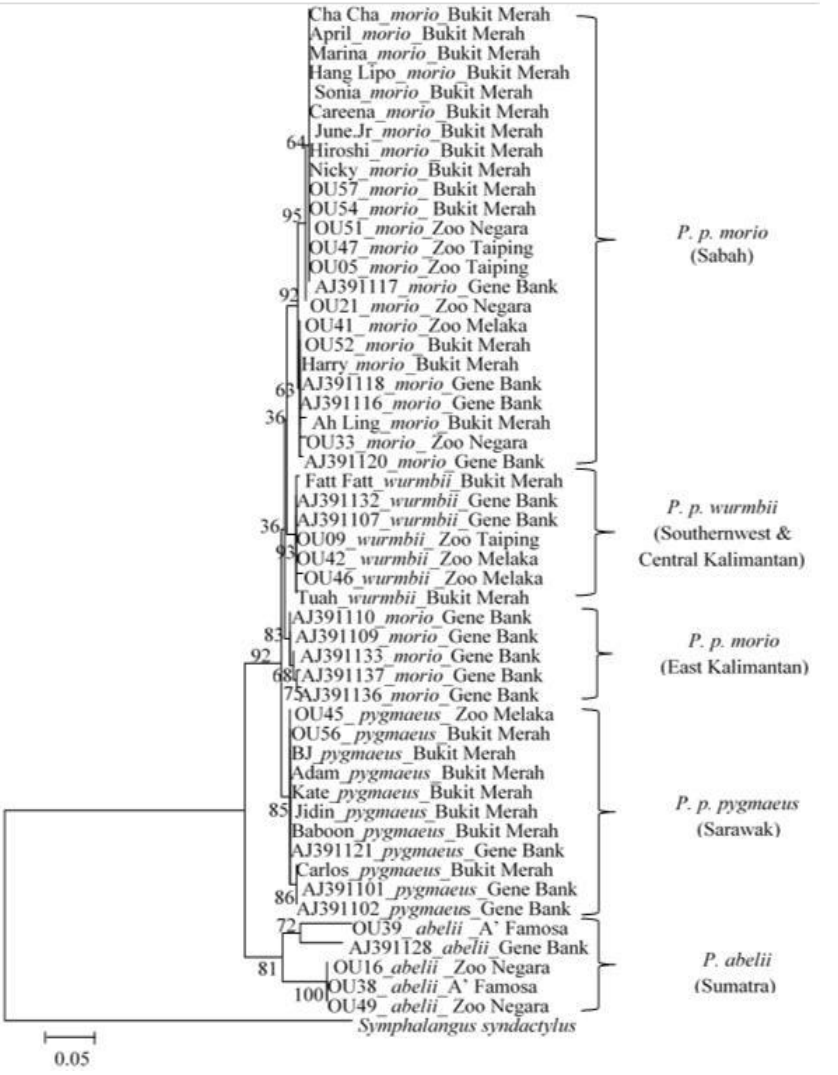

Figure 1: Neighbor-Joining phylogram of D-loop region with bootstrap values

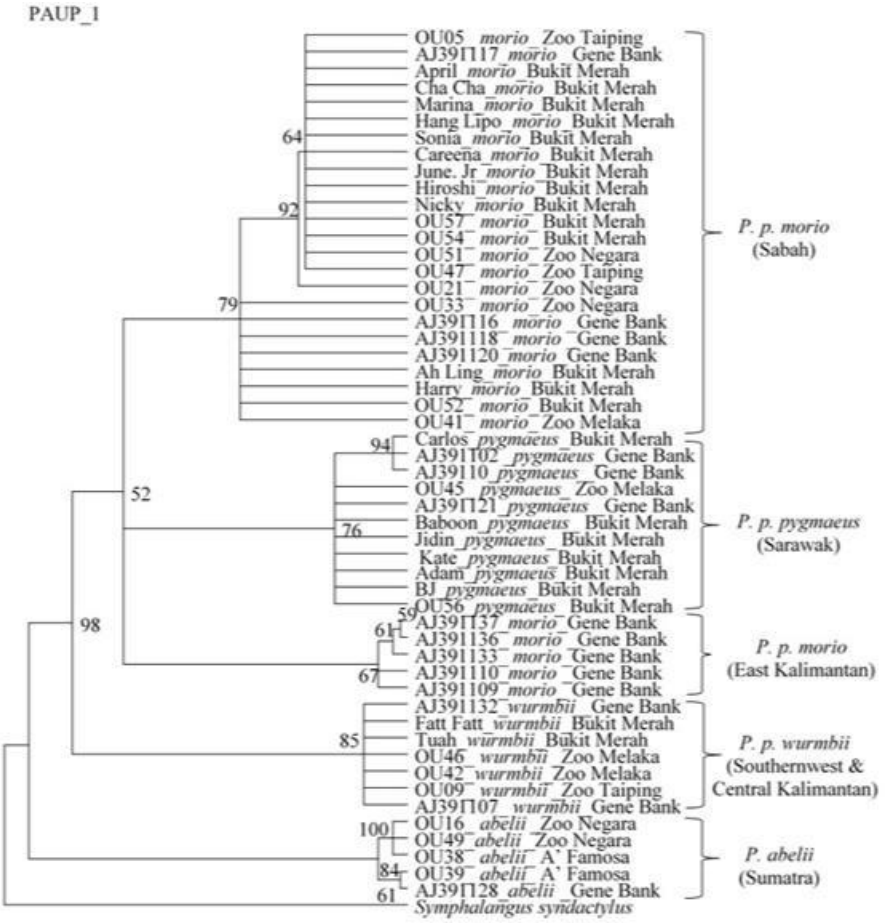

Figure 2: Maximum Parsimony consensus tree of D-loop region DNA sequences obtained from heuristic searches.

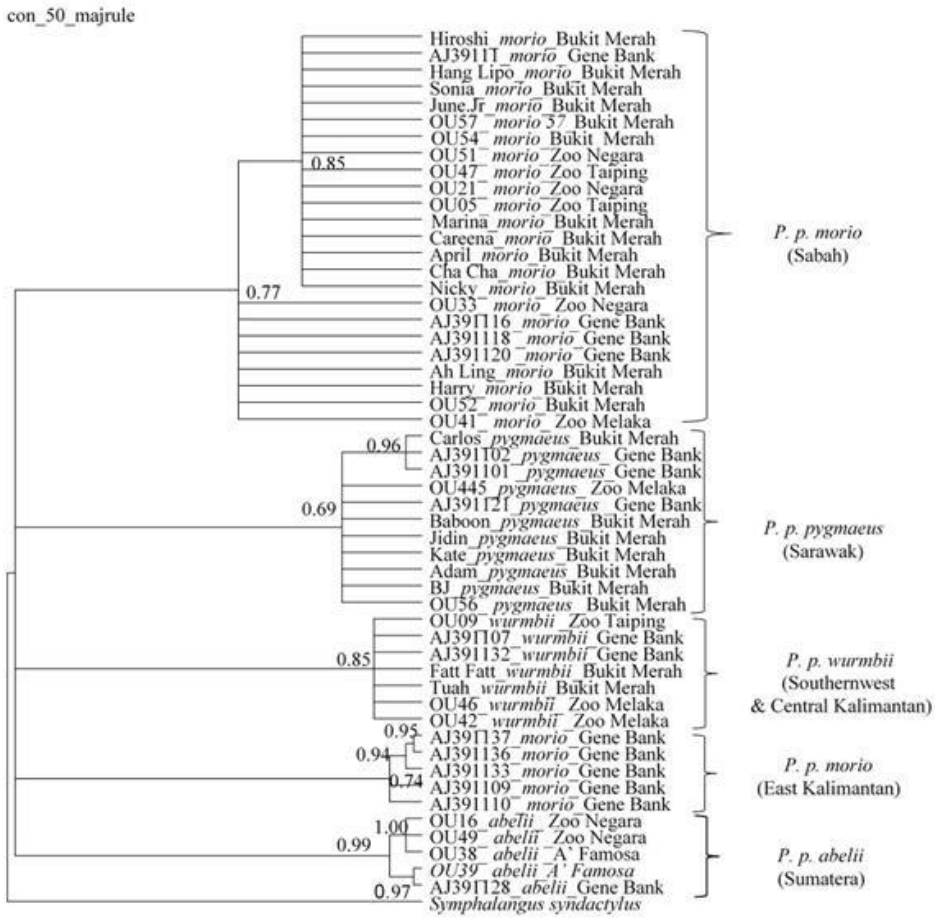

Figure 3: Bayesian inference of the 50% majority rule consensus tree of D-loop region DNA sequences. Bayes-ian posterior probability (PP) are accordingly indicated on the branch.

Discussion

The results showed that three subspecies among the Bornean orangutans were identified from the collected samples. The subspecies identification and geographic origin revealed by mtDNA variation (*P. p. pygmaeus,* Sarawak and northwest Kalimantan; *P. p. morio,* Sabah and east Kalimantan; and *P. p. wurmbii,* southwest and central Kalimantan; Warren *et al.,* 2001; Brandon-Jones *et al.,* 2004). From the phylogenetic analyses, it was determined that all our *P. p. morio* samples originated from Sabah, while the sequences from GenBank originated from east Kalimantan. Our results were also supported by other previous findings (Warren *et al.,* 2001), indicating that our analysis provides strong evidence of geographic clustering. The isolated populations occurred within Bornean orangutans due to geographical barriers, such as ancient rivers and mountains ranges (Rijksen & Meijaard, 1999; Jalil *et al.,* 2008). These factors prevent the subpopulations from simply crossing the territory. Based on mtDNA analysis by Warren et al. (2001), it was determined that the four distinct subpopulations of Bornean orangutans had not undergone a severe bottleneck effect due to high genetic diversity (Rijksen & Meijaard, 1999; Jalil *et al.,* 2008). This could support our genetic distance data, which represents extremely low genetic distance in the four subpopulations of Bornean orangutans.

From of Sumatran orangutans were identifield from Zoo Negara and A' Famosa. These 4 individuals were group in Sumatran orangutans monophyletic clade with 81% bootstrap values to differentiate them from other captive Bornean orangutans. Phylogenetic analyses showed wide genetic variation between these Bornean and Sumatran orangutans, with distinct clades in both orangutan species. Many studies have supported the divergence of Bornean and Sumatran orangutans using different loci, such as COII, ND5, or the whole genome (Zhang *et al.,* 2001; Rauum *et al.,* 2005).

In this study, 37 Orangutan samples from the BMOUI, Zoo Negara, Zoo Melaka, and A' Famosa, were successfully identified with high probability. All samples from the institutions highlighted the effectiveness of mtDNA in genetic identification (Ely *et al.,* 2005). It is necessary to develop identification and management plans based on orangutans' genetic identification at the BMOUI, Zoo Melaka, Zoo Negara, and A' Famosa. In fact, these institutions could play an important role in translocating orangutans back into the wild via the development of studbooks for captive Orang Utan. Hence, the maximum genetic diversity can be preserved and inbreeding will be avoided (Hvilsom *et al.,* 2013).

This research raises at least two crucial implications for orangutans in the BMOUI, Zoo Melaka, Zoo Negara, and A' Famosa. First, the results will allow all zoo and captivity personnel to update and reestablish their sample collections. For example, the new identification of sample OU45 should be recorded. Misidentification of orangutan species or subspecies by zoo personnel or staff will diminish conservation and education efforts (Conde, 2011). Rangers and keepers may had also unintentionally create breeding pairs of mixed species, leading to hybrid species (Hvilsom *et al.,* 2013). Second, a captive conservation program is considered successful when fertile orangutans have been produced in captivity, especially in the BMOUI. In fact, releasing captive orangutans into wild areas could represent a potential benefit for maintaining a stable ex situ population (Goossens *et al.,* 2005). With an effective genetic management plans, the success rate of reintroduction programs will increase (Woodruff, 1990) among the orangutan subspecies.

The classification of geographically isolated populations as separate species or subspecies usually relies on molecular data instead of differences in morphology and behavior (Vignieri *et al.,* 2006). MtDNA sequence variability is one of the important markers for the identification and classification of populations of primates (Abdul-Latiff *et al.,* 2014a). In addition, it is always used as a molecular marker because of the high rate of mutation inherited from the mother's line and the absence of recombination (Rosli *et al.,* 2011; Abdul-Latiff *et al.,* 2014a). The D-loop control region is the most variable part, representing mtDNA that is useful in genetic research (Zhang *et al.,* 2012). The D-loop region functions as an efficient locus for identification of the species and subspecies of captive orangutans, indirectly portrayed their geographic origin were successfully revealed in this study.

Genetic identification of individual orangutans at the species and subspecies level from the BMOUI, Zoo Melaka, Zoo Negara and A' Famosa was successfully carried out in this study. The D-loop region was utilized to understand the phylogenetic relationships of the orangutans in captivity. Future subspecies identification of orangutans in captivity should involve nuclear polymorphic markers instead of a single mitochondrial marker. Genomic characterization of orangutans with alternative markers will encourage the implementation of genetic management plans and may constitute the initial steps for the creation of a studbook for orangutans species and subspecies of the region.

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Melaka, A' Famosa, Zoo Negara. We also thank Bukit Merah Orang Utan Island Foundation (Tan Sri Datuk Mustapha Kamal bin Abu Bakar and Dr Sabapathy Dharmalingam) for providing with the necessary assistance during sample collections. Research methods reported in this research adhered to the legal requirements of Malaysia and was approved by Department of Wildlife and National Parks, Malaysia under research permit (JPHL&TN (IP):100-6/1/14 Jld 2(40). The authors acknowledge Universiti Kebangsaan Malaysia for providing necessary funding, facilities, and assistance. This research was supported by Grants TD-2014-022, DLP-2013-006, UKM-GUP-2017-087 and AP-2015-004.

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