Short Communication:
Effectiveness of nuclear gene in species and subspecies determination of captive orangutans

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Manuscript received: 28 May 2020. Revision accepted: 21 July 2020.

Abstract. *Abdul-Manan MN, Mohd-Ridwan AR, Aifat NR, Osman NA, Abdul-Latiff MA, Dharmalingam S, Md-Zain BM. 2020. Short Communication: Effectiveness of nuclear gene in species and subspecies determination of captive orangutans. Biodiversitas 21: 3665-3669.* Genetic identification of captive orangutans is of paramount importance in providing a correct identity that is essential for captive management. Thus, the utility of nuclear DNA sequences was tested in this study to identify the genetic identity of captive orangutans at Bukit Merah Orang Utan Island. Out of 24 DNA samples that were successfully extracted, only 10 orangutan samples were successfully sequenced for the von Willebrand factor (vWF) gene. From the results, this gene was able to separate the genus *Pongo* at the species level. Distance and character analyses indicated that a clear separation between *P. pygmaeus* and *P. abelii* at the species level. However, the degree of separation at species level was indicated in tree topology with moderate bootstrap values. At the subspecies level of *P. pygmaeus*, this gene was unable to show a clear separation between three Bornean subspecies. All the subspecies were formed clade together with each other. The vMF gene is a good nuclear gene for the study of phylogenetic relationships of orangutans in captivity at the species level, but the genetic identification at subspecies level in the genus level remains unclear. We suggest that future studies should involve multiple independent nuclear markers to increase the probability of getting reliable results.

Keywords: Captive orangutan, nuclear DNA, von Willebrand Factor (vMF) gene, phylogenetic, *Pongo*

INTRODUCTION

The orangutans, genus *Pongo*, are the merely available Hominidae (great apes) that exist in Asia on Borneo and Sumatra Island (Loken et al. 2013; Kamaluddin et al. 2019). This genus consists of three different species (Nater et al. 2017), namely, Bornean orangutan (*Pongo pygmaeus)*, Sumatran orangutan (*Pongo abelii*), and Tapanuli orangutan (*Pongo tapanuliensis*). Roos et al. (2014) classified the Bornean orangutan into three subspecies, namely, *P. p. pygmaeus* (Sarawak and Northwest Borneo), *P. p. wurmbii* (Southwest and Central Borneo), and *P. p. morio* (Sabah and Northeast Borneo). These subspecies of orangutans were classified based on morphology and genetic study of orangutans by primatologists (Warren et al. 2001; Zhang et al. 2001). The Bornean orangutan is presently red-listed as critically endangered under the IUCN (Ancrenaz et al. 2016). Most of the remaining orangutans live outside the protected area (Meijaard and Wich 2007). Over the years, logging, poaching, habitat loss and illegal trades are the major threats to natural populations of orangutans (Freund et al. 2016; Hardus et al. 2012).

There are five primate families found in Malaysia namely Lorisidae, Tarsiidae, Cercopithecidae, Hylobatidae and Hominidae (Md-Zain et al. 2010). Previous molecular systematic studies on Malaysian primates were more focused on the Cercopithecidae, Tarsiidae, and Lorisidae families rather than Hominidae (Md-Zain et al. 2010, 2019; Abdul-Latiff et al. 2019). These previous phylogenetic studies were all based on mitochondrial DNA (mtDNA) markers. All these studies resolved the taxonomy and systematics of Malaysian primates at species and subspecies level using mtDNA markers. In addition, Warren et al. (2001) had also employed mtDNA to identify the distribution of Bornean orangutan. Zhang et al. (2001) utilized microsatellites and mitochondrial DNA sequences to study the genetic divergence of orangutans in Borneo and Sumatra. Zhi et al. (1996) tried to differentiate orangutan subspecies among natural populations using genetic materials, specifically mitochondrial 16S ribosomal RNA sequences, minisatellite loci, and mtDNA restriction fragment length polymorphisms. The findings of this study revealed that Sumatran and Bornean orangutans are largely dissimilar in terms of phylogenetic and genetic distance. However, among isolated populations of Bornean

orangutans, there is little genetic differentiation, suggesting that gene flow might occur among them recently.

The orangutan identity in captivity is doubtful at the subspecies level. Zoos and *ex-situ* management may not be able to separate orangutans in different enclosures due to difficulty in differentiating them based on their morphological appearances. However, Kamaluddin et al. (2018) managed to successfully identify all three orangutan subspecies in the Bukit Merah Orang Utan Island and several zoos in Peninsular Malaysia based on mtDNA marker. We extended their work on the same orangutan individual samples of Bukit Merah Orang Utan Island (BMOUI) by using a nuclear marker. The usability of the nuclear DNA marker to identify the subspecies of orangutan has yet to be tested. The von Willebrand factor (vWF) gene is a non-linked nuclear genomic locus selected as a candidate marker. Chaves et al. (1999) had employed this locus in determining phylogenetic relationships of New World monkeys and used orangutan as their outgroup. The study has strongly supported the taxon Callitrichinae as a monophyletic subfamily within Cebidae. The ability and effectiveness of vWF nuclear locus have yet to be tested in portraying the Bornean orangutan phylogenetic relationships at subspecies level. Thus, we presented here the effectiveness of vWF nuclear locus in portraying orangutan genetic identification in captivity. Bukit Merah Orang Utan Island is located (BMOUI) in Perak state of Peninsular Malaysia was selected as sampling site. Previous genetic identification by Kamaluddin et al. (2018) using mitochondrial D-loop region indicated that BMOUI hosts to all three Bornean subspecies.

MATERIALS AND METHODS

Noninvasive fresh orangutan fecal samples of orangutans were collected at the Bukit Merah Orang Utan Island (Kamaluddin et al. 2018). We also recollected fresh fecal samples to produce good-quality amplification results. Good quality amplification will provide good quality sequence. Usually, non-invasive samples will produce a low DNA quantity (Costa et al. 2017). The collected fecal samples were stored in 70% alcohol solution. Preservation of DNA and storage conditions may be useful to ensure the most reliable yield of DNA. Additional blood samples, including one sample of *P. abelii* (Zoo Negara), were collected based on FTA cards available in the Department of Wildlife and National Parks (DWNP) genebank collection (Kamaluddin et al. 2018). DNA was extracted using the innuPREP Stool Kit (Analytik Jena), the DNeasy® Blood & Tissue Kit, and the innuPREP Forensic Kit (Analytik Jena) following the manufacturer's protocols (Rosli et al. 2011; Aifat et al. 2016).

A 922-bp fragment of the vWF gene introns 11 and 12 has been amplified by polymerase chain reaction (PCR) using Gradient Thermal Cycler (Eppendorf North America, Inc.). PCR was conducted using Red *Taq* Mix with these following parameters: initial denaturation at 95°C for 180 sec, followed by 30 cycles of denaturation at 95°C for 15 sec, annealing at 50°C for 60 sec, extension at 72°C for 10 sec and final extension stage at 72°C for 10 min. The PCR purification kit (QIAGEN) was used for the purification of PCR products. Purified samples have been sent to Apical Scientific Sdn. Bhd (Malaysia) for DNA sequences.

DNA sequences have been viewed and edited using BioEdit sequence alignment editor version 7.2.5. Sequence similarity searches were conducted using GenBank BLASTn application to validate the DNA sequences obtained. Phylogeny trees were reconstructed using both distance (Neighbor-Joining, NJ) and character method (Maximum Parsimony, MP) analysis using MEGA 7 ClustalW multiple alignment algorithms (Kumar et al. 2016) and PAUP 4.0b10 (Swofford 2002). The NJ tree has been reconstructed using the Kimura 2-Parameter model algorithm. The MP tree was obtained using the Tree-Bisection-Regrafting (TBR) search level 1 algorithm, in which the initial trees were obtained by randomly adding sequences (ten replicates). Bootstrap analysis was used to test the strength and reliability of the tree branch with 1000 replicates. Chimpanzee (*Pan troglodytes)* has been selected as an outgroup.

RESULTS AND DISCUSSIONS

After the final editing process, a total of 549 bp of vWF DNA sequences was obtained. Sequence analysis of vWF nuclear gene showed that 543 (87.58%) conserved characters, 5 (0.81%) variable characters, 3 (0.48%) parsimony-informative, and 2 (0.32%) singleton characters. These characters were obtained without including the outgroup. The average nucleotide frequency shows that the G base had the highest average (28.8%), followed by the C base (25.5%), the T base (23.7%), and the A base (22.1%). The pairwise distance of orangutan samples showed an exceptionally low value between a range of 0.000 and 0.001. The pairwise distance between *P. pygmaeus* and *P. abelii* was low at 0.005. In contrast, Kamaluddin et al. (2018) determined a high genetic distance (0.150) between *P. pygmaeus* and *P. abelii* based on mtDNA data. We did not combine both nuclear and mitochondrial markers for further phylogenetic analysis since both these two markers possess a different evolutionary history.

The NJ tree was separated into two main clades, which consisted of subspecies *P. pygmaeus* and *P. abelii* (Figure 1). However, among subspecies samples in the *P. pygmaeus* clade, the NJ tree was not fully resolved as there was no clear separation among them. This clade was supported by low bootstrap value, at 27% confidence level. However, *P. abelii* was clearly separated from the *P. pygmaeus* group, forming a clade with the bootstrap value of 82%. Based on the MP phylogeny tree, the value of CI was 0.800, RI was 0.800, and the tree length was 24. The MP tree portrayed the same result as the NJ tree, showing a clear separation between *P. pygmaeus* and *P. abelii*. The clade of *P. abelii* was supported by 74% bootstrap value. In the MP tree, *P. pygmaeus* subspecies phylogenetic resolution was unresolved with low support of 53% bootstrap value (Figure 2).
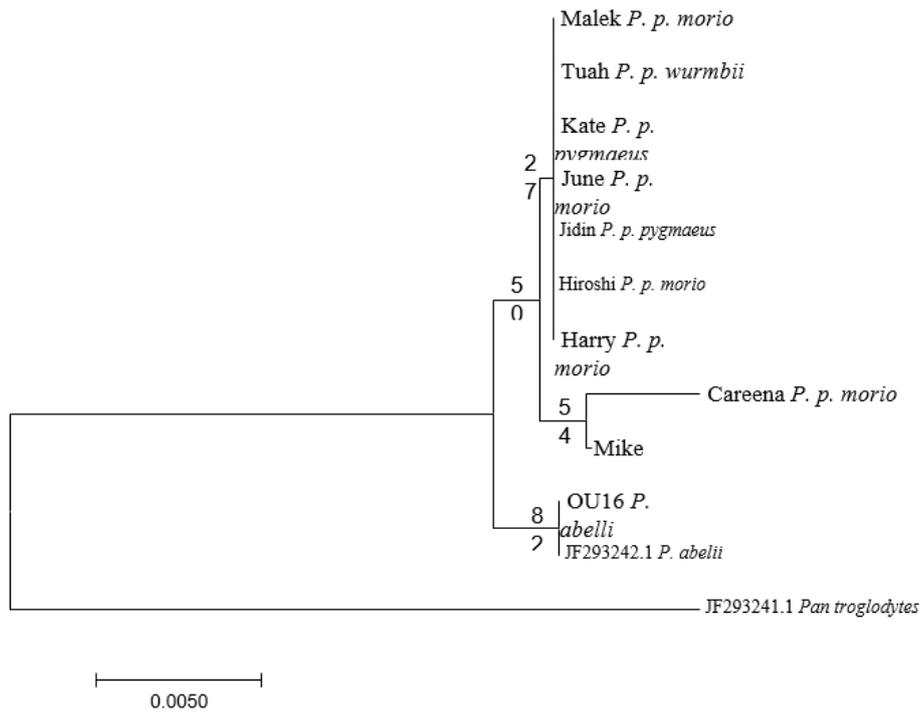
Figure 1. Neighbor-Joining tree generated by Kimura 2-Parameter with bootstrap values on top of branch
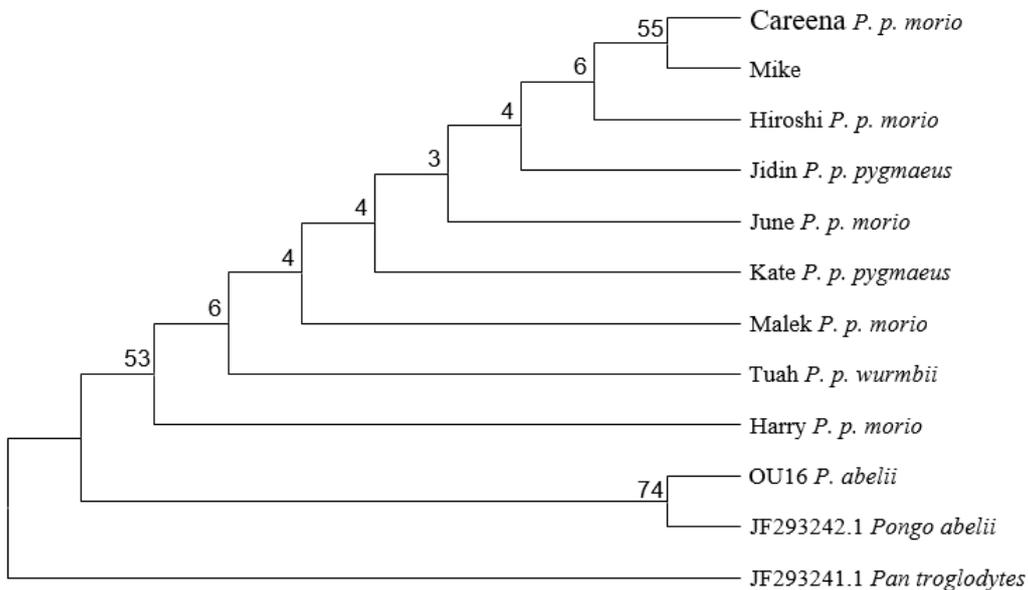
Figure 2. Maximum Parsimony tree generated by heuristic search with bootstrap values on top of branch

Our findings indicated that the nuclear vWF gene is less effective in differentiating subspecies for Bornean orangutan, *P. pygmaeus*. However, this locus can distinguish between the Bornean orangutan and Sumatran orangutan (*P. abelii*). It has been reported that the use of the mtDNA region is more effective in determining the subspecies designation of orangutans (Kamaluddin et al. 2018). The identification of subspecies using the D-loop region on orangutans in captivity had successfully distinguished three subspecies of *P. pygmaeus*, namely, *P.p. pygmaeus* (Sarawak and Northwest Kalimantan), *P. p. wurmbii* (Southwest and Central Kalimantan), and *P.p. morio* (Sabah). The D-loop region has proven to be useful in understanding the phylogenetic relationships between orangutans in captivity, as the control region is the most variable part of mtDNA valuable in systematic molecular

research (Kamaluddin et al. 2018). In addition, the divergence of *P. pygmaeus* and *P. abelii* was also supported by other studies using different mtDNA loci, such as COII, ND5, or the whole genome (Zhang et al. 2001; Steiper 2006; Goossens et al. 2009).

Our findings revealed a low level of intraspecific divergence between *P. pygmaeus* subspecies using the vWF gene. Some samples of *P. pygmaeus* in our study form polytomy and were grouped together in the same clade, but the relationships were not clearly shown. This is supported by Zhang and Hewitt (2003), indicating the difficulty in clarifying the relationship and producing polytomy due to the low evolutionary rate and low divergence of the vWF gene. It was also reported that the rate of evolution of single-copy nuclear sequences in many vertebrates is significantly lower than that of mtDNA (Zainudin et al. 2010; Yaacov et al. 2012; Ma et al. 2019). Thus, the slow rate of evolution of nuclear DNA is one limitation in our study. In other primate studies, Chaves et al. (1999) successfully placed *Callimico goeldii* in the callitrichids phylogenetic tree by assessing the vWF gene of intron 11 obtained from a range of different platyrrhine taxa including numerous marmoset species from both the Amazonian and Atlantic forest groups. Moreover, Opazo et al. (2006) validated the existence of three clades (families Pitheciidae, Cebidae, and Atelidae) and strengthened the connection of the *Aotus–Saimiri–Cebus* group to the callitrichids within the family Cebidae by obtaining orthologous sequences of six nuclear genes including vWF gene and one mitochondrial gene (16S).

Genetic studies of other organisms using nuclear markers have shown similar results to primate studies. For example, the comparison on the effectiveness of mtDNA markers (COI and Cyt *b*) and a nuclear marker (RAGI) in mammalian host identification had shown high genetic variations for COI and Cyt *b* markers compared to RAGI, suggesting that the nuclear marker is less suitable for species delimitation (Mohammadi et al. 2019). The DNA barcoding study on ray-finned fish using a nuclear gene also failed to correctly identify the species (Liu et al. 2017). Nonetheless, nuclear markers are not suited for taxonomic studies and could not separate the closely related cryptic species quickly and appropriately (Dasmahapatra et al. 2010). In phylogenetic studies, nuclear DNA is suitable for addressing the relationships between classes and orders rather than species levels (Steppan et al. 2004).

Although polymorphism exists in nuclear DNA, the divergence in the pairwise sequence is generally small and in most cases is no more than 1–2% (Zhang and Hewitt, 2003). Furthermore, analyses of the orangutan genome indicate that genetic exchange between divergent lineages is restricted to reciprocal gene flow (Rogers and Gibbs, 2014). Therefore, conventional phylogenetic analysis methods are not suitable for the analysis of intraspecific polymorphic data because they cannot clearly resolve the evolutionary relationships in the dataset due to the low degree of divergence. Other limiting factors for nuclear DNA include recombination, selection, heterozygosity, insertion or deletion polymorphism, low divergence and polytomy, gene-specific variation in rate and history, and

PCR and DNA sequencing difficulties (Zhang and Hewitt, 2003; Guo et al. 2016).

In conclusion, this study highlights the effectiveness of the vWF gene in portraying orangutan genetic relationships at the species level but not useful at the subspecies level. Thus, the phylogenetic relationships between orangutan subspecies at BMOUI could not be defined due to the low degree of vWF gene variation in the present analysis. However, our results in genetic relationships at species level help to substantiate the phylogenetics of orangutans reported in previous mtDNA studies (Muir et al. 2010; Ma et al. 2013; Banes and Galdikas, 2016; Kamaluddin et al. 2018). Moreover, this knowledge is a prerequisite to the development of a comprehensive and practical approach for conserving Bornean and Sumatran orangutan genetic resources in captivity. We suggest that future studies should examine multiple independent nuclear markers to increase the probability and effectiveness of subspecies identification. It is recommended to use different gene regions in the nuclear DNA, which are longer sequences and higher sequence variations such as Y-chromosomal locus. Captive orangutan genetic identification needs to be carried out for every *ex-situ* conservation centers. This is to ensure and guiding *ex-situ* management to possibly minimize existing hybrid orangutans cases.

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