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Identification of Diagnostic Mitochondrial DNA Single Nucleotide Polymorphisms Specific to Sumatran Orangutan (*Pongo abelii*) Populations

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ABSTRACT

The hypervariable region I of mitochondrial DNA has frequently been used to distinguish among populations, in particular in species with strong female philopatry. In such cases, populations are expected to diverge rapidly for hypervariable region I markers because of the smaller effective population size and thus increased genetic drift. This rapid divergence leads to the accumulation of mutations exclusively found in one population, which may serve as diagnostic single nucleotide polymorphisms (SNPs). To date, diagnostic SNPs distinctive to Sumatran orangutan populations have not yet been described. However, given the continuously declining numbers of Sumatran orangutans, this information can be vital for effective conservation measures, especially regarding reintroductions of orangutans in rehabilitation centers. Phylogenetic analyses of 54 samples of Sumatran orangutans from nine sampling sites with good provenance, we found five major clades and a total of 20 haplotypes. We propose a total of 52 diagnostic SNPs that are specific to Sumatran orangutan populations. Data can be used to develop restriction fragment length polymorphism assays to carry out genetic assignments using basic laboratory equipment to assign Sumatran orangutan to their population of origin.

1. Introduction

Mitochondrial DNA is transmitted exclusively from females to their offspring and can thus be used to trace matrilineal ancestry (Brown *et al.* 1982). The hypervariable region I (HVR-I) is located within the control region of mitochondrial DNA and evolves rapidly (Lau *et al.* 1998). Thus, the HVR-I is ideally suited to detect genetic variability at the intraspecific level (Tamura and Nei 1993; Brumfield *et al.* 2003). Furthermore, focusing on among-individual variation may improve the success of conservation programs aiming to revitalize declining populations and species (Forsman 2014).

Most mutational events in HVR-I are commonly known as single nucleotide polymorphisms (SNPs) (Fumagalli *et al.* 1989; Morin *et al.* 2004). SNPs displaying unique nucleotide substitutions within a particular population, but not in others, are diagnostic in that they can be used to assign individuals unequivocally to their population of origin (Yang *et al.* 2007; McTavish and Hillis 2015). The use of diagnostic SNPs in mitochondrial DNA has led to a detailed analysis of matrilineal ancestry in humans (der Sarkissian *et al.* 2014; Xavier *et al.* 2015), great apes and monkeys (Sharma *et al.* 2012; Prado-Martinez *et al.* 2013; Baden *et al.* 2014; Kopp *et al.* 2015) and numerous other organisms (Dudgeon *et al.* 2012; Matte *et al.* 2013; Hassanin *et al.* 2014; Shamblin *et al.* 2014). Hereinafter, HVR-I can be helpful in distinguishing populations within a species (Burckhardt *et al.* 1999; Arora *et al.* 2010), as an individual DNA fingerprinting (DeSalle and Amato 2004), especially in cases of strong female philopatry, such as orangutans (van Noordwijk *et al.* 2012; Kopp *et al.* 2014).

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In orangutans (genus: *Pongo*), mitochondrial DNA haplotypes have been used to distinguish between both currently recognized orangutan species (Groves 2001; Brandon-Jones *et al.* 2004), as well as between populations within each island (Borneo: *P. pygmaeus*; Sumatra: *P. abelii*) (Steiper 2006; Nater *et al.* 2011). This is possible due to the remarkably strong natal female philopatry (Goossens *et al.* 2006; Arora *et al.* 2012; van Noordwijk *et al.* 2012) with strongly male biased dispersal (Nietlisbach *et al.* 2012), which has been documented mainly for Bornean orangutans. Although social structure differs between Sumatran and Bornean orangutans (van Schaik *et al.* 2009), general dispersal patterns are the same (Nietlisbach *et al.* 2012). To date, however, it still remains unclear whether the HVR-I alone will be sufficient to assign Sumatran orangutans to their population of origin.

Sumatran orangutans are currently listed as critically endangered (Singleton *et al.* 2008). They occur in the north of the island of Sumatra (tropical forests in the Aceh and North Sumatra provinces) and approximately 6600 individuals are left in the wild (Figure 1; Wich *et al.* 2008). The generally small population sizes of Sumatran orangutan combined with the high habitat fragmentation caused their conservation status to be declared as "critically endangered" (Singleton *et al.* 2008). Here, provenance information based on mitochondrial DNA haplotype information would allow confiscated orangutans to be traced back to their population of origin and would thus significantly enhance conservation efforts. To do so, however, detailed information about the extent and distribution of genetic diversity of the HVR-I in Sumatran orangutan is needed, which we provide in this article.

2. Materials and Methods

2.1. Samples

We used fecal and hair samples of wild Sumatran orangutan from nine sampling locations in Aceh and North Sumatra during 2005–2012 (Figure 2; Table 1). We also used blood from wild-born orangutan held in the Sumatran Orangutan Conservation Program "SOCP" Batu Mbeling rehabilitation center, North Sumatra (Nater *et al.* 2013). All orangutan samples (blood, hair and fecal) were collected under the research permit of the Indonesian Ministry of Forestry, and were preserved in EDTA, ethanol 90%, RNA later or silica gel, respectively (Nsubuga *et al.* 2004; Nater *et al.* 2011). Samples were transported to Bogor Agricultural University and from there to the Anthropological Institute and Museum, University of Zurich, Switzerland, using the Convention on International Trade in Endangered Species permit number 00670/IV/SATS-LN/2013; 09717/IV/SATS-

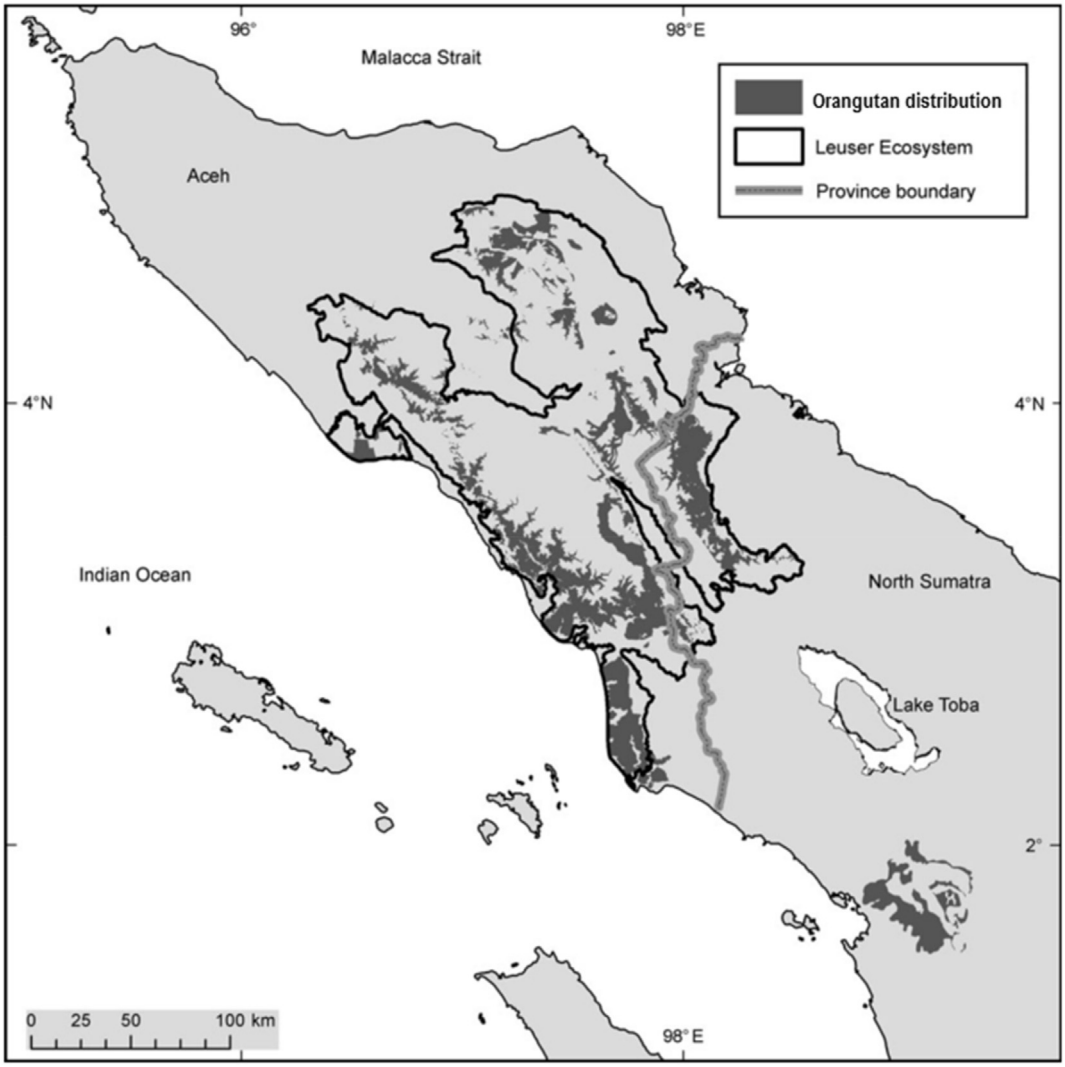

Figure 1. Map of Sumatran orangutan distribution (Wich *et al.* 2008).

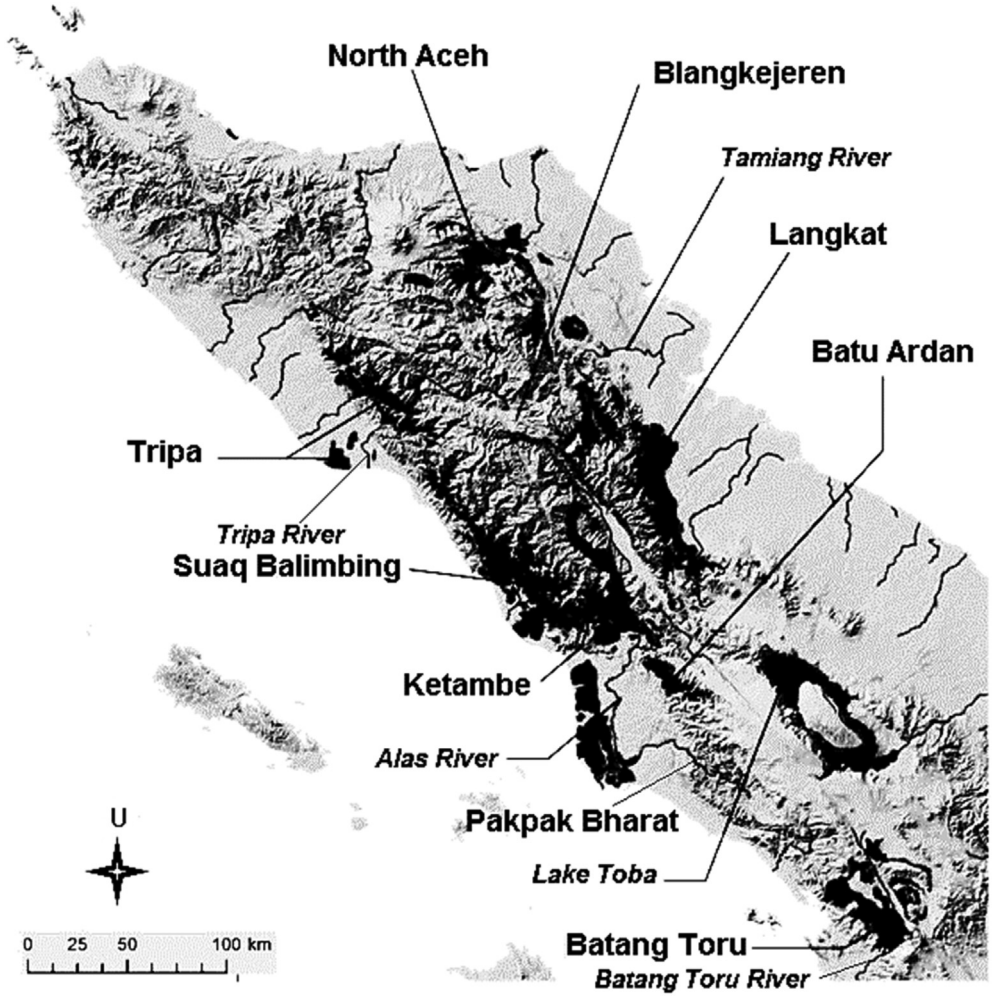

Figure 2. Nine sampling locations of Sumatran orangutan (*Pongo abelii*) populations. North Aceh (north of Tamiang River); Blangkejeren (north of Alas River); Langkat (east of Alas River, south of Tamiang River); BatuArdan (east of Alas River, northwest of Toba Lake); Batang Toru (south of Lake Toba); Pakpak Bharat (southeast of Alas River, southwest of Toba Lake); Suaq Balimbing (Kluet swamp, northwest of Alas River); Ketambe (north of Alas River); and Tripa (north and west of Tripa River).

LN/2010; 07279/IV/SATS-LN/2009; 00961/IV/SATS-LN/2007 and 06968/IV/SATS-LN/2005. In total, we (re)analyzed 54 samples to determine population clades and diagnostic SNPs specific to Sumatran orangutan populations.

2.2. DNA sequencing

Laboratory work was carried out largely as previously described (Arora *et al.* 2010). We amplified the HVR-I of 54 Sumatran orangutans using primers DLF (5′CCTGCCCCTGTAGTACAAATAAGTA3′) and

Table 1. List of Sumatran orangutan samples used in this study

Collector	No. samples	Type	Location	Sample origin (information)	Coll. year
AN	6	Fecal	SQ	Suaq Balimbing	2006–2008
AN	6	Fecal	KE	Desa Tanjung Muda, Desa Rambah Sayang, South East Aceh	2005; 2008
AN	5	Hair	BA	Sidikalang, Perolihen, Sidiangkat	2009
AN	1	Blood	BA	Quarantine (BatuArdan)	2008
AN	5	Blood	BK	Quarantine (Sayo Lues, Blangkejeren)	2004–2005
AN	1	Hair	BK	Blangkejeren	2005–2009
AN	5	Blood	TR	Quarantine(Tripa Swamp)	2009
AN	1	Hair	TR	Tripa Swamp	2007
AN	4	Blood	NA	Quarantine (Takengon, North Aceh)	2007; 2009
AN	2	Hair	NA	Takengon, North Aceh	2007–2009
AN	5	Fecal	LK	Tangkahan, Sampan Getek, aras Napal, Aceh Tamiang	2008
AN	1	Hair	LK	Langkat	2010–2011
PR	5	Hair	PB	Buluh Didi, Lae Meang	2011–2012
PR	1	Fecal	PB	Singkil	2011–2012
PR	6	Fecal	BT	West Batang Toru, East Tapanuli	2011–2012

AN (Nater *et al.* 2013); PR (this study).
SQ = Suaq Balimbing; KE = Ketambe; BA = BatuArdan; PB = Pakpak Bharat; BK = Blangkejeren; TR = Tripa; NA = North Aceh; LK = Langkat; BT = Batang Toru.

D5 (5′TGTGCGGGATATTGATTTCAC3′) (Warren *et al.* 2001; Arora *et al.* 2010; Nater *et al.* 2013). We modified the polymerase chain reaction step by using 45 cycles of 30 seconds at 58°C for annealing. Electropherograms were analyzed using Sequencing Analysis, v5.2, and edited manually. All sequences were assembled using SeqMan (Lasergene v8; DNASTAR).

2.3. Genetic variation and statistical analysis

We aligned all resulting sequences using clustal W algorithm (Thompson *et al.* 1994; Larkin *et al.* 2007) as implemented in Bio-Edit, v7.2.5 (Hall *et al.* 2011). This was followed by DNASp, v5.10.01 (Librado and Rozas 2009) to detect unique haplotypes within sampling locations. To identify diagnostic SNPs and population clades, we carried out a phylogenetic tree reconstruction based on maximum likelihood (ML with 1000 bootstrap replicates) in MEGA6 software (Tamura *et al.* 2013), applying the Tamura-Nei model (Tamura and Nei 1993). Based on the resulting mtDNA clades, we estimated nucleotide substitution rates, polymorphic sites, and nucleotide and haplotype diversity for each clade. We also carried out an analysis of molecular variance and calculated the average population pairwise differences using Arlequin, v3.5.1.2 (Excoffier and Lischer 2010).

3. Results

We sequenced 422 base pairs (bp) of HVR-I mitochondrial DNA sequences from nine Sumatran orangutan sampling locations (54 samples). In total, we found 52 diagnostic SNPs with substitutions specific to single population clades (Table 2). Overall, we observed 20 haplotypes. One haplotype was extremely common and occurred 17 times in our data set in five sampling locations (Figure 3). Most of the other haplotypes were only observed once or twice. Population clades based on the maximum likelihood phylogenetic tree showed five major HVR-I mitochondrial DNA lineages among the nine sampling locations. Two population clades comprised a single sampling location each: Batang Toru (C-BT) and Tripa (C-TR). The other three population clades were heterogeneous with respect to sampling locations. The largest population clade combined HVR-I mitochondrial DNA haplotypes from Pakpak Bharat, BatuArdan, Suaq Balimbing, Ketambe, and Blangkejeren (C-AR). Besides its own unique haplotypes, the North Aceh (C-NA) and Langkat (C-LK) population clades contained haplotypes from Blangkejeren (Figure 3), an area centrally located between C-NA, C-LK and C-AR populations (Figure 2). We also observed an insertion–deletion specific to C-BT.

Overall, there were 91 variable sites within a total of 95 mutations in the aligned sequences (Table 3), indicating high genetic variation. Haplotype diversity (standard deviation) h was 0.880 (0.035) and nucleotide diversity per site (standard deviation) π was 0.088 (0.043). The analysis of molecular variance with fixation index showed high variance among five population clades at 96.43% (FST = 0.964; $p < 0.05$). Our results point to strong inter-population differentiation for HVR-I mitochondrial DNA, demonstrated by the high and significant Φ_{ST} values for all 10 population pairs ($p < 0.05$; Table 4).

Table 2. Number of diagnostic SNPs, insertion and deletion in each clades

Pop clade	No. nucleotide	Diagnostic SNPs	Total
Alas River	3	G	8
	50	C	
	292	C	
	293	A	
	294	C	
	295	A	
	304	A	
	400	T	
Tripa	204	G	4
	232	T	
	304	C	
	332	G	
North Aceh	83	A	3
	174	T	
	225	T	
Langkat	229	A	6
	359	T	
	366	A	
	385	C	
	386	G	
	401	A	
Batang Toru	11	C	31
	48	C	
	78	A	
	81	T	
	84	T	
	112	A	
	133	T	
	156	A	
	169	T	
	184	G	
	188	C	
	192	C	
	205	G	
	209	A	
	211	T	
	279	A	
	284	G	
	296	A	
	298	-(del)	
	299	-(del)	
	306	C	
	307	C	
	313	C	
	324	T	
	329	T (ins)	
	358	A	
	359	A	
	384	C (ins)	
	387	C	
	392	T	
	414	A	
Total	Diagnostic SNPs		52

Pop clade: Population clades which have diagnostic SNPs. No. nucleotide: number of nucleotide where the mutation happens (fixed nucleotide differences occurred). SNP = single nucleotide polymorphisms.

4. Discussion

Our analyses revealed five major genetic matrilineal clades in Sumatran orangutan populations, which can be differentiated by 52 diagnostic SNPs. Given the pronounced tendency of female philopatry in orangutans, as at least documented in Borneo (Arora *et al.* 2012), this information provides a basis for linking Sumatran orangutans currently held in rehabilitation centers to their putative population of origin. Identifying source populations especially in Sumatra is extremely crucial, because the genetic divergence among populations is much deeper compared to Borneo (Warren *et al.* 2001; Arora *et al.* 2010). Moreover, Sumatran orangutans exhibit a large genetic differentiation of populations between north and south area of Lake Toba (Nater *et al.* 2011). However, this is not reflected in the current Sumatran orangutan taxonomy, which still considers all Sumatran orangutans to be member of a single species without any division into subspecies. Because of this, caution should be exerted when releasing Sumatran orangutans of unknown origin in the same area, due to potential mixing of individuals from different gene pools, which might lead to outbreeding depression (Moritz 1999; Frankham 2010). It can also present problems in terms of social interactions, for instance when unrelated females

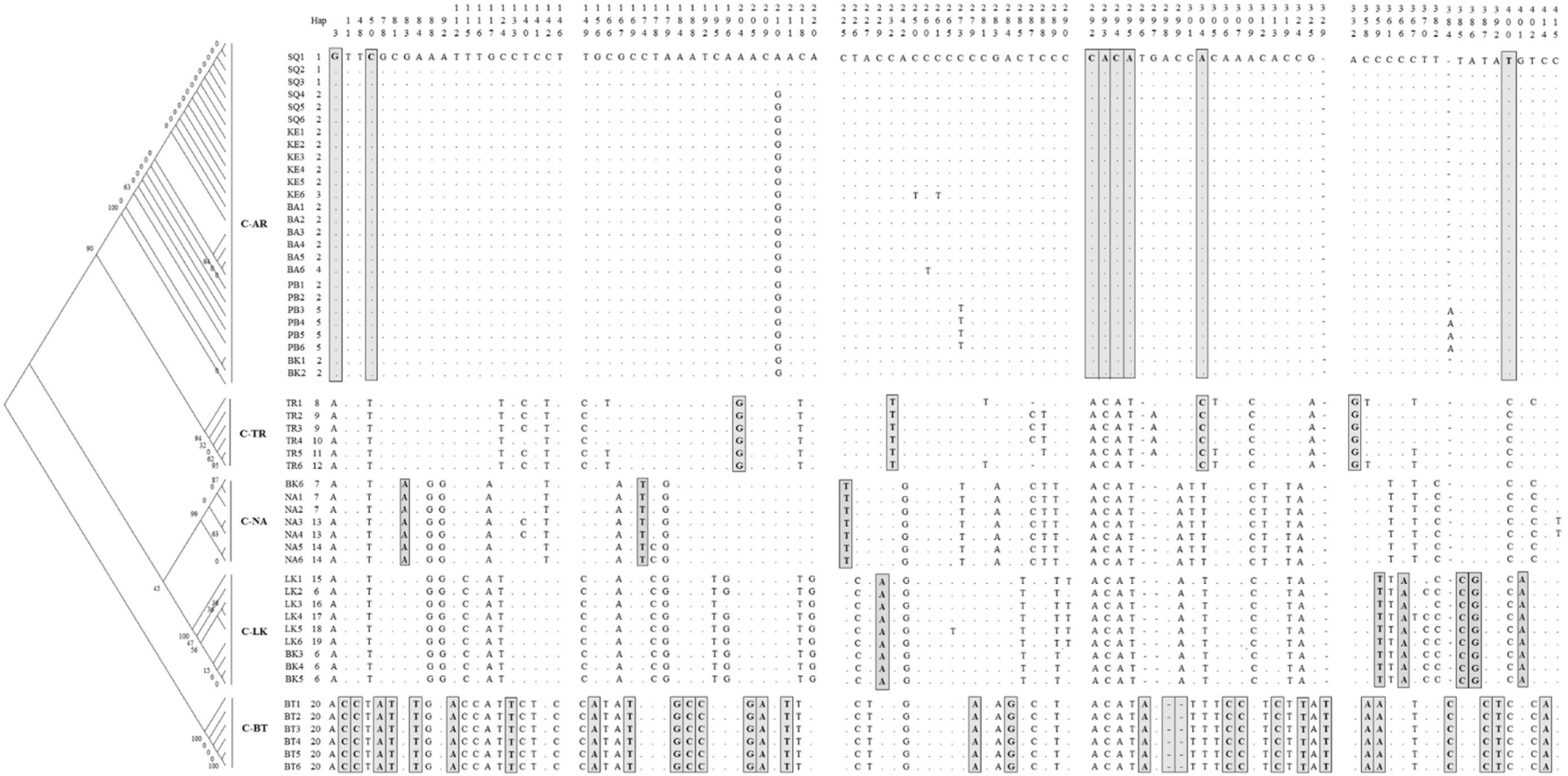

Figure 3. Hypervariable region I (HVR-I) mitochondrial DNA haplotypes. Maximum likelihood tree of partial HVR-I mitochondrial DNA sequences, with bootstrap support values in percent (Tamura-Nei model; bootstrap = 1000). C-AR: Alas River population clade (Pakpak Bharat, BatuArdan, Ketambe, Suaq Balimbing and part of Blangkejeren sampling locations); C-TR: Tripa population clade; C-NA: North Aceh population clade; C-LK: Langkat population clade; C-BT: Batang Toru population clade (left). Haplotype variations of partial HVR-I mtDNA sequences (422 bp) with diagnostic single nucleotide polymorphisms in bold character and gray squares (right). SQ = Suaq Balimbing; KE = Ketambe; BA = BatuArdan; PB = Pakpak Bharat; BK = Blangkejeren; TR = Tripa; NA = North Aceh; LK = Langkat; BT = Batang Toru.

Table 3. Nucleotide substitution rates (maximum likelihood estimate) and polymorphic sites in two, three and four alleles

Nucleotide (substitution rates)	A	T	C	G	Polymorphic sites	Singleton variable sites	Parsimony informative sites
Adenine (A)	–	*1.88*	*3.50*	**7.40**	Two variants	4	83
Thymine (T)	*2.88*	–	**33.27**	*0.92*	Three variants	0	4
Cytosine (C)	*2.88*	**17.91**	–	*0.92*	Four variants	0	0
Guanine (G)	**23.05**	*1.88*	*3.50*	–	Total	4	87

Each entry on nucleotide substitution shows the rates of substitution (r) from one base (row) to another base (column); Rates of different transitional substitutions are shown in **bold** and those of transversional substitutions are shown in *italics*; sum of r values = 100.

are forced into the home ranges of a group of related females they may be chased away.

One of the ultimate goals of genetic conservation is to ensure the long-term persistence of species, mainly through the maintenance of intra-population genetic diversity (Kahilainen *et al.* 2014), which still appears to be at appreciable levels in Sumatran orangutans. In contrast to previous studies where four geographically distinct haplogroups were reported (Nater *et al.* 2011; Nater *et al.* 2013), our results indicate an additional fifth mitochondrial DNA clade (Tripa location), which is different from the remaining Alas River clade (called "West Alas" in the Nater *et al.* 2013). Tripa is a peat-swamp forest on the west coast of Aceh, with an estimated census size of approximately 280 individuals in a 140 km^2 habitat (Wich *et al.* 2008). This location has encountered a massive oil palm conversion, with only 23.53% of its original size remaining relatively undisturbed (van Schaik *et al.* 2001; Gaveau *et al.* 2009), given the extensive habitat loss and anthropogenic pressure this location is currently facing (van Schaik *et al.* 2001; Wich *et al.* 2008; Nater *et al.* 2013). Therefore, further investigation is needed to address whether more detailed work is required to assess the conservation status of orangutans from this area.

We found one sampling location (Blangkejeren, a dry highland area at an altitude up to 1000 m, which is located in Central Aceh), sharing haplotypes with three other populations (North Aceh, Langkat and Alas River). Blangkejeren is located at the northern headwaters of the Alas River (Barber *et al.* 2005), which does not act as an effective barrier to gene flow in the area. It is also centrally positioned among the populations of North Aceh, Langkat and Alas River (Ketambe), to all of which it is roughly equidistant. Further sampling, ideally involving samples from wild orangutans or samples from rehabilitation centers with good provenance, is required to investigate the status of the Blangkejeren highland area due to its central location and unique ecology.

An earlier study based on mitochondrial genes suggested that the Batang Toru lineage diverged from all other orangutan lineages in both Sumatra and Borneo around 3.5 million years ago (Nater *et al.* 2011). Based on this and more recent autosomal evidence (Nater *et al.* 2013), the Batang Toru population is genetically the most isolated in Sumatra. Therefore, it needs further investigation whether this population might comprise a new subspecies, although currently no obvious morphological differences have been documented. Nonetheless, the Batang Toru orangutan population requires a dedicated conservation effort, due to its isolation (small census size with approximately 550 individuals in an area of ca. 975 km^2 at about 200–1500 m above sea level) (Wich *et al.* 2008). This density is much higher than that thought to be sustainable for semi-solitary orangutans (Singleton and van Schaik 2001). An adult male of Sumatran orangutan require ca. 4.50 km^2 of home range with ca. 1 km^2 daily travel distance for both sexes (Campbell-Smith *et al.* 2011). Sumatran orangutans need large habitats close to streams, rivers and swamps with abundant availability of soft-pulp fruit as main food sources (Delgado and van Schaik 2000; Wich *et al.* 2011a). We are questioning the possibility of the habitat supporting such high densities of orangutans, within this geographically isolated area, without natural chances of dispersal in any way.

Table 4. Population average pairwise differences from five population clades of HVR-I Sumatran orangutan

Population	C-AR	C-TR	C-NA	C-LK	C-BT
Alas River (C-AR)	0.801	24.661	38.124	43.936	71.345
Tripa (C-TR)	21.779	4.963	35.127	41.045	58.650
North Aceh (C-NA)	36.996	31.917	1.457	32.692	57.378
Langkat (C-LK)	42.716	37.744	31.143	1.640	64.932
Batang Toru (C-BT)	70.945	56.168	56.649	64.112	0.000

Average number of pairwise differences inter-population (above diagonal). Average number of pairwise differences intra-population (diagonal elements; gray shading color). Average corrected number of inter-population pairwise differences, computed with Tamura-Nei without gamma correction (below diagonal).

From a genetic diversity perspective, orangutans have the highest genetic diversity among all great apes (Steiper and Young 2006; Prado-Martinez *et al.* 2013). Orangutan populations in Sumatra are thought to be very old, based on the deep phylogenetic splits which date back hundreds of thousands to millions of years (Nater *et al.* 2011). This is in stark contrast to Borneo, where for mitochondrial DNA all populations appear to consolidate around 176 thousands years ago (Arora *et al.* 2010). This remarkable stability of Sumatran populations is reflected in our data set in the higher diversity estimates in Sumatra compared to Borneo (Arora *et al.* 2010). However, these idiosyncrasies are not yet taken into account by the current orangutan taxonomy, which suggests three subspecies for Borneo, but none for Sumatra (Groves 2001). Thus, there is an urgent need to revise the taxonomy of Sumatran orangutans by taking genetic information into account.

In conservation genetics, methods like genetically informed demography-based approaches, cladistics diversity measures, nested clade analysis and diagnostic SNPs are frequently used to assist in comprehensive decision-making (DeSalle and Amato 2004; Morin *et al.* 2004). The haplotypes described in this study provide a first step at documenting the genetic diversity of all extant Sumatran orangutan populations. However, our data are not sufficient yet to advocate taxonomic revisions. Broader genetic population analyses and different marker systems will be needed to do so. A pilot survey to the southern area of Batang Toru and historic DNA analysis using museum samples will sharpen the status recommendation. Based on this present study, restriction fragment length polymorphism assays can be developed to carry out genetic assignments using basic laboratory equipment. Our study also addresses the requirements by the "Strategi dan Rencana Aksi Konservasi Orangutan Indonesia 2007–2017" (Indonesian National Orangutan Conservation Strategy and Action Plan 2007–2017; Soehartono *et al.* 2007). This Indonesian regulation on orangutan conservation already requires to take action for stabilizing the minimum population in the wild by educating the local communities as well as public and strengthening the national conservation law to limit hunting, illegal trading and primary land forest conversion (Indonesia 1990; Meijaard *et al.* 2011; Wich *et al.* 2012). The regulation also specifies the reintroduction of all individuals in captivity, back to their natural habitat by 2015. Our study supports the latter task by giving compiled genetic information, allowing the

identification of orangutan management units, particularly for the conservation efforts of Sumatran orangutan in its natural habitat.

Conflicts of interest

The authors declare no conflict of interest.

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