Molecular Ecology (2008) **17**, 2898–2909 doi: 10.1111/j.1365-294X.2008.03793.x

Riverine effects on mitochondrial structure of Bornean orang-utans (*Pongo pygmaeus*) at two spatial scales

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Abstract

We examined mitochondrial DNA control region sequences of 73 Kinabatangan orang-utans to test the hypothesis that the phylogeographical structure of the Bornean orang-utan is influenced by riverine barriers. The Lower Kinabatangan Wildlife Sanctuary contains one of the most northern populations of orang-utans (*Pongo pygmaeus*) on Borneo and is bisected by the Kinabatangan River, the longest river in Sabah. Orang-utan samples on either side of the river were strongly differentiated with a high Φ_{ST} value of 0.404 ($P < 0.001$). Results also suggest an east–west gradient of genetic diversity and evidence for population expansion along the river, possibly reflecting a postglacial colonization of the Kinabatangan floodplain. We compared our data with previously published sequences of Bornean orang-utans in the context of river catchment structure on the island and evaluated the general relevance of rivers as barriers to gene flow in this long-lived, solitary arboreal ape.

Keywords: control region, noninvasive genetics, phylogeography, population structure, primates, riverine barrier hypothesis

Received 13 December 2007; revision received 21 March 2008; accepted 7 April 2008

Introduction

The orang-utan (*Pongo pygmaeus*), the largest arboreal ape, is found on Sumatra and Borneo in Southeast Asia and has traditionally been classified into two subspecies, *P. pygmaeus pygmaeus* in Borneo and *P. p. abelii* in Sumatra. However, recent molecular data have led to the reclassification of the orang-utan into two distinct species, *P. pygmaeus* and *P. abelii* (see Xu & Arnason 1996; but see Muir *et al*. 1998, 2000; Zhang *et al*. 2001). Based on mitochondrial control region DNA data, Warren *et al*. (2001) identified four putative evolutionary groups within the Bornean orang-utan, corresponding to populations living in (i) Sabah, (ii) Sarawak and northwest Kalimantan, (iii) southwest and central Kalimantan, and (iv) east Kalimantan. Warren *et al*. (2001) recommended that these subpopulations should be treated as separate units for conservation with their genetic integrity maintained. They also suggested that rivers may have influenced current patterns of mitochondrial DNA (mtDNA) variation within orang-utans in Borneo. This hypothesis was recently supported by our microsatellite data (Goossens *et al*. 2005), which indicated that immigration and gene flow across the Kinabatangan River is extremely unlikely to have occurred over recent demographic timescales. Therefore, for comparison, we studied the genetic structure of Lower Kinabatangan orang-utan population using mtDNA control region sequences to provide a phylogeographical perspective to this study and to allow us to make inferences on longer term spatio-temporal demographic processes affecting the genetic diversity of northern Bornean orang-utans.

Phylogeographical studies are often used to examine the effects of biogeographical barriers, such as rivers (e.g. Telfer *et al*. 2003; Erikkson *et al*. 2004) and mountain ranges (Hewitt 2000), which may disrupt gene flow and shape genetic structure over long timescales. For widespread species, genetic divergence may nevertheless occur primarily as a function of distance due to limited gene flow (Avise *et al*. 1987). Nevertheless, support for the importance of rivers

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in shaping genetic diversity in the wet tropics (the so-called 'riverine barrier hypothesis', e.g. Salo *et al*. 1986) has been found within primates (Ayres & Clutton-Brock 1992) and lizards, *Gymnodactylus darwinii* (see Pellegrino *et al*. 2005) and *Liolaemus monticola* (see Torres-Perez *et al*. 2007). However, support for the hypothesis in other fauna such as birds is more equivocal; for example, some avian groups may be more influenced by rivers than others (Hayes & Sewlal 2004), and even within primates, Collins & Dubach (2000) did not find any indication of rivers as a barrier when studying the genus *Ateles*. Watercourse width can be an important additional factor affecting dispersal and gene flow across rivers, as has been shown in a number of groups (e.g. Ayres & Clutton-Brock 1992; Hayes & Sewlal 2004).

Among the Great Apes, rivers correlate with genetic differentiation in a number of cases but not in others. For example, bonobos show high genetic diversity across their range within the Congo and can be loosely divided into two major clades (Eriksson *et al*. 2004). The greatest differentiation was observed between populations divided by Congo and Lomami rivers, respectively. In chimpanzees, Gonder *et al*. (1997, 2006) identified the Sanaga River in Cameroon as the most likely critical barrier separating two chimpanzee subspecies. However, in western lowland gorillas, Clifford *et al*. (2004) did not find either the Sanaga or the Cross rivers to delineate the boundaries of present-day mitochondrial control region haplogroups, in contrast to current taxonomic designations. Thus, the role of rivers in shaping genetic structure in Great Apes cannot be generalized, although rivers are likely to present a barrier to gene flow in some species.

If rivers do act as barriers to gene flow, then predictions can be made as to their role in shaping population genetic structure along their course. The interaction between riverine barriers, Pleistocene refugia and postglacial expansion routes may produce patterns of genetic structure which reflect the increased likelihood of gene exchange among populations residing at the headwaters of river systems (e.g. Peres *et al*. 1996) and genetic structure better reflecting historical refugia than contemporary gene flow in species with limited dispersal (e.g. Patton *et al*. 1994). The expected patterns of genetic diversity in regions where riverine barriers and other putative Pleistocene refugia occur will depend on their relative geographical orientation, proximity and ecological factors (such as the requirement for the presence of closed canopy forest, e.g. Clifford *et al*. 2004). The most likely glacial refuge for Kinabatangan orang-utans is the Crocker range hills and Mount Kinabalu, which at an altitude of approximately 4100 m above sea level is the highest mountain between the Himalayas and the New Guinean highlands. Mount Kinabalu is approximately 150 km west of the Kinabatangan River and it is hypothesized that forest species recolonized the Kinabatangan floodplain from this refuge, potentially establishing parallel clines in genetic diversity from east to west along either side of the river. Such phylogeographical patterns have not been previously explored in orang-utans, either in Borneo or Sumatra.

Genetic studies on orang-utans to date have mainly focused on the taxonomic status of the Bornean and the Sumatran taxa (Xu & Arnason 1996; Zhi *et al*. 1996; Muir *et al*. 2000; Zhang *et al*. 2001), with rather fewer studies focusing on population genetic structure (Warren *et al*. 2000, 2001; Goossens *et al*. 2005). Most of these studies have utilized invasive samples (see Kanthaswamy *et al*. 2006) taken from individuals in zoo and rehabilitation centres with very limited (necessarily noninvasive) sampling from wild animals (Kanthaswamy & Smith 2002). Only recently have completely noninvasive samples (i.e. hair and faeces) been utilized, and used to study wild orang-utans in the Lower Kinabatangan floodplain (Goossens *et al*. 2005, 2006a, b).

In the current study, the structure and historical demography of the fragmented orang-utan populations previously analysed by Goossens *et al*. (2005, 2006a, b) were re-examined using mitochondrial control region sequences. As mitochondrial genomes are maternally inherited in a single copy, an absence of increased dispersal rates in females (as has been inferred by Utami *et al*. 2002 and Goossens *et al*. 2006b) is predicted to lead to an increased structuring of mitochondrial lineages. Such demographic effects are also predicted to result in a decrease in intrapopulation mitochondrial variability and to increase mitochondrial differentiation between subpopulations. Recently, Goossens *et al*. (2006b) using microsatellite markers showed males and females to be equally philopatric in the Kinabatangan, raising the question as to whether mtDNA would show a different structure to nuclear DNA. Here, we analysed samples from the northern and southern side of the river to investigate phylogeographical structure and gene flow along an almost linear habitat. In addition, sequences from Warren *et al*. (2001) from known localities in Borneo were used to analyse the phylogeographical relationships between Lower Kinabatangan Wildlife Sanctuary samples and localities elsewhere in Borneo.

Materials and methods

DNA samples

Faecal samples ($n = 73$) used in the current study were collected in 2001 from all 10 lots in the Lower Kinabatangan Wildlife Sanctuary (LKWS), Sabah (Fig. 1). Details of sampling and DNA extraction protocols are given in Goossens *et al*. (2005). DNA was extracted in a class 1 microbiology safety hood. As an added precaution during each polymerase chain reaction (PCR) amplification,

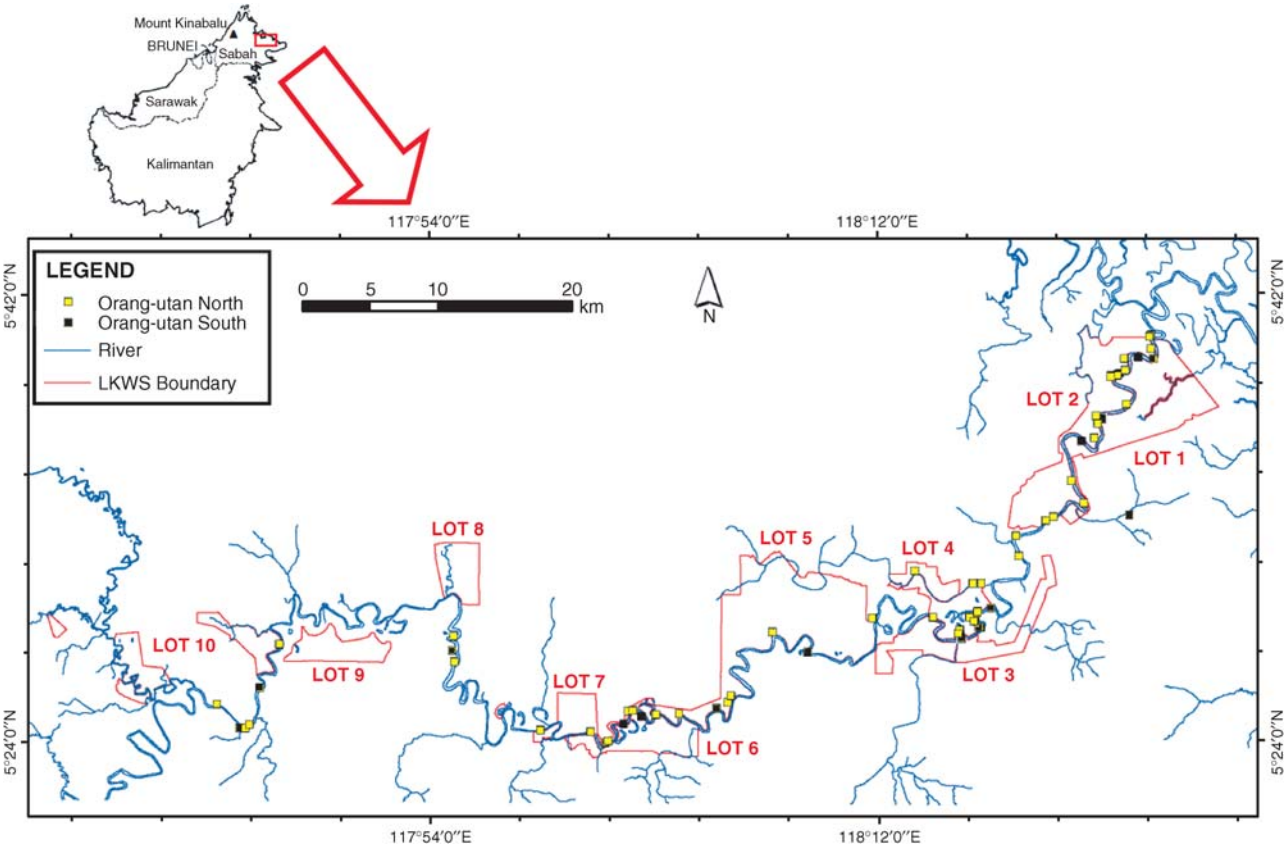

Fig. 1 Map showing Borneo and the red square indicates Lower Kinabatangan Wildlife Sanctuary. Distribution of orang-utan faecal samples (yellow and black squares) collected along the Kinabatangan River. Red line indicates the Lower Kinabatangan Wildlife Sanctuary boundaries for each Lot and blue lines indicate the Kinabatangan River and its tributaries.

experiment samples of all personnel involved were used as a positive control. All 73 faecal samples represent 73 different individuals as all have previously been genotyped using 14 human-derived microsatellites (Goossens *et al*. 2005). As only a single sample was collected from lot 7, this sample was combined with those from the closest lot (5) for analysis. Information on LKWS lot size and forest types can be obtained from Ancrenaz *et al*. (2004a). Unique DNA sequences have been submitted to GenBank under Accession nos EU547189–EU547201.

Orang-utan sequences from Warren *et al*. (2001; $n = 29$; see Supplementary material) were combined with sequences from the current study to further investigate the relationships between orang-utan sequences in Borneo. Warren *et al*.'s sequences (GenBank AJ391095–AJ391141) were derived from several populations from the Malaysian States of Sarawak and Sabah, and the Indonesian province of Kalimantan. Sequences from the current study were shortened (by 89 bp) to match those from Warren *et al*. (2001). The combined data set (78 Sabah and 102 Borneo sequences) comprising 234 bp of sequence was analysed as described below.

Control region sequencing

The left hypervariable domain of the control region (323 bp) was amplified by PCR using primers Pp-5′ (5′-GCACTTAACTTCACCATC-3′) and Pp-3′ (5′-AAACAAGGGACCACTAAC-3′) designed during the current study specifically to amplify orang-utan mtDNA. The PCR mix contained 1.5 μL 4 mg/mL BSA, 2 μL 10× PCR Buffer, 1.5 μL 25 mM $MgCl_2$, 1 μL 10 mM of dNTP mix, 0.2 μL of 50 pM of each primer, 0.2 μL of Ampli*Taq* Gold (Applied Biosystems), 11.4 μL of ultrapure water and 2 μL template DNA in a final volume of 20 μL. PCRs, carried out in a Perkin Elmer 9700 thermocyclers, were performed following an initial denaturation for 12 min at 94 °C followed by 40 cycles of 94 °C for 40 s, at 61 °C for 30 s and 1 min at 72 °C with a final extension step of 10 min at 72 °C. Negative controls (with DNA template replaced with ultrapure water) were included with each PCR. Prior to sequencing, 5 μL of each PCR product was electrophoresed on a 1.5% agarose gel to verify amplification. Each 10 μL PCR product was cleaned by the addition of 1 μL 1:1 ratio of Exonuclease I (10 U/μL) (USB Corp.) and Shrimp Alkaline Phosphatase 1 U/μL

(USB Corp.) (Hanke & Wink 1994). The product was incubated at 37 °C for 1 h and at 80 °C for 15 min to deactivate the enzymes. Sequencing PCR was performed using ABI BigDye Terminator version 1 (Applied Biosystems). Each PCR product was sequenced in both directions. PCR products were precipitated by adding 90 μL of 63% isopropanol to each PCR tube. The product and isopropanol were vortexed for 20 s, left to stand for 15 min, then centrifuged for 30 min at 13 000 *g*. The supernatant was discarded and 150 μL of 70% isopropanol was added to each PCR tube. The PCR strips were then centrifuged for 1 min at 500 *g* and the pellets dried at 52 °C for 2 min. Sequencing was performed in an ABI3100 automated sequencer. The mtDNA control region sequences were aligned using the program SEQUENCHER 3.1.2 (GeneCodes) with correction by eye. A BLAST (basic local alignment search tool) nucleotide search was performed on each sequence. The DNA amplified is extremely similar in phylogenetic analysis to those in GenBank, and two completely match previous orang-utan control region mtDNA sequences published by Warren *et al*. (2000). Furthermore, the DNA was amplified using several sets of other primers (such as Kocher *et al*. 1989 and Palumbi 1996), all producing the same sequences (although with different lengths). We also did not encounter any sequencing artefacts.

Phylogenetics, mtDNA diversity and population structure

Sequences were collapsed into unique haplotypes using DAMBE 4.2.13 (Xia & Xie 2001). Intraspecific gene genealogies were inferred using minimum spanning in ARLEQUIN version 3 (Excoffier *et al*. 2005) and NETWORK version 4.1.1.1 (Bandelt *et al*. 1999), respectively. Intraspecific nucleotide level (π) and haplotype diversities (h) were estimated using ARLEQUIN (Tajima 1983; Excoffier *et al*. 2005). Analysis of molecular variance (AMOVA) was used to analyse how genetic variability was partitioned within and between riversides using Φ-statistics in ARLEQUIN. For the purposes of the analysis, we categorized two 'regions' as comprising all population fragments north and south of the river, respectively. AMOVA can incorporate information on the absolute number of differences among haplotypes and haplotype frequencies. The significance of variance was tested by 1000 random permutations. In order to test the sequences for deviation from the expectations based on neutral theory, Tajima's D (Tajima 1989), Fu's F_S (Fu 1997), Fu and Li's D^* (Fu & Li 1993), and Fu and Li's F^* (Fu & Li 1993) were calculated using ARLEQUIN and DNASP 4.10.3 (Rozas *et al*. 2003). Past demography was also assessed by mismatch distribution (distribution of pairwise sequence differences; Rogers & Harpending 1992) in ARLEQUIN based on a spatial and sudden expansion models for orang-utan samples at three different levels; for each riverside, for Sabah and for Borneo. A coalescent-based simulation method to test for evidence of population expansion was also carried out, implemented in FLUCTUATE version 1.4 (Kuhner *et al*. 1998). The model was run five times to ensure convergence of the estimates.

Results

Lower Kinabatangan Wildlife Sanctuary

From all 73 DNA samples, 323 bp of the left hypervariable domain of the control region was successfully amplified (Table 1). From the 323 nucleotides, 314 were invariant and nine were variable (2.8%) with eight transitions and one transversion.

Among the 13 haplotypes identified (Table 1), OU11 and OU12 were dominant and found in 28 (38.3%) and 25 (34.2%) samples, respectively. OU11 was identified in eight out of nine lots (not in lot 1) and OU12 in six lots (absent from lots 2, 4 and 5). Only haplotypes OU10 (north = 4; south = 1), OU11 (north = 21; south = 7) and OU12 (north = 5; south = 20) were found on both sides of the river. Haplotypes OU01, OU02, OU07, OU08 and OU13 were present exclusively on the northern side, whereas OU03, OU04, OU05, OU06 and OU09 were only on the southern bank. Besides haplotypes OU11 and OU12, only OU05 and OU10 were recorded in more than one Lot (OU05 = lots 1, 6 and 9; OU10 = lots 2 and 9) (see Table 2).

A minimum-spanning network connecting the 13 haplotypes in a linear form revealed a partial separation into two groups, largely corresponding to populations on the northern and southern sides of the river (Fig. 2). Haplotypes OU12 and OU11, distinguished by 10 substitutions, were the most common haplotypes on each riverside. There were four haplotypes between OU11 and OU12, two haplotypes radiating from OU12 and three haplotypes from OU11. Based on AMOVA, the genetic variation in LKWS orang-utans was mostly attributable to differences among populations between riversides ($\Phi_{ST} = 0.404$; $P < 0.001$), suggesting that the river is a significant barrier to gene flow. Within the northern riverside, the Φ_{ST} value was higher ($\Phi_{ST} = 0.388$, $P < 0.001$) than for the southern riverside ($\Phi_{ST} = 0.067$, $P = 0.123$), indicating genetic structure on the northern but not on the southern riverside. Nucleotide diversity (π) ranged from 0.005 ± 0.003 on the northern riverside to 0.008 ± 0.005 in the whole of LKWS. Overall haplotype diversity (h) within LKWS was 0.734 ± 0.035. Comparing the north and south populations, the latter had the highest nucleotide (π, 0.006 ± 0.004) and haplotype diversity (h, 0.690 ± 0.071) (Table 2). The overall high haplotype and low nucleotide diversity in the LKWS indicates a population bottleneck event followed by rapid population growth and accumulation of mutations.

Table 1 Condensed matrix displaying variable sites of the 323-bp alignment of the mtDNA control region for 13 haplotypes found in Lower Kinabatangan orang-utan. Haplotype codes and nucleotide position are displayed on the left, and haplotype frequencies for each Lot (L) are given on the right

	Variable sites									North					South			
	9 9	1 7 5	2 0 9	2 2 6	2 4 3	2 5 5	2 6 1	3 9 3	3 0 8	L2	L4	L5	L8	L10	L1	L3	L6	L9
OU01	C	A	C	C	A	G	T	C	T			1						
OU02	.	.	.	.	.	.	.	A	C	1								
OU03	.	G	.	T	.	.	C	.	C								1	
OU04	T	.	.	.	G	A	.	.	C						2			
OU05	T	.	.	.	.	A	.	.	C						1		1	1
OU06	.	.	.	.	.	A	.	A	C						1			
OU07	.	G	T	.	.	.	.	.	C			1						
OU08	.	G	T	.	.	A	.	.	.		1							
OU09	.	.	.	T	.	A	.	.	C							3		
OU10	.	G	.	.	.	.	.	.	.	4								1
OU11	.	G	T	.	.	.	.	.	.	3	10	5	2	1		3	3	1
OU12	.	.	.	.	.	A	.	.	C				1	4	8	4	5	3
OU13	T	.	T	.	.	.	.	.	.			1						
TOTAL										8	11	8	3	5	12	10	10	6

Table 2 Number of sequences and haplotypes, nucleotide diversity (π), haplotype diversity (*h*), test of selective neutrality (Tajima's *D*, Fu's F_S, Fu and Li's *D**, and Fu and Li's *F**) and population parameters of theta (θ) and growth parameter (g) of orang-utan mtDNA control region sequences for LKWS, Sabah and Borneo

Samples	All LKWS	LKWS north	LKWS south	Sabah	Borneo
Sequences	73	35	38	78	102
Haplotypes	13	8	8	17	40
h	0.736 ± 0.035	0.620 ± 0.086	0.690 ± 0.071	0.768 ± 0.034	0.865 ± 0.025
π	0.007 ± 0.005	0.005 ± 0.003	0.006 ± 0.004	0.011 ± 0.007	0.026 ± 0.014
D	0.789 (*P* = 0.837)	0.162 (*P*= 0.591)	–0.157 (*P* = 0.471)	–0.798 (*P* = 0.236)	–1.582 (*P* = 0.030)
F_S	–2.586 (*P* = 0.170)	–1.920 (*P* = 0.120)	–0.811 (*P* = 0.371)	–5.147 (*P* = 0.023)	–18.897 (*P* = 0.000)
*D**	—	—	—	—	–3.140 (*P* < 0.05)
*F**	—	—	—	—	–3.046 (*P* < 0.02)
θ	0.041 ± 0.003	0.008 ± 0.001	0.006 ± 0.001	0.053 ± 0.004	0.177 ± 0.019
g	305.44 ± 36.70	170.68 ± 173.35	119.42 ± 130.52	526.12 ± 56.03	147.54 ± 16.96

Neutrality tests were performed to detect for additional evidence of population expansion. Negative values indicate the presence of either some form of selection (unlikely at the noncoding control region) or population expansion. All data sets (LKWS, north and south) had negative values for Fu's F_S (Table 2), indicating population expansion, although these values were not significant at the 95% level. In contrast, Tajima's *D* results were much lower (< 1.0) and only one data set (south) showed a negative value, but again this was not significant (Table 2).

Bimodal mismatch distributions (not shown) for LKWS, the northern and southern populations indicate two population expansions or the presence of two or more mixed populations that have subsequently expanded. The latter interpretation is clearly supported by the network in which the haplotypes OU11 and OU12 were found on both sides of the river. A coalescent approach to detect population expansion using FLUCTUATE indicated positive estimates for the growth parameters for all sample groups (LKWS, northern and southern) (Table 2). Estimates of theta (θ ± SD) were 0.041 ± 0.003 (LKWS), 0.008 ± 0.001 (north) and 0.006 ± 0.001 (south). The growth parameters (g ± SD) were 305.44 ± 36.70 (LKWS), 170.68 ± 173.35 (north) and 119.41 ± 130.52 (south) which indicates population growth. Both positive theta values and growth parameters high values support the results of the neutrality test and mismatch

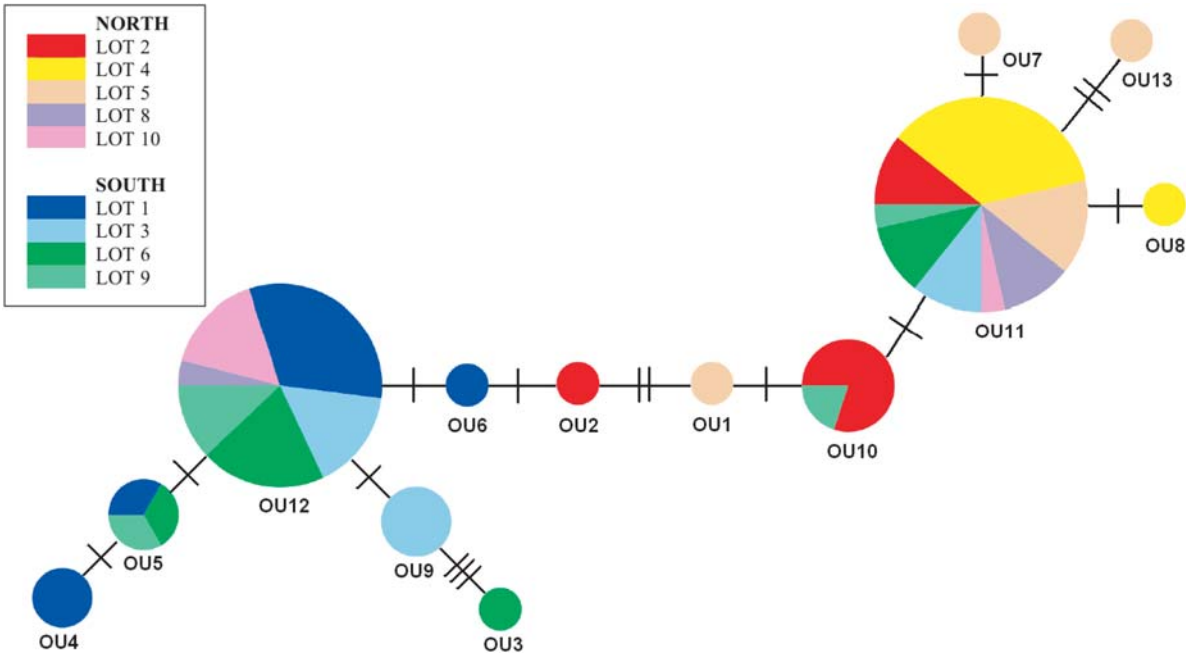

Fig. 2 Minimum-spanning network (MSN) of the Kinabatangan orang-utan sequences. Each circle represents a haplotype and the diameter scales to haplotype frequency. The smallest circles represent singletons. Mutational steps are represented by black bars on lines connecting haplotypes (MSN).

distribution, that the Kinabatangan orang-utan population has experienced relatively recent demographic expansion.

Sabah

The LKWS samples were combined with five Sabah samples from Warren *et al*. (2001) and reanalysed. From 78 sequences, there were a total of 17 haplotypes with 18 polymorphic sites. There were 17 transitions and one transversion in the data set with a haplotype diversity h of 0.768 ± 0.034 and nucleotide diversity π of 0.011 ± 0.007. Of the five Warren samples, only one (SB70, AJ391119, Lahad Datu, Sabah), matched an LKWS haplotype, OU08. Four other haplotypes grouped within the dominant northern haplotypes, OU11 (SB57-AJ391117) or OU12 (SB372-AJ391116; SB71-AJ391120; SB60-AJ39118). For the Sabah data set, both networks showed a similar topology to the LKWS data set (not shown) with the addition of four new haplotypes, radiating from OU11 (SB57, SB70) and OU12 (SB372, SB60, SB71).

Both tests of selective neutrality (Fu's F_S and Tajima's D) revealed negative values. However, the values obtained from Fu's F_S (–5.147 $P = 0.023$) were significant and far greater than Tajima's D (–0.798 $P = 0.236$) which was not significant. A bimodal pattern was also found for mismatch distribution for the Sabah data set (Fig. 3). Growth estimate (g ± SD) for the Sabah data set indicated positive growth of 526.12 ± 56.03 and theta (θ ± SD) of 0.053 ± 0.004 (Table 2). These results indicate that the Sabah population, like the Lower Kinabatangan population, has been expanding in the recent past.

Examining the major Sabahan haplotypes, OU11 and OU12, reveals some evidence for colonization from the west to the east (Fig. 5). Figure 2 revealed that both haplotypes, OU11 and OU12 are the most common haplotypes in Sabah and may be the two ancestral sequences given that that other, less common haplotypes are usually only separated by one or two mutational steps. On the northern bank of the river, OU11 increases in frequency from west to east (Fig. 5) becoming fixed in lot 5, the largest population unit within the sanctuary. OU12 is the dominant haplotype on the southern bank and is fixed only in lot 1 (Fig. 5). In contrast, on both sides of the river, the western edge of the sanctuary possesses a mixture of both dominant haplotypes. However, statistical and spatial analysis of this pattern is uninformative, given the number of sampling stations and such a pattern can only be confirmed by extensive additional sampling further upriver, closer to the source of the Kinabatangan, but outside the limits of the wildlife sanctuary.

Borneo

The combined data set between LKWS samples and Warren *et al*. (2001) yielded a total of 40 haplotypes (13 LKWS and 27 Warren) with 62 polymorphic sites (Table 2) and 65 substitutions, 57 of which were transitions and eight transversions. The haplotype diversity obtained for the

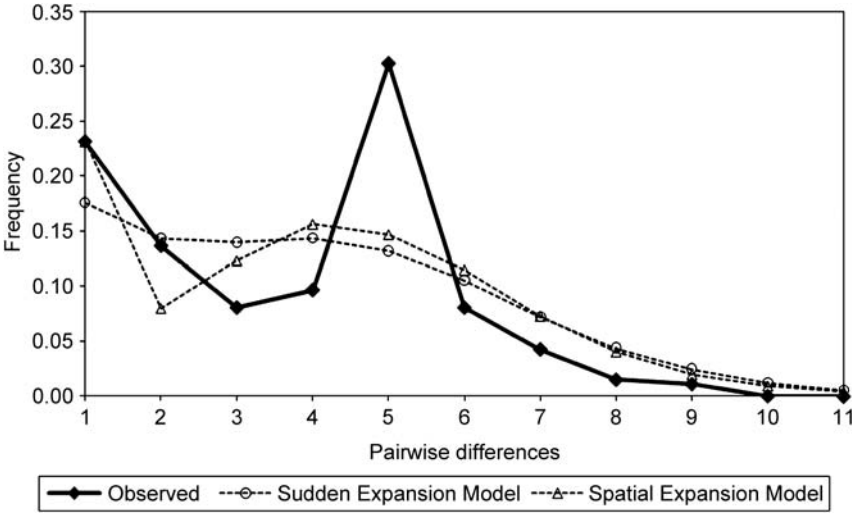

Fig. 3 Mismatch distribution for Sabah orang-utan (LKWS and five Warren samples). Solid line represents the observed and dashed lines represent the expected for each model.

Bornean data set was h: 0.865 ± 0.025 and nucleotide diversity was π: 0.026 ± 0.014. Both networks revealed four groups corresponding to (i) Sabah, (ii) Sarawak and northwest Kalimantan, (iii) southwest and central Kalimantan, and (iv) east Kalimantan (Fig. 4 — median-joining network shown). The most similar haplotypes to the Sabah samples was the east Kalimantan group, which was separated by four substitutions.

Both Fu's F_S and Tajima's D revealed negative values for the neutrality test which were significant at the 95% significant level (D: –1.582 and F_S –18.973). To further examine the Bornean data set, Fu and Li's D^* and F^* were calculated as these tests are more sensitive to singletons and both gave significant negative results of –3.140 ($P < 0.05$) and –3.046 ($P < 0.02$), respectively. Based on a coalescent simulation, FLUCTUATE indicated moderate growth estimates (g ± SD) of 147.54 ± 16.96 and a large theta (θ ± SD) of 0.177 ± 0.019. All these results indicate population expansion and the presence of several Bornean populations.

Discussion

This study shows highly significant mitochondrial differentiation of *Pongo pygmaeus* populations on either side of the Kinabatangan River in Sabah. This provides support for the suggestion, based on microsatellite data, that this river is the major barrier for dispersal of orang-utans in the LKWS (Goossens *et al*. 2005), and highlights the pivotal role of the river in structuring genetic variation over a long period. In the current study, both AMOVA and median-joining networks clearly indicate two major genetic groups of orang-utans on either side of the Kinabatangan River. These findings, together with those reported by Warren *et al*. (2001), indicate that rivers play an important role in shaping the genetic structure of Bornean orang-utans. The low nucleotide and high haplotype diversity exhibited by LKWS orang-utans suggests population expansion by a few founder lineages. The two haplotypes (OU11 and OU12) exhibiting the highest frequencies are most likely to represent the cofounders of the current population. This interpretation was further supported when Sabah samples (Warren *et al*. 2001) were also shown to cluster with OU11 and OU12. The occurrence of two common haplotypes coupled with rarer haplotypes, differentiated by single mutational steps suggests that these sequences could be potential ancestral or founding lineages in populations which recently experienced expansion (Posada & Crandall 2001).

Weak structuring among haplotypes despite significant division between barriers can be possibly caused by a relatively recent demographic event, such as population growth or expansion. The high haplotype diversity, low nucleotide diversity, unimodal mismatch distribution of mtDNA haplotypes and the tests of selective neutrality (Tajima's D and Fu's F_S) observed in the whole LKWS population and in the north and south populations, all indicate a possible historical expansion. The coalescent-based estimator (θ) and growth parameters (g) also strongly support a historical population expansion.

The present population of orang-utans in the LKWS is small, about 1000 individuals (Ancrenaz *et al*. 2004b), and we sequenced 73 individuals, about 7% of the total population. However, of this 7%, we identified 13 haplotypes of which 12 were new to Sabah. Warren *et al*. (2001) reported only five haplotypes from their Sabah study and only one of these matched our haplotypes. Among the few studies on orang-utans, our work identifies the largest number of haplotypes from one study site (27 000 ha). We also found evidence that the population of orang-utans in Borneo has not been static, but was until very recently expanding after a series of bottlenecks. Zhi *et al*. (1996) found only nine haplotypes using 16 sRNA, and they also described

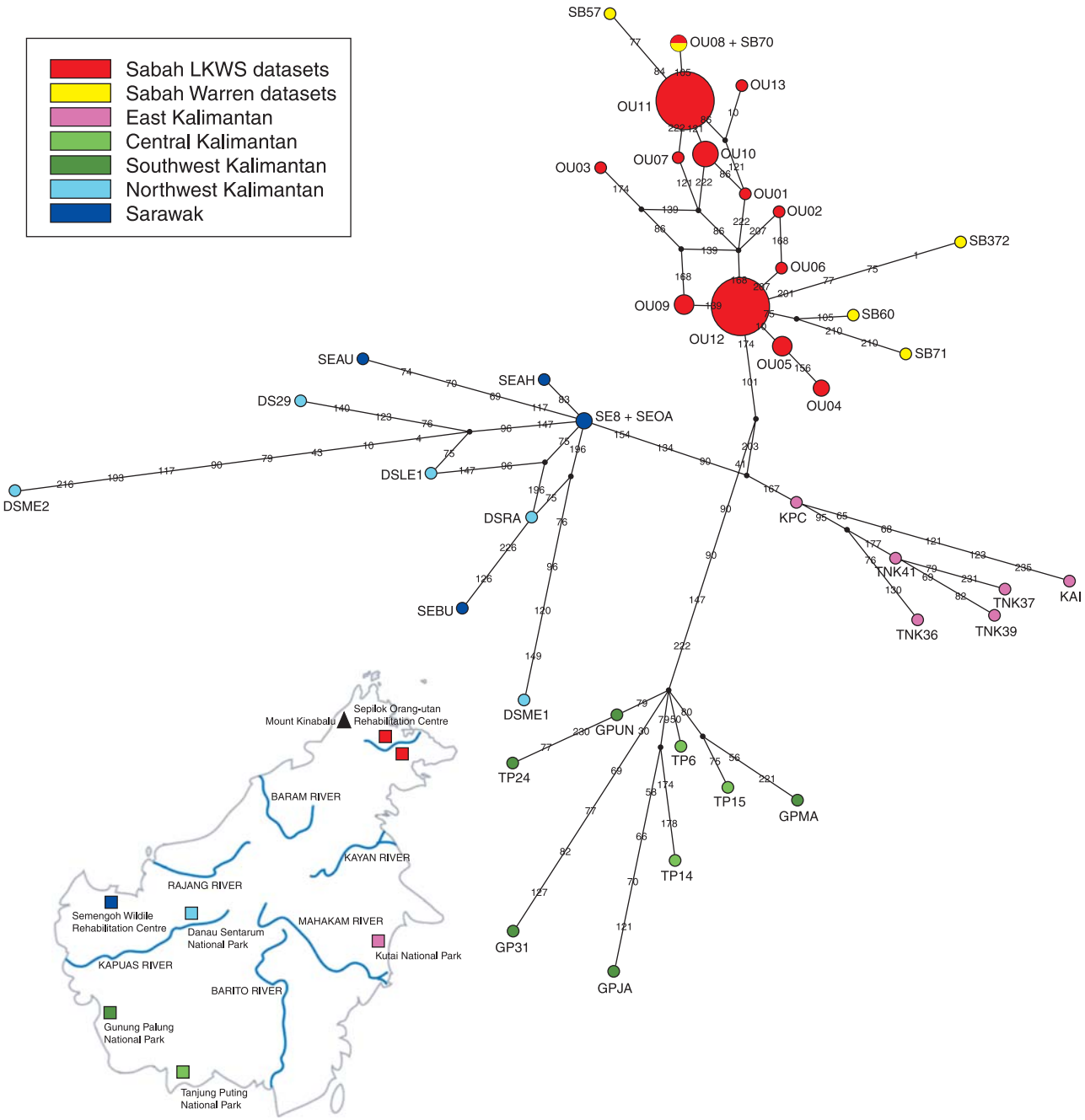

Fig. 4 Median-joining network of Bornean orang-utan samples. Black dots are median vectors presumed unsampled or missing intermediates. The number on the line indicates the site, which has undergone mutations.

complete isolation between Sumatran and Bornean orang-utans. However, they also reported very little differentiation within the Bornean orang-utan and, contrary to our data, found no evidence for past bottlenecks or founder effects (Zhi *et al*. 1996). Muir *et al*. (2000) identified three orang-utan lineages, two in Sumatra and one in Borneo. The latter consisted of just four cytochrome *b* and five NADH haplotypes. In contrast to Zhi *et al*.'s (1996) finding of complete dichotomy between Sumatran and Bornean orang-utans, Muir *et al*. (2000) found one Sumatran individual with a haplotype that was identical to the most widespread Bornean haplotype. However, both of these studies were based on limited geographical coverage. Warren *et al*. (2001) had access to all subspecies from Borneo and Sumatra, and using the control region, identified 37 haplotypes from 41 Borneo individuals that clustered into four distinct

subpopulations. These were distinct from four haplotypes identified from five Sumatran individuals (Warren *et al*. 2001). The current study is the only comprehensive population genetic study using mtDNA of orang-utans in a single area. Despite the restricted size (27 000 ha), we show that orang-utans in the Kinabatangan retain relatively high levels of mtDNA diversity. This complements the findings of Goossens *et al*. (2005, 2006a) who identified high levels of nuclear genetic diversity in the same orang-utan populations.

During the Early and Middle Pleistocene, orang-utans were widely distributed throughout mainland Southeast Asia (Harrison *et al*. 2006). When the Sunda Shelf was exposed, orang-utans are thought to have colonized Sumatra, Borneo and Java. Although a land bridge existed between mainland Southeast Asia and its islands, the movement of orang-utans might have been disrupted by a drier arid landscape of seasonal woodlands and grasslands that bordered the eastern edge of the Malay Peninsula and continued onto the lowland areas between Sumatra and Borneo, through southern Kalimantan and eastern Java and the Lesser Sunda Islands (Bird *et al*. 2005; Harrison *et al*. 2006). This almost completely isolated the orang-utan populations within Borneo, probably contributing to their morphological and molecular separation from Sumatran orang-utans, where there is no current evidence of population subdivision (Harrison *et al*. 2006). Brandon-Jones (1998) described a severe glacial drought around 190 000 years ago and a second less severe drought 80 000 years ago. Contraction and expansion of rainforest distribution has been the prime mediator of extant primate distribution in Borneo (Brandon-Jones 1998). The first severe glaciation might have caused a population decline and fragmentation of orang-utan populations due to contraction of the forest habitats. Orang-utans were previously considered highly dependent on primary forest (Delgado & van Schaik 2000), and being solitary animals, the effects of fragmentation and drought during glaciation might have resulted in local extinctions. However, Ancrenaz *et al*. (2004b) suggested that orang-utan populations can survive in small degraded forest, based on the finding that within a restricted area (Kinabatangan), these primates can utilize a wide range of foods, from fruits and leaves to insects and bird eggs. This could explain why the contraction of rainforest during the last glaciation did not wipe out all orang-utan populations as some might have survived in rainforest remnants (Brandon-Jones 1998). If gene flow was subsequently severed between isolated populations, this might have resulted in allopatric speciation (Slatkin 1987).

Based on fossil records, the Kinabatangan floodplain forest is a relatively recent habitat (Noad 2001), which is most likely to have been colonized by orang-utans from the glacial refugia of Mount Kinabalu, a known forest refugium for many species, including orchids (Barkman & Simpson 2001), termites (Garthorne-Hardy *et al*. 2002) and oaks (Cannon & Manos 2003). Assuming orang-utans colonized from this refugium, which lies to the west of the Kinabatangan headwaters, we predicted greater genetic diversity of orang-utans in the west compared to the east. If the Kinabatangan populations were founded by very few lineages, then a small number of haplotypes would dominate (Lawler *et al*. 1995) as detected in the current study. Haplotypes OU11 and OU12 were the most common haplotypes, each dominating one riverside and being more common in the east compared to the west (see Fig. 5, OU11, 1–10; OU12, 1–8). However, based on haplotype (h) and nucleotide diversities (π), a general trend of western populations having a higher diversity compared to the east was not apparent, possibly due to sampling effects. Nevertheless, this process of colonization is likely to have been slow, considering that orang-utans have a long lifespan, very low fecundity and occur at low densities (Delgado & van Schaik 2000). Recently, Goossens *et al*. (2006b) showed that orang-utans in Kinabatangan are highly philopatric; this further supports the hypothesis of relatively few founders colonizing the Kinabatangan. Further sampling of populations farther upriver towards the source of the Kinabatangan in the Crocker Range may clarify whether the predicted cline in genetic diversity from headwaters to the sea as part of the riverine barrier hypothesis (Peres *et al*. 1996; Patton *et al*. 1994) applies to the Kinabatangan orang-utan.

Currently, Bornean orang-utan mtDNA clusters into four haplogroups, although many more samples need to be analysed to confirm this pattern. Mapping the locality of samples used for genetic analyses reveals the possible separation effects of five major rivers, the Rajang, Kapuas, Barito, Mahakam and Kayan (Fig. 4). Warren *et al*. (2001) hinted that geographical barriers might be responsible for isolating the four separate subpopulations of Bornean orang-utans. As described above for the Kinabatangan, the other three haplogroups (east Kalimantan, Sarawak and northwest Kalimantan, central and southwest Kalimantan) might have originated in a similar fashion during the last glaciation. The Crocker mountain range on which Mount Kinabalu is located, is considered to consist of several separate refugia (Tanaka *et al*. 2001). In addition to Mount Kinabalu in the north, there is a second refugium in the east and a third in the west (Garthorne-Hardy *et al*. 2002) from which the different orang-utan haplogroups might have expanded. Such divergence within a species has also been shown in other species of Great Ape, such as the bonobo (*Pan paniscus*), which occurs within the Democratic Republic of Congo (Eriksson *et al*. 2004), and the western lowland gorilla (Clifford *et al*. 2004).

Groves (1986, 2001), Groves *et al*. (1992) and Uchida (1998) suggested that Bornean orang-utans could be separated into three subspecies based on cranial and dental (post

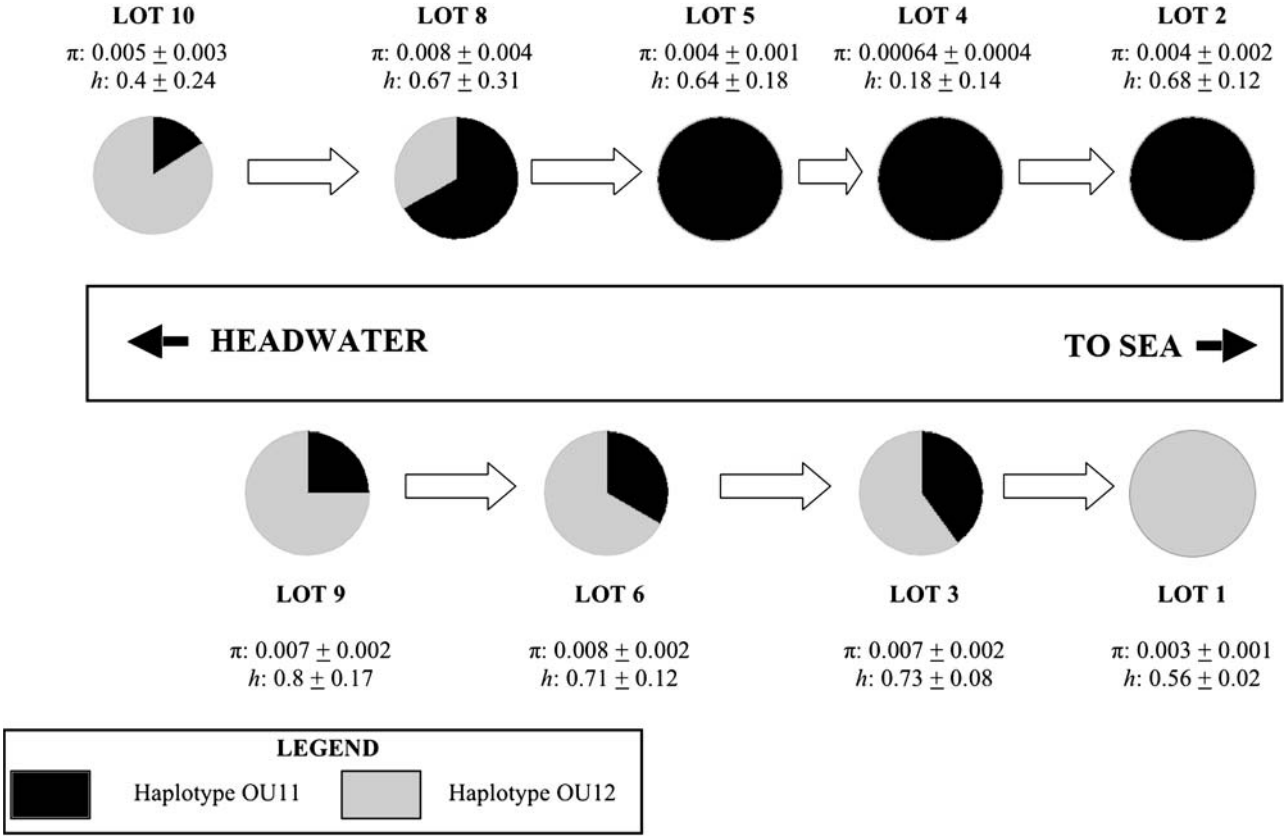

Fig. 5 The possible sequential event of lineage sorting in orang-utan populations on the northern and southern sides of the Kinabatangan River. Haplotype diversities shown indicate the whole haplotypes found within the Lot. Haplotype OU11: L10 = 1, L8 = 2, L5 = 5, L4 = 10, L2 = 3. Haplotype OU12: L9 = 3; L6 = 5; L3 = 4; L1 = 8.

canine) morphologies: (i) *P. p. morio* found in Sabah and east Kalimantan, (ii) *P. p. pygmaeus* found in Sarawak and northwest Kalimantan, and (iii) *P. p. wurmbii* found in central and southwest Kalimantan. Only recently has this hypothesis of three subspecies been accepted (Goossens *et al*. 2005; Harrison *et al*. 2006); in fact, Warren *et al*. (2001) and the current study suggest four taxa or evolutionary significant units. However, the current data cannot be explained by Groves' (1986, 2001) morphological subspecies because our Sabah and east Kalimantan clades do not form reciprocally monophyletic groups, unlike the Sarawak and northeast Kalimantan (*P. p. pygmaeus*) and central and southwest Kalimantan (*P. p. wurmbii*) which do correspond to subspecies groupings. Instead, the divergence between the Sabah and east Kalimantan populations could be attributed to the Kayan River that separates them. However, more samples south of Kinabatangan and north of Kayan River are required to substantiate this hypothesis. Unlike the other three great rivers of Kalimantan (Kapuas, Barito and Mahakam), the Kayan River is relatively short, and there is a possibility of gene flow around the headwaters of this catchment in the highlands. Orang-utans have been reported in the highlands (i.e. Mount Kinabalu; see Ancrenaz *et al*. 2005), and although they cannot swim, they can travel over large areas, providing suitable habitat is available. However, more research is needed to resolve the relationship between the east Kalimantan and Sabah subspecies.

The current study has demonstrated the importance of rivers in shaping the genetic structure of orang-utan populations. Previously, Warren *et al*. (2001) suggested that geographical barriers are responsible for partitioning of Bornean orang-utan populations, and here we postulate these geographical barriers to be rivers. This inferred that geographical structuring in the Bornean orang-utan population poses an immediate issue for conservation and further study is needed to explore the extent of geographical structuring in wild, geo-referenced Bornean orang-utan samples to detect distinct populations for conservation purposes. Populations that are highly divergent must be protected to safeguard the genetic diversity of the dwindling Bornean orang-utan, especially in Kalimantan. Further research with a larger coverage of samples and different molecular markers (i.e. Y-chromosomes; microsatellites, see Kanthaswamy & Smith 2002) could provide alternative views on population genetics of this species.

Acknowledgements

We thank the Sabah Wildlife Department and especially Patrick Andau and Laurentius Ambu for permission to conduct research in the LKWS. We also thank Maryati Mohamed and Menno Schilthuizen for their support throughout the study. We extend our thanks to the KOCP research assistants for all their hard work and their dedication to the conservation of the orang-utan and its habitat in the Lower Kinabatangan Wildlife Sanctuary and to the following sponsors for their financial support of the KOCP: zoos of Pittsburgh, Cleveland, Columbus, Houston, Chester, Philadelphia, Apenheul, Beauval and La Palmyre, USFWS GACF, WWF, AOF, BOS USA and Disney. This work was funded by the Darwin Initiative for the Survival of Species (Grant no. 09/016, DEFRA, UK), Universiti Malaysia Sabah (short-term grant no. B-0804–11-PR/U009), Cardiff University and by a PhD scholarship to M.F.J. from SLAB UMS. Finally, we would like to thank Sheena James, Carlos Fernandes, Yoshan Moodley, Mireille Johnson-Bawe and Gabriela Juma for their help and support at different stages of the study. Orang-utan faeces were exported to the UK under export permit from Federation of Malaysia (CITES Certificate no. 0467, security stamp no. MY 9123707) and under import permit from the UK (CITES Certificate no. 236719/02).

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Supplementary material

The following supplementary material is available for this article:

Table S1 Orang-utan samples from Warren *et al*. (2001) analysed in the current study.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-294X.2008.03793.x
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