Terrestrial Nest-Building by Wild Chimpanzees (*Pan troglodytes*): Implications for the Tree-to-Ground Sleep Transition in Early Hominins

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ABSTRACT Nest-building is a great ape universal and arboreal nesting in chimpanzees and bonobos suggests that the common ancestor of *Pan* and *Homo* also nested in trees. It has been proposed that arboreal nest-building remained the prevailing pattern until *Homo erectus*, a fully terrestrial biped, emerged. We investigated the unusual occurrence of ground-nesting in chimpanzees (*Pan troglodytes*), which may inform on factors influencing the tree-to-ground sleep transition in the hominin lineage. We used a novel genetic approach to examine ground-nesting in unhabituated chimpanzees at Seringbara in the Nimba Mountains, Guinea. Previous research showed that ground-nesting at Seringbara was not ecologically determined. Here, we tested a possible mate-guarding function of ground-nesting by analyzing DNA from shed hairs collected from ground nests and tree nests found in close proximity. We examined whether or not ground-nesting was a group-level behavioral pattern and whether or not it occurred in more than one community. We used multiple genetic markers to identify sex and to examine variation in mitochondrial DNA control region (HV1, HV2) sequences. Ground-nesting was a male-biased behavior and males constructed more elaborate ("night") nests than simple ("day") nests on the ground. The mate-guarding hypothesis was not supported, as ground and associated tree nests were built either by maternally-related males or possibly by the same individuals. Ground-nesting was widespread and likely habitual in two communities. We suggest that terrestrial nest-building may have already occurred in arboreally-adapted early hominins before the emergence of *H. erectus*. Am J Phys Anthropol 000:000–000, 2012.

Every weaned great ape builds a new nest, or bed, to sleep in every night, and sometimes nests are built during the day as a place to rest. Chimpanzees (*Pan troglodytes*), bonobos (*Pan paniscus*), and orangutans (*Pongo pygmaeus*) generally sleep in arboreal nests, whereas gorillas (*Gorilla gorilla*) often nest on the ground (see review in Fruth and Hohmann, 1996). As nest-building occurs in all living great apes, parsimony suggests that the common ancestor also built nests (Sept, 1992; Sabater Pi et al., 1997). Arboreal nesting in chimpanzees and bonobos further suggests that the last common ancestor of *Pan* and *Homo* slept in trees. Australopiths and early *Homo* may have continued to use trees for overnight sleep in safe, inaccessible locations from ground predators (Sabater Pi et al., 1997; Stewart, 2011). These early hominins likely used bipedal terrestrial travel as the main form of locomotion (Ward, 2002; Haeusler and McHenry, 2004), but their morphology suggests they were still able to climb trees (McHenry and Coffing, 2000). The shift to a more fully terrestrial life style in *Homo erectus* (Ruff, 2009) has been proposed to include a transition from arboreal to terrestrial sleeping (Coolidge and Wynn, 2009), possibly facilitated by the controlled use of fire (Wrangham, 2009).

Nests are made of woody plant materials and therefore are not preserved in the fossil record. Hence, it is impossible to establish whether terrestrial sleep emerged with *H. erectus*, or if some australopiths occasionally nested on the ground, based on fossils or artifacts. The study of nest-building in our closest living relative, *Pan* sp., and in particular of what motivates some populations of chimpanzees to sleep in nests on the ground, can inform on environmental or social factors that may have influenced the transition from tree to ground sleep in early hominins.

The West African chimpanzees (*Pan troglodytes verus*) in the Nimba Mountains, Guinea, build an unusually high percentage of nests on the ground (Matsuzawa and Yamakoshi, 1996; Humle, 2003; Koops et al., 2007, in press). Chimpanzees usually make arboreal nests (Fruth and Hohmann, 1996), but occasional ground nests

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have been recorded at some study sites (Reynolds and Reynolds, 1965; Goodall, 1968; Boesch, 1995; Furuichi and Hashimoto, 2000; Maughan and Stanford, 2001; Pruetz et al., 2008; Hicks, 2010). However, ground-nesting is rare in most populations (i.e., less than 5% of nests), especially for night-time use (but see Hicks, 2010). Furthermore, the identity of ground-nesters at these study sites, as well as the function of ground-nesting remain unknown. In contrast to the general chimpanzee pattern, the unhabituated chimpanzees in the Seringbara region of the Nimba Mountains make up to 20% of nests on the ground, including both elaborate ("night") and simple ("day") nests. This behavioral pattern is stable across seasons and years (Koops et al., 2007, in press).

The Nimba population has few predators (Koops et al., in press), which may allow ground-nesting to occur. However, lack of predators does not explain *why* chimpanzees decide to nest on the ground. Previously, systematic investigation of the influence of environmental factors on ground-nesting in Nimba showed no effect of climate, or of the availability of suitable trees for nesting (Koops et al., 2007). Furthermore, preliminary sexing results showed that males built most ground nests. Ground-nesting was hypothesized to reflect a male mate-guarding strategy (Koops et al., 2007). That is, males may nest on the ground, in order to guard an estrous female in a tree above. Furthermore, as ground-nesting in Nimba cannot be explained by eco-environmental factors, it is possible that this represents a cultural variant in this population. To date, it remains unknown whether this behavioral pattern is widespread (i.e., is a group-level pattern) or if it is restricted to only a few individuals.

In the present study, we set out to further test the mate-guarding hypothesis and to investigate nest-builder identity by analyzing DNA (deoxyribonucleic acid) samples obtained from nests. Molecular genetic analyses provides valuable information about features such as relatedness (Nsubuga et al., 2008), individual identity (McGrew et al., 2004), and social structure (Bradley et al., 2007) of primate groups not (yet) habituated to the presence of humans. Genetic material, such as shed hairs and feces, can often be found in, under, or close by, a fresh sleeping nest. To assign nest builder identity with certainty, individual identification with microsatellites is optimal, requiring high quality and quantity DNA from fecal samples (Morin et al., 2001). However, at Nimba, feces were not reliably and consistently associated with chimpanzee nests, so we relied on shed hairs found within nests. Unfortunately, shed hairs are problematic sources of DNA, because they contain both low-quality and low-quantity nuclear DNA (Gagneux et al., 1997; Morin et al., 2001). Consequently, shed hairs have very low polymerase chain reaction (PCR) amplification success rates and high error rates when using markers in the nuclear genome (Gagneux et al., 1997; Taberlet et al., 1997; Morin et al., 2001; Jefferey et al., 2007).

In order to maximize the amount of information obtained from low-quality DNA extracted from shed hairs, we adopted a new approach using multiple genetic markers. First, sex identification of samples was essential, in order to test our hypothesis. Second, we used mitochondrial DNA (mtDNA) to make a conservative estimate of the number of ground-nesting individuals. MtDNA, as opposed to nuclear DNA, is present in many copies per cell and so is useful when analyzing shed hairs. In addition, maternally-inherited mtDNA has a high rate of evolution and is therefore extremely variable. The hypervariable control region of mtDNA evolves most quickly and is commonly used in studies of intraspecies variation (Saccone et al., 1991). Within the control region, two hypervariable segments have been described (Greenberg et al., 1983), i.e., hypervariable region 1 (HV1) and hypervariable region 2 (HV2). In primate research, mtDNA analyses have focused almost exclusively on HV1, even though HV2 has proven highly informative in human genetic research (Budowle et al., 1999; Salas et al., 2000). In the current study, we developed a protocol for HV2 sequencing in the chimpanzee. To distinguish between individuals belonging to different matrilines, we sequenced both HV1 and HV2. With the combined information regarding sex, HV1 and HV2 haplotypes, we addressed four research questions: 1) is ground-nesting a male-biased behavior? 2) Does ground-nesting reflect a male mate-guarding strategy? 3) Is ground-nesting a group-level behavioral pattern? 4) Does ground-nesting occur in more than one community?

METHODS

Study site

The Seringbara study site (N 07.37°; W 08.28°) is in the Nimba Mountains in the south-eastern part of the Republic of Guinea. The study site covers about 25 km^2 of steep hills and valleys and is 6 km from Bossou, where a community of 12 to 23 chimpanzees has been studied for over 30 years (Matsuzawa et al., 2011). The Seringbara region is characterized by evergreen forest of medium altitude (Guillaumet and Adjanohoun, 1971) and the highest peak is at 1,752 m. The foothills are covered by primary tropical forest. Above about 900 m the vegetation changes into montane forest interspersed with patches of terrestrial herbaceous vegetation and high-altitude grasslands. The Nimba Mountains harbor several groups of chimpanzees and the region has been surveyed periodically since 1992 (Matsuzawa and Yamakoshi, 1996; Shimada, 2000; Humle and Matsuzawa, 2001; Humle, 2003), both in Seringbara and Yealé (Ivory Coast), on the other side of the Nimba range at 10 km from Seringbara. Since 2003, researchers or field assistants have been based at the Seringbara study site. The study population remains largely unhabituated to human observers, but at least six individuals were identified and regularly observed. Koops (2011a,b) gives additional information on the Seringbara research site.

Sample collection

Hair samples were collected during four field seasons totaling 18 months: April to August 2006; December 2006; November 2007 to June 2008; September to December 2008. Chimpanzee hairs were collected from ground nests and accessible tree nests (i.e., possible to access by free-climbing) that were no more than 1 month old. Nest age was assigned as: 1) fresh ($\leq$2 days): leaves still green and fresh; 2) recent ($>$2 days and $\leq$1 week): leaves still green, but wilted and droopy leaves and branches; and 3) old ($>$1 week and $\leq$1 month): nest mainly made up of dead brown leaves, but still intact (*sensu* Tutin and Fernandez, 1984). We considered a tree nest to be "in close association" with a ground nest if it was the closest tree nest to that ground nest in the nest

group. A nest group was defined as "nest of same age within 30 m of any other nest of the nest group" (Humle, 2003). Nest status was assigned as: 1) day nest: simple in construction and thought structurally too weak to support a chimpanzee's weight overnight; 2) night nest: elaborate in construction, often associated with the presence of feces below or near the nest; 3) unknown status. Hairs were stored in 95% ethanol at ambient temperature in the field and transferred to a refrigerator (+4°C) upon return to the laboratory.

DNA extraction

Each hair was treated separately and DNA was extracted from the follicle using a modified version of the Chelex 100 method (Walsh et al., 1991). Each hair sample (~0.5 cm including follicle) was washed with distilled water, air-dried, and put into a 2 ml tube containing 200 μl 5% (wt/vol) Chelex resin (Bio-Rad, Richmond, CA). Next, 2 μl of Proteinase K solution was added. Samples were incubated at 56°C overnight and subsequently vortexed at high speed for 10 s and incubated in a 100°C heat-block for 15 min (samples collected in 2006) or boiled for 8 min (samples collected in 2007–2008). Next, samples were vortexed for 10 s and centrifuged for 3 min at 13,000 rpm. The DNA extracts were stored frozen at −20°C and vortexed and centrifuged before each use. For each nest, we extracted and analyzed DNA from one to four hairs separately, depending on availability of hairs and PCR amplification success rates.

Sex identification

We used two PCR sexing methods: 1) amelogenin (AMG) nested PCR, and 2) Y-linked sex-determining region (SRY) PCR. The AMG nested PCR was based on the widely used amelogenin assay (Sullivan et al., 1993; Bradley et al., 2001; Ensminger and Hoffman, 2002). This assay uses the X-Y homologous gene amelogenin, which generates different length products from the X-and Y chromosomes (X: 106 base pairs (bp) and Y: 112 bp). Due to limited amplification success with this primer set alone, we designed a nested PCR protocol. The primer set for the outside PCR was Amel-C and Amel-D and for the inside PCR Amel-A and Amel-B (Sullivan et al., 1993; Table 1). We used both primer sets, sequentially, in total reaction volumes of 25 μl, containing 2.0 μl DNA templates (outside PCR) or 2.0 μl PCR product (inside PCR), 2.5 μl 10× PCR buffer, 0.75 μl 50 mM $MgCl_2$, 2.0 μl 2.5 mM dNTPs, 0.4 μl of each primer, 1.0 μl BSA (1 mg/ml) and 0.3 μl *Taq* polymerase. Amplification conditions for the outside PCR were: 94°C for 2 min, 40 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s, and 72°C for 10 min. Amplification conditions for the inside PCR were: 94°C for 1 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and 72°C for 10 min. PCR products were electrophoresed on an 8 to 10% polyacrylamide gel for 105 to 120 min at 300 V and visualized with UV light following EtBr or Sybr Gold staining.

The second sexing method was based on the AMG-SRY method (Di Fiore, 2005). This method uses a multiplex PCR to amplify, at the same time, fragments of the amelogenin X gene (~200 bp) and the Y-linked sex-determining region (SRY) gene (~165 bp). Following limited amplification success, we adapted the method and amplified SRY without AMG in a two-round nested PCR. The SRY primer set was SRY-F1 and SRY-R1 (Di Fiore, 2005; Table 1). This first PCR amplification was carried out in a total volume of 12.5 μl consisting of 4.0 μl DNA template in the first round and 2.0 μl PCR product in the second round. In addition, we used 1.25 μl 10× PCR buffer, 0.375 μl 50 mM $MgCl_2$, 1.0 μl 2.5 mM dNTPs, 0.5 μl BSA (10 mg/ml), 0.4 μl SRY-F1 primer, 0.4 μl SRY-R1 primer, and 0.15 μl *Taq* polymerase. Amplification conditions for the first and second round PCR were 94°C for 2 min, 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and 72°C for 5 min. PCR products were visualized as described above.

In all PCRs, we included both a positive control (plucked hair from a chimpanzee with known sex) and a negative control (ddH_2O) to ensure detection of PCR contamination. Multiple nested AMG and SRY PCRs were conducted for each hair extract and whenever possible we analyzed multiple hairs per nest. Sex was assigned as male when at least two observations of the Y chromosome were made, and as female following at least three observations of only the X chromosome. Sex was considered unknown when only one result (male or female) was obtained or when different PCRs resulted in contradictory outcomes for the same nest. During optimization of the PCR-based sex typing, we directly sequenced both male and female samples to ensure that amplification yielded amelogenin and SRY sequences.

mtDNA control region sequencing—HV1 and HV2

We amplified part of HV1 region of the mitochondrial genome. Initially, we used PCR to amplify a region of ~293 bp. However, following low success rates, we designed a nested PCR procedure which first amplified a ~509 bp region followed by a second PCR which amplified the ~293 bp region. We amplified this region of the mtDNA HV1 since it falls within the 605 bp region amplified by Shimada et al. (2004) for samples collected between 1999 and 2000 in the Bossou-Nimba region (i.e., Seringbara, Bossou, Yealé). Thus, this allowed comparison of HV1 haplotypes found at Seringbara in our study with those previously reported for Seringbara, Bossou, and Yealé. In all PCRs, we included both a positive and negative control (see above). Whenever possible, we analyzed two hairs from the same nest.

The outside primers (Table 1) used to amplify the ~509 bp region were L15989 (Steighner et al., 1999) and H16498 (Kocher and Wilson, 1991). The total PCR reaction volume for the outside PCR was 12.5 μl, consisting of 2.0 μl DNA templates, 1.25 μl 10× PCR buffer, 0.75 μl 50 mM $MgCl_2$, 1.0 μl 2.5 mM dNTPs, 0.5 μl of each primer, and 0.125 μl *Taq* polymerase. PCR amplification consisted of 94°C for 1 min, 40 cycles of 94°C for 20 s, 54°C for 30 s, 72°C for 90 s, and 72°C for 10 min. The inside primers (Table 1) used to amplify the ~293 bp region were 16108Rm13 (this study) and H16401m13 (Vigilant et al., 1989). Total PCR reaction volume was 25 μl, consisting of 1.5 μl PCR product, 2.5 μl 10× PCR buffer, 1.5 μl 50 mM $MgCl_2$, 2.0 μl 2.5 mM dNTPs, 0.8 μl BSA (1 mg/ml), 0.5 μl of each primer and 0.25 μl *Taq* polymerase. PCR amplification consisted of 94 °C for 1 min, 32 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 10 min. Products of the inside PCR were separated on a 1% agarose gel (100 V, 15 min) and visualized using EtBr and UV light. PCR products were sent for sequencing to Macrogen (Korea). Sequences were aligned with the Clustal W function (MEGA 4.0.2.) and chromatograms were visually checked for ambiguous

TABLE 1. Primers used for sex determination and sequencing of HV1 and HV2

Name	Nucleotide sequence	Source
Amel-A	5′-CCCTGGGCTCTGTAAAGAATAGTG-3′	Sullivan et al., 1993
Amel-B	5′-ATCAGAGCTTAAACTGGGAAGCTG-3′	Sullivan et al., 1993
Amel-C	5′-ACCTCATCCTGGGCACCCTGG-3′	Sullivan et al., 1993
Amel-D	5′-AGGCTTGAGGCCAACCATCAG-3′	Sullivan et al., 1993
SRY-F1	5′-AGTGAAGCGACCCATGAACG-3′	Di Fiore, 2005
SRY-R1	5′-TGTGCCTCCTGGAAGAATGG-3′	Di Fiore, 2005
L15989	5′-CCCAAAGCTAAGATTCTAAT-3′	Steighner et al., 1999
H16498	5′-CCTGAAGTAGGAACCAGATG-3′	Kocher and Wilson, 1989
L16108Rm13	5′-**CAGGAAACAGCTATGAC**CGTATTTCGTACATTACTGC-3′	This study
H16401m13	5′-**TGTAAAACGACGGCCAGT**TGATTTCACGGAGGATGGTG-3′	Vigilant et al., 1989
CE16498	5′-TGTATCYGRCATCTGGTTC-3′ with Y = C/T and R = A/G	This study
CEH5	5′-CACTGAAAATGRRTKGARGGG-3′ with R = C/T and K = A/C	This study
L00056	5′-GAGCTCTCCATGCATTTGGTA-3′	Krings et al., 1999
H00397	5′-CATACCGCCAAAAGATAAAT-3′	Krings et al., 1999
PtHV2FA	5′-GGGCCATGAAGTTCAAAAGTCTC-3′	This study
PtHV2RA	5′-TGAAATYTGAAGTCTGGC-3′ with Y = C/T	This study

bases. For each nest, we sequenced one sample with the forward primer and, whenever possible, we sequenced a second sample with the reverse primer. All samples with novel or ambiguous sequences were always sequenced in both directions. We aligned and compared all sequences obtained with published sequences of the whole chimpanzee mitochondrial and nuclear genomes (Genbank) to identify inconsistencies indicating nuclear insertions of mtDNA, or "numts" (Thalmann et al., 2004).

We amplified part of the HV2 region for those nests with successfully assigned sex and HV1 haplotype. Whenever possible, we analyzed two hairs per nest. We designed a nested PCR in which the outside primers (Table 1) amplified a ~664 bp region: CE16498 and CEH5 (this study). The inside primers (Table 1) amplified a ~334 bp region: L00056 and H00397 (Krings et al., 1999). As for HV1, we aligned all sequences with published sequences of the whole chimpanzee mitochondrial and nuclear genomes (Genbank). For HV2, we initially identified "numts" in some samples. We therefore designed a second set of inside primers (Table 1) which amplified a ~411 bp region: PtHV2FA and PtHV2RA (this study). We used this primer set to confirm sequences found with the first nested primer set. PCR amplification conditions and sequencing procedures were the same as for the HV1 region (see above). For HV2, all samples were sequenced with both the forward and reverse primers.

Data analyses

The proportion of ground nests belonging to males and females and proportions of ground nests of different status ("day" vs. "night"), were compared using χ^2 tests. All analyses were two-tailed and significance levels were set at 0.05. Statistical tests were performed in SPSS 14.0. Mitochondrial DNA sequences (HV1 and HV2) were analyzed in Mega 4.0.2 (Kumar et al., 2001). For HV1, a phylogenetic tree was generated with the neighbor-joining (NJ) function in this program. We used ARLEQUIN 3.1 (Schneider et al., 2000) to calculate nucleotide diversities, pairwise F_{ST} values (Weir and Cockerham, 1984) and to perform exact tests for population differentiation (Raymond and Rousset, 1995).

RESULTS

During 18 months of field study we recorded 634 nests (in 151 nest groups), of which 90 nests (~14%) were built on the ground. We obtained hair samples for 46 ground nests (in 21 nest groups) and for seven tree nests built in close association with one or more ground nests. The seven tree nests in association with ground nests were recorded over 5 months in 2006 (April–December), in seven different nest groups distributed across the study area. In addition, we collected hairs from 15 accessible tree nests (in nine nest groups) and one hair sample from the ground at a day-time resting place (Table 2).

TABLE 2. Number of hair samples and nests for which HV1 and HV2 were successfully amplified in relation to number of hair samples and nests analyzed (in parenthesis)

	HV1		**HV2**	
	Samples	Nests	Samples	Nests
Tree nests	24 (59)	19 (22)	9 (20)	7 (16)
Ground nests	45 (125)	30 (46)	28 (43)	23 (29)
Resting place	1 (1)	x	0 (1)	x
Total	**70 (185)**	**49 (68)**	**37 (64)**	**30 (45)**

Amplification success rates

The amelogenin sexing method had a PCR amplification success rate of 63% (140 of 222). For successfully amplified samples, the dropout rate for the Y-chromosome allele was 37% and 19% for the X-chromosome. The SRY sexing method had a lower PCR amplification success rate at 40% (61 of 151). The (nested) PCR amplification success rate for the HV1 region was 53% (91 of 171). However, there was great variation in HV1 amplification success between samples collected in 2006 (16% or 13 of 83) versus samples collected in 2007 to 2008 (89% or 78 of 88). Only hair samples from which the HV1 region was successfully amplified were analyzed for HV2. Samples analyzed for HV2 had a (nested) PCR amplification success rate of 66% (47 of 71).

Sex of nest-builders

We analyzed hair samples from 46 ground nests, of which 30 (65%) were assigned to males, 4 (9%) to females, and 12 (26%) to individuals of unknown sex. The proportion of ground nests constructed by males compared with females (30:4) was significantly higher than a 50:50 level, or a female-biased ratio typical for West African chimpanzees (Sugiyama, 1984; Boesch and Boesch-Achermann, 2000), as would be expected if males

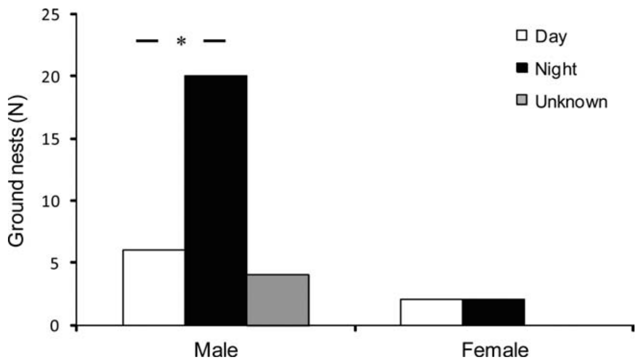

Fig. 1. The number of "day," "night," and "unknown" status ground nests constructed by males (n = 30) and females (n = 4). *χ^2 test: $P < 0.05$.

and females constructed ground nests equally often ($\chi^2 = 19.9$, df = 1, $P < 0.0001$).

Male chimpanzees constructed more elaborate "night" nests than simple "day" nests on the ground ($\chi^2 = 7.5$, df = 1, P = 0.009, Fig. 1). Of ground nests assigned to females two were "night" and two were "day" nests. Ground nests made by males labeled as "unknown" were deemed ambiguous, as they did not clearly fall into either "day" or "night" nest categories. It remains to be confirmed by direct observations if elaborate nests are used at night and simple nests during the day.

HV1 and HV2 regions

We calculated mtDNA diversity for the HV1 region based on sequences obtained for 49 nests and one resting place during 2006 to 2008 (Table 2). The 293 bp HV1 region showed 43 polymorphic sites (15%). Since the exact number of individuals for which hair samples were collected was unknown, we calculated minimum and maximum values for nucleotide diversity. Minimum values were calculated under the assumption that each sample came from a different individual and maximum values under the assumption that each individual bears a different mitochondrial variant (*sensu* Shimada et al., 2004). Minimum nucleotide diversity for HV1 was 0.037 and maximum was 0.068. When we combined data from samples collected in 1999 to 2000 and 2006 to 2008, minimum nucleotide diversity was 0.042 and maximum 0.065.

We successfully sequenced 293 bp of the HV1 region for 30 of the 46 of ground nests (Table 2). Five of the seven haplotypes reported by Shimada et al. (2004) for Seringbara were also found in this study (S-1, S-2, S-4, S-6, S-7; Table 3). In addition, we found two haplotypes not previously described (S-New 8, S-New 9). Six out of seven haplotypes were identified in male samples (n = 21), S-New 8 was of unknown sex, and three haplotypes were observed in females (n = 3). Hence, HV1 data showed at least 10 individuals built ground nests (Fig. 2A).

We obtained HV1 sequences for samples collected from 19 tree nests to compare relative frequencies of mtDNA haplotypes between tree and ground nests (Fig. 2B). Ground and tree nests showed a similar distribution of haplotypes. Haplotype S-6 was more common for tree nests and S-1 more common for ground nests, but this was not statistically testable due to low numbers of nests per haplotype. Although the frequency of haplotypes did

TABLE 3. Nucleotide sequences of HV1 haplotypes at Seringbara for 1999 to 2000 (S1–S7) and 2006 to 2008 (in bold)

Nucleotide positions corresponding to Anderson Sequence (J01415)

Name	16124	16129	16134	16130	16148	16146	16157	16166	16170	16177	16173	16178	16179	16182	16191	16222	16223	16225	16230	16233	16248	16263	16261
												*			*								
S-1	t	g	c	c	t	g	c	c	-	c	c	t	t	a	c	c	t	t	a	a	c	c	t
S-2	.	a	.	.	c	.	.	t	.	.	t	.	.	.	.	.	c	c	.	g	.	t	.
S-3	c	a	t	.	.	.	t	t	c	t	.	.	c	g	.	.	c	c	g	g	t	.	c
S-4	.	.	.	t	.	a	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	t	.
S-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S-6	.	.	.	t	.	a	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	t	c
S-7	c	a	t	.	.	.	t	t	c	t	.	.	c	g	.	t	c	c	g	g	t	.	c
S-New 8	.	a	.	.	.	a	.	t	c	t	.	c	c	.	.	.	.	c	g	g	.	.	c
S-New 9	c	a	.	.	.	.	t	t	c	t	.	.	c	g	t	t	c	c	g	g	t	.	c

Name	16273	16274	16278	16284	16292	16293	16296	16303	16304	16310	16320	16323	16348	16352	16353	16355	16360	16362	16374	16375	16387	16399	16400
			*									§						*		*			
S-1	c	g	c	a	c	c	-	a	a	g	t	c	c	t	c	a	t	c	a	t	a	a	c
S-2	t	a	.	.	.	t	c	.	.	a	.	.	.	.	.	.	.	.	.	.	g	.	.
S-3	.	.	.	g	t	.	c	g	.	.	c	t	.	c	.	c	c	.	c	.	.	g	t
S-4	.	.	.	.	.	.	c	.	.	.	.	.	.	.	.	.	c	.	.	.	.	.	.
S-5	.	.	.	.	.	.	-	.	.	.	.	.	t	.	.	.	.	.	.	.	.	.	.
S-6	.	.	.	.	.	.	c	.	g	.	.	.	.	.	.	.	c	.	.	.	.	.	.
S-7	.	.	.	g	t	.	c	g	.	.	c	t	.	c	.	c	c	.	c	.	.	g	t
S-New 8	.	.	t	g	.	.	c	g	.	.	.	t	.	.	t	t	c	t	c	c	.	g	.
S-New 9	.	.	.	g	t	.	c	g	.	.	c	t	.	c	.	c	c	t	c	.	.	g	*t*

* Nucleotide position found to be polymorphic in Seringbara in 2006 to 2008.
§ Nucleotide position found to be polymorphic in Bossou-Nimba region in 2006 to 2008.

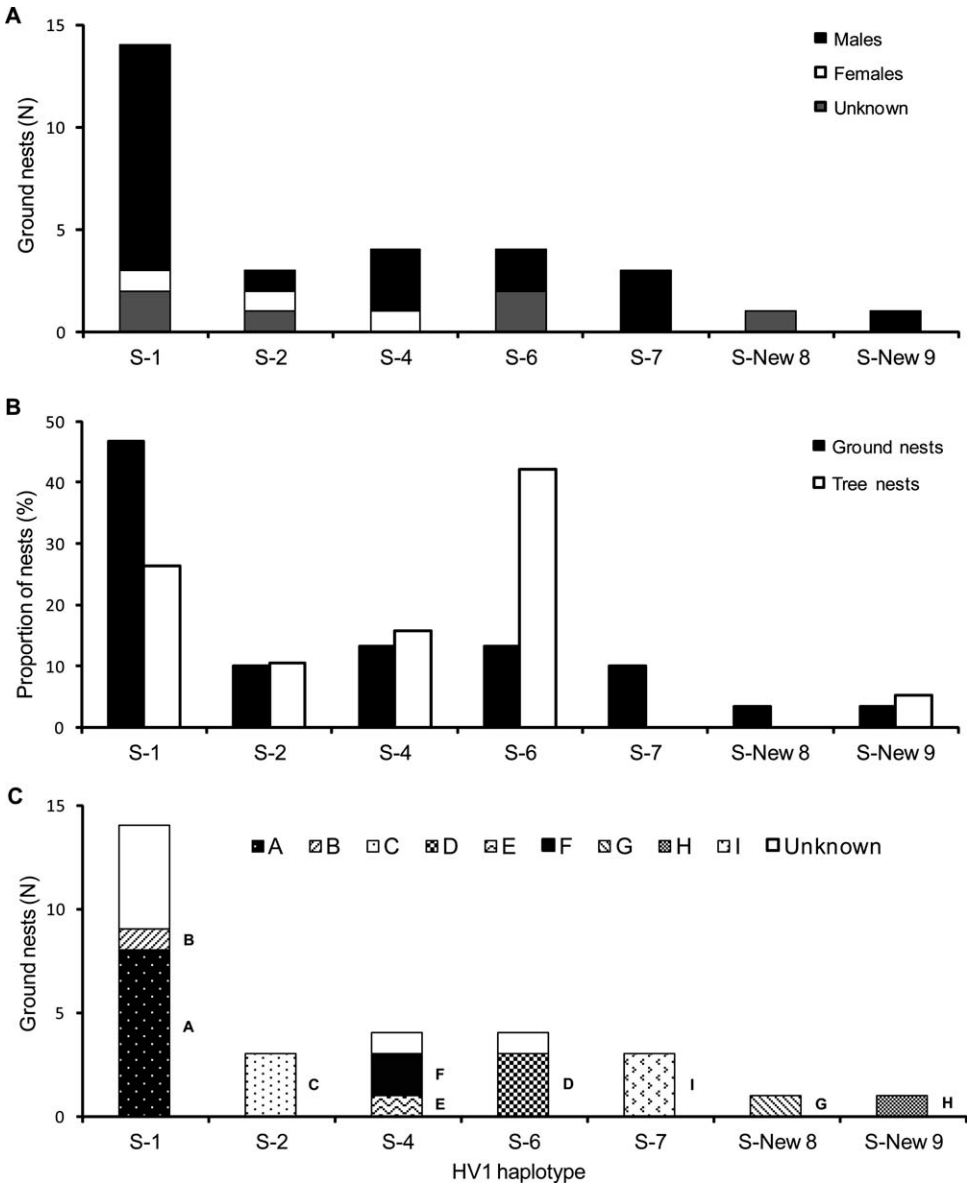

Fig. 2. Ground nests (n = 30) and tree nests (n = 19) attributed to the different mtDNA haplotypes. **A**: Number of ground nests attributed to mtDNA HV1 haplotypes and sex class. **B**: Percentage of ground and tree nests attributed to mtDNA HV1 haplotypes. C: Number of ground nests attributed to mtDNA HV1 and HV2 haplotypes.

not control for possible pseudo-replication of individuals making multiple nests, it did show that ground-nesting was part of the behavioral repertoire of chimpanzees belonging to all HV1 haplotypes present at Seringbara and was thus not restricted to particular matrilines.

The mtDNA diversity of the HV2 region was calculated based on a total of 30 nests (n = 23 ground nests, n = 7 tree nests; Table 2). The HV2 region was 334 bp long and had 31 polymorphic sites (9%; Table 4). Minimum nucleotide diversity for this region was 0.026 and maximum was 0.036. We identified one "numt" sequence for samples obtained from six different nests. This sequence was not an optimal match for the mitochondrial genome, but was a better match for a region in the chimpanzee nuclear genome. We thus interpreted this sequence as a "numt". Phylogenetic analyses demonstrated that all but the "numt" sequence clustered together with published mtDNA HV2 sequences (unpublished data). Following re-analysis of the samples with the second nested primer set, we were able to identify and confirm the authentic HV2 sequences for all samples. Hence, numt sequences were never used in the analyses.

We successfully sequenced part of the HV2 region of the D-loop for 23 of the 30 ground nests analyzed. These ground nests had known HV1 haplotypes and HV2 sequences were used to differentiate individuals further. We found a total of nine different HV2 haplotypes (Table 4). The HV2 region thus improved our ability to distinguish between individuals. For HV1 S1 we found two HV2 haplotypes (HV2-A and HV2-B). HV1 S4 had either HV2-E or HV2-F (Fig. 2C). Combined results for sexing, HV1 and HV2 analyses showed that a minimum of 12 individuals nested on the ground.

TABLE 4. Nucleotide sequences of HV2 haplotypes for the Seringbara region (2006–2008)

	Nucleotide positions corresponding to Anderson Sequence (J01415)																													
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3
	4	4	5	5	5	5	5	7	8	8	8	8	9	9	9	0	1	2	2	4	5	5	5	6	9	0	1	4	7	8	8
	3	6	0	1	2	3	9	3	2	4	5	7	5	6	8	0	4	5	6	1	2	3	8	2	1	9	0	0	3	6	4
Name																															
A	g	c	t	a	c	a	c	t	t	g	t	t	c	c	t	g	a	g	t	g	c	t	c	t	-	c	c	c	a	a	a
B	.	.	.	.	.	.	.	.	.	.	.	.	t	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C	a	t	.	.	.	.	t	.	c	.	c	c	.	t	.	.	.	.	.	.	t	.	.	c	.	.	.	.	.	.	g
D	.	.	.	.	.	.	t	.	.	.	c	.	.	t	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	g
E	.	.	.	.	.	.	t	.	.	.	c	.	.	t	c	a	.	.	.	.	.	.	.	.	.	.	.	.	.	.	g
F	.	.	.	.	.	.	t	.	c	.	c	.	.	t	c	a	.	.	.	.	.	.	.	.	.	.	-	.	.	.	g
G	.	.	.	.	.	g	t	.	c	.	.	c	.	t	c	a	.	.	.	a	.	.	t	.	.	.	t	a	.	.	.
H	.	t	.	g	t	.	t	c	c	a	c	.	.	t	c	a	g	a	c	.	.	c	t	.	a	-	t	a	g	g	.
I	.	.	c	g	t	.	t	t	c	a	.	.	.	t	c	a	g	a	c	.	.	c	t	.	a	-	t	a	g	g	.

TABLE 5. Sex, HV1 and HV2 haplotypes for ground and tree nest associations

	Ground			Tree		
Nest association	Sex	HV1	HV2	Sex	HV1	HV2
Pair 1	?	?	?	F	S-6	?
Pair 2	X	X	X	M	?	?
Pair 3	M	?	?	M	?	?
Pair 4	M	S-4	E	M	S-4	E
Pair 5	F	?	?	F	?	?
Pair 6	M	S-6	?	M	S-6	D
Pair 7	M	S-6	D	M	S-6	D

?, Unsuccessful; X, no hairs available.

For six pairs of closely associated ground and tree nests, we analyzed sex, HV1, and HV2 haplotype for both nests (Table 5). For pair 2, we were unable to obtain hairs from the ground nest. Tree nests associated with ground nests were most often constructed by males (five of seven), as were associated ground nests (four of five). The five successfully sexed pairs consisted either of two males ($n = 4$) or two females ($n = 1$). The three pairs for which HV1 was successfully sequenced had identical HV1 haplotypes. Furthermore, the two pairs for which also HV2 was successfully sequenced had identical HV2 haplotypes.

HV1 haplotype distribution

The HV1 haplotypes of ground and tree nests showed local clustering when mapped onto the study site (Fig. 3). Haplotype S4 was exclusively found in the north-east, whereas haplotypes S6, S7, and S-New 8 occurred only in the south-west. The remaining three haplotypes were found in both areas. The successfully amplified ground nests were found in both the north-east ($n = 5$) and the south-west ($n = 25$). The presence of two communities was previously hypothesized based on direct observations of known individuals at different locations (Koops, 2011a). To evaluate patterns of population differentiation we calculated minimum and maximum pairwise F_{ST} values for the two potential communities (*sensu* Shimada et al., 2004). We used an Exact Test for population differentiation at the haplotype level (Raymond and Rousset, 1995). Minimum values (see above for definitions) showed population differentiation (exact test: $P < 0.0001$), but a nonsignificant F_{ST} value ($F_{ST} = 0.01$, $P = 0.297$). Maximum values were not significant for either test ($F_{ST} = -0.177$, $P = 0.958$; Exact Test: $P = 1$). The actual F_{ST} value is likely more similar to minimum than to maximum values, as HV1 variants were shared between individuals. When data reported by Shimada et al. (2004) from 1999 to 2000 ($n = 20$ nests) were combined with our data from 2006 to 2008 ($n = 50$ nests), we found a minimum estimate of $F_{ST} = 0.049$, which showed a trend toward significance ($P = 0.075$). The exact test indicated that the communities differ significantly ($P < 0.0001$).

The phylogenetic relationship between the Seringbara HV1 mtDNA variants and the variants reported by Shimada et al. (2004) for the neighboring populations of Bossou and Yealé showed no clustering according to geographic region (Fig. 4). The two newly described haplotypes for Seringbara fell within mitochondrial clades and subclades formerly unrepresented in Seringbara (Fig. 4). The lack of geographic clustering for the Bossou-Nimba region suggests that chimpanzees have moved around in the past and that too little time has elapsed since gene flow decreased for geographically distinct population structures to emerge.

DISCUSSION

This study was the first to investigate a rare nest-building mode in chimpanzees, i.e., ground-nesting, by using multiple genetic markers to analyze DNA from shed hairs. To gain insight into the possible function of ground-nesting, we determined the sex of ground- and tree-nesting individuals in close proximity. Based on sex, HV1, and HV2 sequencing, we also estimated the minimum number of individuals nesting on the ground. Ground nests were built consistently across years in 2006 to 2008, although proportions of ground nests varied significantly across study periods (Koops et al., in press). The overall percentage of ground nests in 2006 to 2008 (14.2%) was higher than the proportion of ground nests previously recorded in 2003 to 2004 (6.1%) at the Seringbara study site (Koops et al., 2007).

Our results showed that ground-nesting is a strongly male-biased behavioral pattern, albeit not exclusively male. Preliminary results from samples collected in 2003 to 2004 suggested a male-bias in ground-nesting, as 15 out of 16 successfully sexed nests were attributed to males (Koops et al., 2007). However, too few nests were analyzed successfully then to draw definitive conclusions. We have shown here that males built the great majority of ground nests and constructed more elaborate ("night") nests than simple ("day") nests on the ground. Females made many fewer ground nests, of which equal

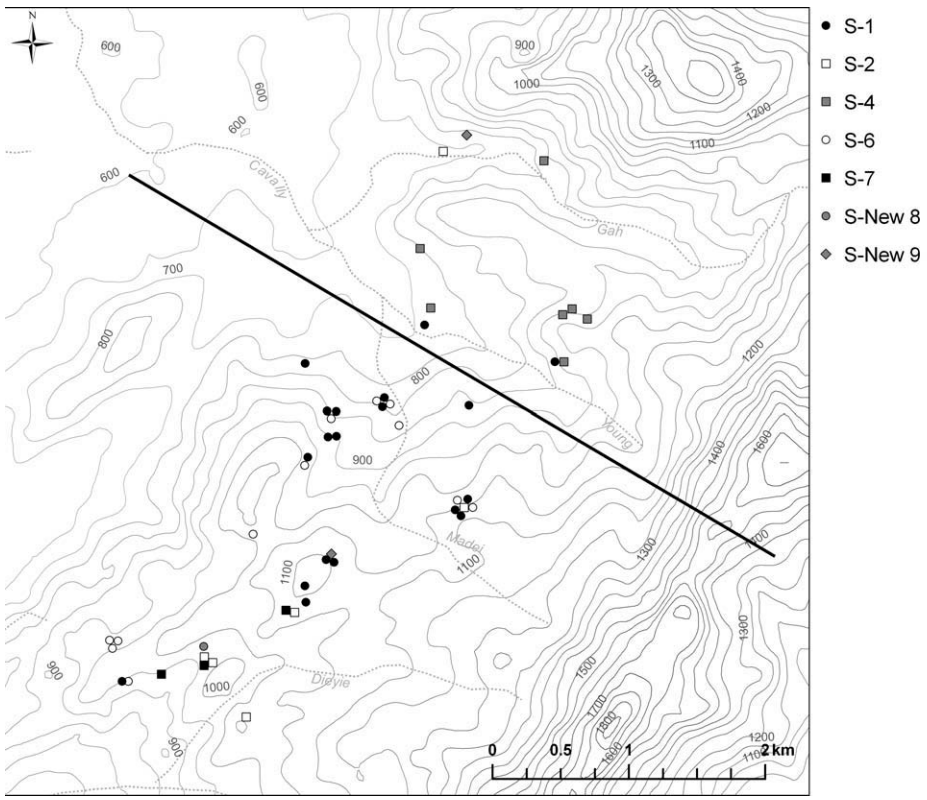

Fig. 3. Distribution of mtDNA HV1 haplotypes (see legend) across the Seringbara study site (2006–2008). The black line represents a schematic border between the two putative communities based on direct sightings of identified chimpanzees.

amounts were elaborate or simple in construction. If we assume that elaborate nests were used at night and simple nests during the day (Brownlow et al., 2001), our findings support the idea that "day" and "night" nests are functionally different. Day-time ground nests were used by both sexes, possibly for comfortable resting, whereas night nests on the ground were predominantly made by males.

The mate-guarding hypothesis predicts, first, that elaborate ground nests most often are grouped with (elaborate) tree nests. This prediction was previously confirmed (Koops et al., 2007). Second, it predicts that when ground and tree nests are made close together, males nest on the ground and females in the trees above. For the ground-tree nest associations for which sex was successfully determined, none showed the predicted pattern. In most cases, both nests were constructed by males. Furthermore, matching haplotypes for nest pairs suggested that either the same males nested both on the ground and in the tree, or that maternally-related males nested in close association. We cannot exclude the possibility that Seringbara chimpanzees occasionally make both a ground and a tree nest during the same night. Thus, these findings provide no support for the mate-guarding hypothesis. However, we successfully obtained hair samples only from tree nests closest to the ground nests. For most (3 of 4) of male-male nest pairs, there were other tree nests higher in the trees, so more than one (related) male may have guarded a female in a nest in the tree above. To test conclusively the mate-guarding hypothesis, we need to analyze DNA samples from all nests present in a group that also has a ground nest. Male chimpanzees have been found to nest lower than females in Budongo, Uganda (Brownlow et al., 2001). One proposed explanation for this difference in nest height was that males are heavier, which may increase the risk of branches giving way under their weight. By nesting lower they may reduce the risk of injury. Similarly, a male-bias in ground-nesting could be due to the larger body-size of adult males and the associated risk of tree-nesting, especially overnight.

Ground-nesting was suggested to be a potential cultural behavior in the Nimba chimpanzees (Koops et al., 2007). The key question is whether ground-nesting is found in only a few individuals belonging to one or two matrilines or whether it represents a group-level behavioral pattern. Sexing results showed that at least one male and one female nested on the ground, but gave no indication as to how widespread ground-nesting was. Sequencing of HV1 dramatically increased the level of resolution, as ground-nesting occurred in individuals belonging to seven different haplotypes or matrilines. Information on HV1 haplotypes combined with sexing results showed that at least 10 individuals nested on the ground. Furthermore, all haplotypes found among tree-nesting individuals also were found among ground-nesters, which further suggests ground-nesting was not restricted to certain matrilines.

Sequencing of HV2 for ground-nesting individuals with known HV1 haplotypes provided an even higher level of resolution for distinguishing between individuals. Based on sex, HV1, and HV2, we confirmed ground-nesting behavior for at least 12 individuals. The study area covers about 25 km^2 and the density of nest-building chimpanzees was previously estimated (by Distance 5.0 software) to be 1.42 chimpanzees/km^2 (*sensu* Plumptre and Reyn-

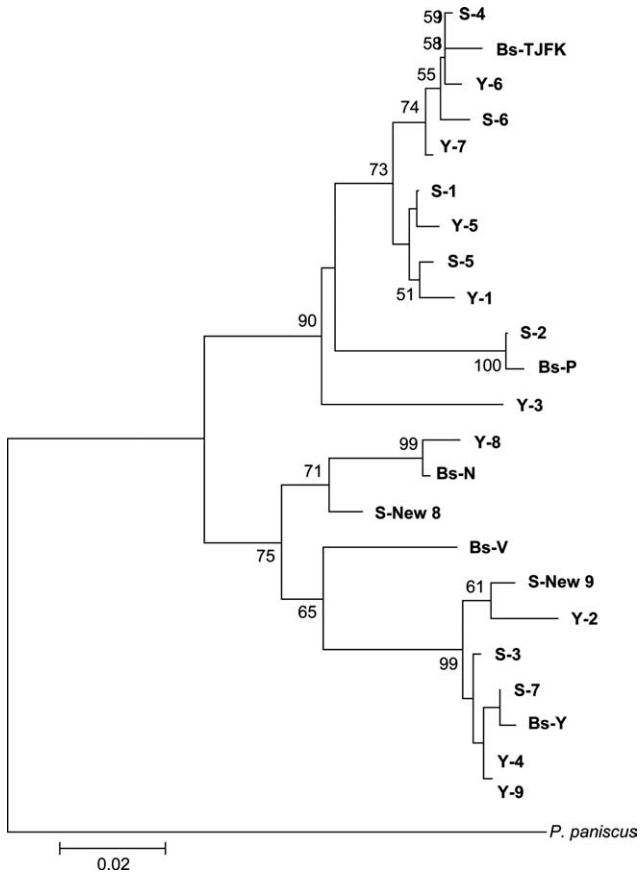

Fig. 4. Neighbor-joining tree of HV1 mtDNA variants found in Seringbara (S) and neighboring communities of Bossou (Bs) and Yealé (Y), adapted from Shimada et al. (2004). *Pan paniscus* is used as out-group and bootstrap values above 50 are shown. Variant Y4 and Y9 differ by 1 bp at nucleotide position 16538 (Shimada et al., 2004) outside the 293 bp region we analyzed and therefore appear to overlap in this tree.

olds, 1997). This density estimate predicts approximately 36 nest-building individuals in the 25 km^2 study area. We therefore conclude that at least one third of nest-building chimpanzees in the area we surveyed constructed nests on the ground. Ground-nesting is thus an habitual behavioral pattern in this population (*sensu* Whiten et al., 1999). To assess whether ground-nesting represents a cultural variant, future research should compare nest-building techniques between different matrilines and between individuals to establish whether the behavior is indeed socially-learned.

Regional clustering of haplotypes was congruent with the prediction that there are two communities at Seringbara. However, the study area did not cover the entire home-ranges of both communities and the total number of chimpanzees likely exceeded 36 individuals. The north-east community was estimated to have at least 17 chimpanzees, based on maximum nest group size (n = 17) and maximum party size observed (n = 10). The south-west community was estimated to have a minimum of 19 members, based on maximum nest group size (n = 19) and maximum party size observed (n = 16). Both potential communities included individuals who nested on the ground. Population differentiation between the communities was supported by the exact test, but F_{ST} values failed to reach significance. However, Shimada et al. (2004) reported nonsignificant F_{ST} values ranging from 0.01 to 0.06 for communities known to be different from each other. This suggests that within the Seringbara population, there may be two communities, despite a nonsignificant and relatively low F_{ST} value (0.05). Chimpanzees share mtDNA variants among neighboring communities (Langergraber et al., 2007), even over large distances (Morin et al., 1994), which makes distinguishing between communities based on mtDNA difficult. To differentiate between individuals and communities more confidently, microsatellite genotyping of DNA from fecal samples is needed (Langergraber et al., 2007; Guschanski et al., 2009).

In the current study, we found no shared mtDNA variants with neighboring populations in Bossou and Yealé based on data reported by Shimada et al. (2004), despite our prolonged sampling effort at Seringbara. The absence of shared haplotypes strongly suggests that there has been no exchange of individuals between Seringbara and neighboring chimpanzee groups in recent times (Shimada et al., 2004). At Bossou, chimpanzees rarely nest on the ground, and never at night, whereas ground-nesting is common in both chimpanzee populations in the contiguous forest of the Nimba Mountains, i.e., Seringbara in the West and Yealé in the East (Matsuzawa and Yamakoshi, 1996; Humle, 2003; Koops et al., 2007). Ground-nesting may have been part of the behavioral repertoire of a continuous population in the Bossou-Nimba region and may have gone extinct in the Bossou community following habitat separation between Bossou and Nimba.

Our study is the first to provide evidence of widespread ground-nesting in a population of chimpanzees, which contrasts with the general pattern of arboreal nest-building in *Pan*. We showed that terrestriality is not a necessary condition for habitual ground-nesting, which raises the possibility that ground sleep may have been practiced in some populations of pre-*erectus* hominins. Arboreal nest-building in apes has been proposed to function as anti-predation strategy (McGrew, 2004). Safety from terrestrial predators may have been especially important in early hominins living in open habitats with a high predation pressure (Sabater Pi et al., 1997; Stewart, 2011). The question therefore is whether or not these hominins would have risked sleeping on the ground? In chimpanzees, predation pressure is generally low in populations where ground-nesting occurs (Furuichi and Hashimoto, 2000; Maughan and Stanford, 2001; Koops et al., 2007; Pruetz et al., 2008), but at Bili (Democratic Republic of Congo) chimpanzees nest on the ground (10.9% of nests, n = 273) despite the presence of leopard (Hicks, 2010). Hence, presence of predators in paleohabitats of early hominins does not preclude terrestrial sleeping, in nests or otherwise. For *H. erectus*, sleeping on the ground may have been facilitated by the use of fire to protect against large terrestrial predators (Wrangham, 2009). However, ground-nesting in chimpanzees, especially at Bili, suggests that use of fire may not have been a prerequisite for ground sleep. Furthermore, chimpanzees at Seringbara habitually nest on the ground despite living in an evergreen tropical rainforest with plentiful nest trees. This suggests that a limited availability of nest trees as a result of exploitation of increasingly open habitat by *H. erectus* (Cerling et al., 2011) may not have been a necessary condition for the transition from tree-to-ground sleep. Terrestrial sleep may thus have been present in species of hominins before the emergence of *H. erectus*,

which suggests a gradual transition from tree-to-ground sleep in the hominin lineage.

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