

**The effects of landscape features on the
distribution and genetic structure of forest
duikers (Cephalophinae) in the tropical
forest of Moukalaba, Gabon**

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2015**

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Acknowledgements

I would like to first of all thank the God for the gift of life and His immense and sustaining Grace. God has granted me good health to be able to conduct this study. I ascribe Him all glory and honour.

My heartfelt thanks to Dr. E. Inoue, for his wonderful support and encouragement throughout my study period in Japan. His concern and support included not only academics but my general wellbeing of which I am profoundly grateful. I wish to express my deepest gratitude to my supervisors Drs. J. Yamagiwa, N. Nakagawa, M. Inoue-Murayama and R. Koda for their helpful suggestions and comments on this study. I would like to thank Dr. Y. Nakashima for his cooperation at the field site and helpful discussion on my study. I am extremely grateful for the valuable assistance and advice provided throughout this study by Dr. A.K. Basabose. I also thank Drs A. Ngomanda and C. Atteke for providing administration support. I am indebted to Mr. S. Hongo for valuable comments and advices.

I am immensely grateful to the following people who assisted me in various ways as far as my research, wellbeing and administration procedures; Mr. T. Tajima, Ms. C. Nze-Nkogue, Ms. K. Tsubokawa, Ms. M. Nishikawa, Mr. P.P. Mbeyang-Nguema, Ms. H. Taniguchi, Ms. Y. Shima, Ms. N. Hiramatsu, Ms. C. Ando and Mr. T. Ichiyama.

I would like to sincerely acknowledge the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT), the Japan Science and Technology Agency (JST), the Japan International Cooperation Agency (JICA) and Science and Technology Research Partnership for Sustainable Development (SATREPS) for providing funds and resources for this study.

Finally, I would to express my gratitude to my family especially my mother H. Mekina Oyono and my kids (Jovany, Giraud, Vianey, Akomessa and Rayan) for their moral support and encouragement throughout the study.

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Chapter I

General introduction

The issues of what landscape features influence the distribution of mammals have been of interest to ecologists. Landscape ecology has advanced our understanding of how landscape heterogeneity affects ecological processes (Turner et al. 2001). Similarly, the emerging field of landscape genetics, which combines landscape ecology and population genetics (Manel et al. 2003), has provided a framework for examining how the physical landscape affects genetic characteristics of populations. Determining the landscape features that lead to patterns of distribution can provide new insights about the ecology of species.

Tropical forests in Africa are concentrated in the Congo Basin. The Congo Basin is one of the world's great wilderness areas containing the second largest tropical forest block on earth, and is well known for the high diversity and high biomass in large mammals (White 1994). Vertebrate communities in the Congo Basin are dominated by frugivores (Gautier-Hion et al. 1980, Flenning et al. 1987). Duikers, which are mainly frugivorous (Gautier-Hion et al. 1980; Dubost 1984; Newing 2001), contribute to species richness in mammals in the Congo Basin (White 1994). In contrast with other fruit-eating species, such as gorilla, chimpanzee and elephant, studies on duiker species are few.

Duikers are ungulate species of the subfamily Cephalophinae, found only in Africa and primarily inhabiting forested areas (Kingdon 1997). Duikers form an important group including three genera and twenty species all weighing less than 80 kg (Kingdon 1997; Kingdon and Hoffmann 2013). The genus *Philantomba* comprises 3

species with small size (4-9 kg in body weight): Maxwell duiker (*P. maxwellii*), blue duiker (*P. monticola*) and Walter's duiker (*P. walteri*). The genus *Sylvicapra* includes one species common duiker (*S. grimmia*; 12-19 kg in body weight). The genus *Cephalophus* comprises 16 species. Among them, 13 medium sized duikers are sometimes called red duikers (12-30 kg in body weight): Ader's duiker (*C. adersi*), bay duiker (*C. dorsalis*), Peter's duiker (*C. callipygus*), Harvey's duiker (*C. harveyi*), white-bellied duiker (*C. leucogaster*), Natal red duiker (*C. natalensis*), black duiker (*C. niger*), black-fronted duiker (*C. nigrifrons*), Ogilby's duiker (*C. ogilbyi*), Rwenzori red duiker (*C. rubidus*), red-flanked duiker (*C. rufilatus*), Weyn's duiker (*C. weynsi*), zebra duiker (*C. zebra*). Three other duikers are relatively large (50-80 kg in body weight): Jentink's duiker (*C. jentinki*), yellow-backed duiker (*C. silvicultor*) and Abbott's duiker (*C. spadix*) (Johnston and Anthony 2012; Kingdon and Hoffmann 2013). All 20 species of duikers are not continuously distributed throughout African forests. Most species dwell in the tropical forests of central and west Africa, creating overlapping regions among different species of forest duikers (Kingdon and Hoffmann 2013).

All duiker species are thought to be territorial and live solitary or pair (Dubost 1980; Feer 1989). Most duikers are diurnal species, except for the larger duikers which are cathemeral species and the bay duiker which is nocturnal (Feer 1989; Newing 2001). Moreover, it is not uncommon for 3–7 duiker species to live sympatrically in African forests (Dubost 1979; Newing 2001). For example, in central African forest, at least six duiker species coexist, namely, blue duiker, Ogilby's duiker, white-bellied duiker, black-fronted duiker, bay duiker, and yellow-backed duiker (Kingdon 1997; Christy et al. 2008; Kingdon and Hoffmann 2013). The approach of landscape ecology might be useful to

understand how several mammal species belonging to the same subfamily coexist in the same forest.

The study of duikers is also important for conservation purposes. In west and central Africa, bushmeat is the most important source of animal proteins for people and duikers are the common prey species (Foerster et al. 2011; Mockrim et al. 2011; van Vliet et al. 2012). On average, over 60% of species currently hunted and used in the wild meat industry in the Congo basin comprises duiker and rodent species (Thibault and Blaney 2003; MacDonald et al. 2011; Mockrim et al. 2011). Although conservation status of duikers globally is least concern, apart from Ader's duiker, Jentink's duiker, Abbott's duiker and zebra duiker, unfortunately duiker populations are decreasing throughout African forests (IUCN 2012). Therefore, information on abundance and ecology of duikers in wild is needed for future conservation decisions as the hunting pressure on those species is very high.

Some studies of forest duikers furnish important indicators as which factors might predict their habitat quality (Dubost 1979; White 1994; Blom et al. 2006; Nakashima et al. 2013). All duiker species are thought to be territorial and most primarily inhabiting forested areas (Dubost 1984; Feer 1989), but some species have adapted to regenerating forest (Kingdon 1997; Newing 2001). The distribution of duiker species are influenced by vegetation types such as primary and secondary forests, understorey vegetation density, canopy cover and flooded surface (Dubost 1979; White 1994; Newing 2001; Clark et al. 2009; Poulsen et al. 2011; Nakashima et al. 2013). Abundances of duiker species were reported to be high in primary forest (Clark et al. 2009; Poulsen et al. 2011; Nakashima et al. 2013). Moukalaba-Doudou National Park, Gabon (hereafter, Moukalaba) is comprised of several vegetation types: old secondary forests, swamp

forests, gallery forests, young secondary forests, and savannahs. The park has rivers and Doudou Mountain with a maximum altitude of 900 m. This environmental conditions may affect duiker species diversity and distribution.

Studies of duikers present particular challenges. First, it is difficult to discriminate duiker species based on morphological characteristics (Kingdon 1997; Kingdon and Hoffmann 2013), second in wild condition duiker species are difficult to observe because of their cryptic behaviour (Breuer and Hockemba 2012), and finally duiker species identification based on morphological characteristics of dung is very confuse and difficult (White 1994; van Vliet et al. 2008; Bowkett et al. 2009). Recent advances of noninvasive sampling can allow to assess wildlife population abundance and genetic differentiation in protected area (Waits and Paetkau 2005; Inoue et al. 2007). Therefore, noninvasive sampling such as fresh dungs for genetic analysis is more appropriate for study cryptic forest species such as duikers (van Vliet et al. 2008; Bowkett et al. 2009).

Moreover, a greater insight and improved understanding of the genetic structure of duikers within study area can provide an insight into the effects of landscape features on their distribution. Movement of individuals is often restricted by physical barriers in wild animals and geographic gaps such as rivers, valleys, and mountain have been known to contribute to the distribution and genetic structure of mammals (Eriksson et al. 2004; Pérez-Espona et al. 2008; Vigilant and Guschanski 2009). For estimating gene flow within or between populations, mitochondrial displacement (mtDNA D-loop) and microsatellite markers are useful (Inoue et al. 2011; Bowkett et al. 2014; Gueta et al. 2014; Coster et al. 2015). The D-loop is non-coding part of mitochondrial genome which is known to be hypervariable (Arif et al. 2012). Microsatellite markers are a class of short nucleotides (2 – 6 bp) that are repeated in tandem; they are usually ubiquitous in the

genome of both eukaryotes and prokaryotes and are highly mutable and therefore show high level of intraspecific variation (Buschiazzo and Gemmel 2006). The above characteristic of microsatellite and mitochondrial D-loop, make them the excellent markers for conducting population genetic studies.

In this study, I conducted dung counts combined with genetic species identification of duikers in thirteen 2-km line transects across several types of vegetation in Moukalaba forest. I investigated the different patterns of duiker species distribution and the effect of landscape features on their abundance. I also developed microsatellite markers that can characterize the genetic structure of duikers in order to understand the dispersal of duiker populations in the wild. So far, this study is the first attempt to develop original microsatellite markers for duikers and to provide first-hand genetic structure on duiker species inhabiting central African forest using mitochondrial and microsatellite markers.

This thesis is divided in two parts. In the first part, I provide basic information on the differences in abundance of duiker species among habitats and vegetation with different level of disturbance in Moukalaba. Moukalaba forest is a suitable study site because it comprises several vegetation types and the density of duikers in the park is the highest in the African forest (Nakashima et al. 2013). In the second part, I develop novel microsatellite markers for blue duiker with cross-amplification in bay, Ogilby's and yellow-backed duikers, and I determine the genetic diversity and structure of duiker species using those loci and mitochondrial D-loop marker. Moukalaba forest has Doughetsi River and Doudou Mountain with a maximum altitude of 900 m, I investigated the effects of landscape features on duiker gene flow. Finally, I discussed the different effect of landscape features among coexisting duiker species and implications for their

conservation. Finding of this study is useful for basic understanding of the population dynamic of duiker species.

Chapter II

Differences in the abundance of duiker species among habitats in Moukalaba forest

2.1 Abstract

I investigated the relative abundance of four duiker species and the environmental effect including human impacts on their abundance in the forest of Moukalaba, Gabon. I counted and collected fresh faeces from duikers in thirteen 2-km line transects established in different types of vegetation. Genetic species identification showed that blue duiker, Ogilby's duiker, which might include the sister species Peter's duiker, bay duiker, and the yellow-backed duiker coexisted. Genetic analyses successfully identified Ogilby's and bay duiker, which are difficult to discriminate using other methods. The blue, Ogilby's, and the yellow-backed duikers were found in all transects. The dung abundance of bay duiker was quite low compared to other duiker species. This result suggests the importance of accumulating information regarding the abundance of each duiker species for updating their conservation status. The dung count method with genetic species identification may be useful for this purpose. The dung abundance of Ogilby's duikers and blue duikers was affected by human impacts, while that of yellow-backed duikers was not affected by human impacts. The dung abundance of Ogilby's duikers was lower in young secondary forests, which are regenerating after significant human disturbance. In contrast, generalized liner mixed model showed that the dung abundance of blue duikers was not affected by vegetation type and was lower in transects nearer to the

villages, which might be due to past hunting activity. The difference in human impacts among duiker species should be considered for their conservation management.

2.2 Introduction

Duikers (Cephalophinae) are widely distributed throughout African forests and several species of duiker, belonging to the same subfamily, normally live sympatrically in African forests (Kingdon 1997). For example, in southern Gabon, at least five duiker species coexist, namely, blue duiker, Ogilby's duiker, white-bellied duiker, bay duiker, and yellow-backed duiker (Christy et al. 2008). Duikers occupy all forest types and their abundance is reportedly influenced by the vegetation type (Kingdon 1997). The densities of blue and red duikers (Ogilby's, white-bellied and bay duikers) are known to be higher in primary forests than in secondary forests (White 1994; Clark et al. 2009; Poulsen et al. 2011; Remis and Kapnou 2011). Blue duikers are regularly hunted (Foerster et al. 2011; Mockrim et al. 2011; van Vliet et al. 2012) and have been reported to be less abundant where hunting occurs (Topp-Jørgensen et al. 2009; Nielsen and Treue 2012). However, most studies use a limited number of transects and typically examine a single transect per forest type to evaluate the abundance of duikers across different types of vegetation. Therefore, information regarding distinctive distribution patterns and factors that affect the distribution of these territorial species is not fully understood. More extensive study is required to elucidate the effect of environment, including human impact, on their abundance.

In African forests, visual surveys and dung counts have been used to estimate the abundance and density of duikers (White 1994; Hoppe-Dominik et al. 2011; Viquerat et

al. 2012; Jimoh et al. 2013). However, visual surveys are difficult to conduct in dense forests because of the cautious nature of duikers (White 1994; Breuer and Hockemba 2012). Moreover, it is difficult to distinguish red duiker species in the wild by utilizing visual surveys (White 1994; Nakashima et al. 2013). Although the dung count method is relatively easy to conduct, species identification of duikers on the basis of only the morphological characteristics of dung is difficult (van Vliet et al. 2008; Bowkett et al. 2009, 2013). The dung count method combined with genetic species identification is considered one of the most appropriate methods for studying duikers, in particular red duikers (van Vliet et al. 2008; Bowkett et al. 2009); however, despite its relevance, the method has not been effectively applied in extensive studies.

Moukalaba consists of several types of vegetation including mountain areas with topographic slopes. The abundance of duikers at Moukalaba was assessed using a line transect method via direct observation, and the density of duikers in the park was the highest in the African forest (Nakashima et al. 2013). Similar to other reports, the encounter rate of red duikers was higher in primary forests than in young secondary forests at Moukalaba. However, the encounter rate of blue and yellow-backed duikers did not vary among vegetation types, and this result was most likely due to the limited size of the surveyed transects (Nakashima et al. 2013). To obtain robust information about each vegetation type, additional spatial transect replicates are needed to assess the Moukalaba region, which varies in vegetation type, elevation, and topographic slope. In addition, from the 1960s to the 1980s, most of the study area was logged, and logging intensity, slash and burn agriculture, and hunting pressure were high near the villages (Blaney and Thibault 2001). Moukalaba is a suitable site for assessing the effects of environmental conditions including human impact on duiker species.

In this study, I conducted dung counts combined with genetic species identification of duikers in thirteen 2-km line transects across several types of vegetation in the Moukalaba forest. I investigated the dung abundance of each duiker species, especially red duiker species and discussed the effectiveness of the method for red duikers. I also surveyed the effect of various environmental factors and then discussed the effect, including human impact, on their abundance.

2.3 Materials and Methods

2.3.1 Study site

The study was conducted in the northeastern part of the Moukalaba-Doudou National Park. At the research station, from 2004–2006, the annual rainfall fluctuated from 1582 to 1886 mm, and the mean monthly minimum and maximum temperatures ranged from 21.3 to 24.1°C and 29.3–33.7°C, respectively (Takenoshita et al. 2008).

Thirteen 2-km line transects were established within three forest types: three transects in young secondary forests (YS1, YS2, and YS3), three transects in swamp forests (S1, S2, and S3), and seven transects in old secondary forests (OS1, OS2, OS3, OS4, M1, M2, and M3) (Figure 2–1). The classification of vegetation was based on Landsat radar and aerial imagery (Ministere des Eaux et Forêts et du Reboisement, Tecsalt International, Quebec Canada; provided by WWF Gamba Gabon). The old secondary forest has been selectively logged between the 1960s and the 1980s, and has closed canopy, and ground vegetation tends to be sparse. The young secondary forest is regenerated from a recent slash-and-burn practices in agriculture, and is dominated by *Musanga cecropioides*. In young secondary forest, the density of ground vegetation is

high and the canopy cover is comparatively low. The swamp forest also has closed canopy but the ground is inundated during the rainy season. Although the previous study set one transect in primary forests (Nakashima et al. 2013), I did not set transects because the area of primary forests is limited in our study area. In this study, according to altitude (> 250 m where steepness of mountain slopes increases), I considered M1, M2, and M3 transects mountain forest regions. I calculated the Euclidean distance from the nearest village to the centre point of each transect based on GPS coordinates. I also calculated the standard deviation (SDV) of elevation obtained every 30-m in each transect as an indicator of topographic slope.

2.3.2 Dung count surveys and genetic species identification

From January 2011 to August 2012, mainly in the dry season, I visited each transect twice at an interval of at least five days. Sampling was conducted five times in the M3 transect due to the small number of samples. During the reconnaissance survey, one researcher and one field assistant walked together with 15-m belts on both sides of the transect for 2.5–3.5 hours. Census speed was adjusted based on vegetation structure and topography to ensure the detection of the maximum number of dung pellets.

The encounter rate of fresh pellets might be less vulnerable to the environmental condition than that of decayed pellets. Therefore, I counted and collected only fresh dung piles of ungulate faeces using cotton swabs in order to avoid affecting the variance of the dung decay rate (van Vliet et al. 2009; Breuer et al. 2010) and to maintain a high probability of dung detection. Faecal samples were preserved in lysis buffer containing

0.5% SDS, 100 mM EDTA (pH 8.0), 100 mM Tris-HCl (pH 8.0), and 10 mM NaCl (Hayaishi and Kawamoto 2006).

DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Tokyo, Japan). A portion of the mitochondrial control region (approximately 650 bp) was amplified with forward CRF1: 5'-CTCCCTAAGGCTCAAGGAAGC-3' and reverse primers CRR3: 5'-CCTGAAGRAAGAACCAGATGTC-3' (Ntie et al. 2010a). Polymerase chain reaction (PCR) amplifications, were carried out in a total volume of 15 μ L containing 2 μ L of template, 150 μ M of each dNTP, 200 nM of each primer, 0.2 μ L of T4 gene 32 protein (Nippon Gene, Tokyo, Japan), and 0.52 U Expand High Fidelity enzyme (Roche Diagnostics, Tokyo, Japan) mixed with PCR buffer supplied by the manufacturer. After an initial incubation at 95°C for 5 min, 45 amplification cycles were performed with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. This was followed by a final extension at 72°C for 30 min. PCR products were purified using ExoSap IT (USB, Cleveland, USA). Direct sequencing was carried out with the ABI3130xl genetic analyser (Applied Biosystems, Foster City, USA), and the fragments were aligned and edited with MEGA5 (Tamura et al. 2011).

In west and central African forests, duiker species live sympatrically with a similar-sized ungulate named water chevrotain (*Hyemoschus aquaticus*) (Dubost 1978; Newing 2001; Christy et al. 2008). I utilized NCBI BLAST to identify the sequences of water chevrotain by comparing the target sequence to the first and second closest species in the BLAST results. I concluded the sample belonged to water chevrotain when there was high confidence (98-100% identity) with the first species (water chevrotain) and the identity with the second closest species was not greater than 90% (Inoue and Akomo-Okoue 2015).

Previous research indicates that phylogenetic tree-based analyses using control region sequences are the best methods to identify duiker species (Ntie et al. 2010a). I aligned all of the sequences that were similar to the duiker sequences, and then constructed a neighbour joining phylogenetic tree, which included additional reference duiker sequences; suni (*Neotragus moschatus*) sequences were used as the outgroup (Ntie et al. 2010a; Inoue and Akomo-Okoue 2015).

Although Ogilby's and Peter's duikers may occur in Moukalaba (Christy et al. 2008), the molecular techniques could not discriminate between Ogilby's and Peter's duikers (Johnston et al. 2011; Hassanin et al. 2012; Johnston and Anthony 2012). Hassanin et al. (2012) suggested that the two species might be subspecies rather than formal species, which may be due to possible hybridization between them (Johnston and Anthony 2012; Kingdon and Hoffmann 2013). Therefore, in this study, I considered Ogilby's and Peter's duikers as a single taxon (hereafter referred to as Ogilby's duiker).

2.3.3 Data analysis

All statistical analyses were performed using R 2.14.0 (R Core Development Team 2011). The correlation between dung pellet abundance among the transects was assessed using the Kendall tau rank correlation test. In order to survey the similarity of duiker species abundance between transects, principal component analyses (PCA) and cluster analyses were conducted using the number of duiker dung pellets in each transect per visit. Additionally, a one-way analysis of variance with Tukey HSD multiple comparisons was utilized to test the differences between each duiker species across vegetation types.

To examine the effect of environmental factors on the dung abundance of each duiker species, a generalized linear mixed model (GLMM) was tested using the glmmML function of the glmmML package (Broström and Broström 2009). Three factors were identified as explanatory variables (vegetation type, distance from the nearest village, and topographic slope index); however, vegetation type was subsequently omitted from the explanatory variables. The models were fitted with a Poisson error distribution and a log link function, and transect was set as a random effect. The model selections were performed based on an information theoretical approach using the Akaike information criterion (AIC). AIC values and Akaike weights were computed for all alternatives, and were compared to select an adequate model. According to the principal of parsimony, when alternative models have indistinguishable AIC values ($\Delta AIC < 2$), the best model is selected based on the lowest AIC value (Burnham and Anderson 2002). Model selections were carried out using the dredge function in the MuMIn package (Barton 2011).

2.4 Results

2.4.1 Dung abundance and distribution patterns of duiker species

I analysed 349 dung pellets, and the PCR success rate ranged from 75–92% with an average of 81%. The phylogeny showed that 146, 83, 35, and 6 faecal samples belonged to blue, Ogilby's, yellow-backed, and bay duiker clusters, respectively. Blue, Ogilby's, and yellow-backed duikers were found in all transects (Table 2–1). The abundance of dung pellets from bay duikers was relatively low and the samples were mainly found in old secondary forests. DNA analyses revealed that I also collected 14

samples of dung from water chevrotain, dung piles of which were difficult to discriminate from those of duikers. However, the data on water chevrotain were not used in further analyses.

I investigated the correlation of the dung abundance of three duiker species (blue, Ogilby's, and yellow-backed), from which I collected numerous dung pellets. A significant positive correlation was found between the number of blue and Ogilby's duiker dung piles ($P < 0.01$, $\tau = 0.58$; Fig. 2–2a); however, the ratio of Ogilby's duiker to blue duiker dung pellets was relatively low in four transects (YS1, YS2, YS3, and S1), three of which were located in the young secondary forests. Moreover, a marginal positive correlation between the number of blue duiker and yellow-backed duiker pellets was identified ($P = 0.06$, $\tau = 0.42$; Figure 2–2b); but, the ratio of yellow-backed duiker to blue duiker faeces was relatively high in three transects (S1, S2, and S3) that were located in swamp forests. There was no correlation between the number of Ogilby's and yellow-backed duiker dung pellets ($P = 0.20$, $\tau = 0.37$; Fig. 2–2c).

2.4.2 Factors affecting duiker abundance

The PCA indicated two factors that explained 81.2% of the sample variance. The first factor was characterized by high loadings of blue (0.88), Ogilby's (0.86), and bay duikers (0.76). The second factor was characterized by high loadings of yellow-backed duikers (0.94). Transects were plotted based on the first two principal components (Figure 2–3), and, except for transect YS1, transects located in the same vegetation type were clustered (Figure 2–4). The number of dung pellets of the three major species (blue, Ogilby's, and yellow-backed) differed among vegetation type (Figure 2–5). Regarding

blue duikers, the number of faecal samples in old secondary forests was significantly higher than that in mountain forests ($P < 0.01$). For Ogilby's duikers, the number of faecal samples in old secondary forests was significantly higher than in the mountain ($P < 0.05$) and young secondary forests ($P < 0.01$). With regard to yellow-backed duikers, the number of faecal samples in the swamp forest was significantly higher than that of any other vegetation type (Figure 2–5).

GLMM analyses showed that the distance from the nearest village and topographic slope index contributed to the variations in dung abundance of blue duikers (Table 2–2a). The dung abundance of Ogilby's and yellow-backed duikers was explained by vegetation type. However, when vegetation type was excluded from the set of explanatory variables, the GLMM analyses indicated that the dung abundance of blue and Ogilby's duikers could be explained by the distance from the nearest village and topographic slope index (Table 2–2b). Moreover, under these conditions, the distribution of yellow-backed duikers was explained only by topographic slope index (Table 2–2b).

2.5 Discussion

2.5.1 Dung counts combined with genetic species identification

Although dung counts have been used to estimate the abundance of ungulates (Forsyth et al. 2011; Alves et al. 2014), detection rate (dung decay rate and defecation rate) may bias the results of density using dung counts (Viquerat et al. 2012). Some reports showed that the dung counts method is useful for habitat use of other ungulates (Månsson et al. 2011; Alves et al. 2014). However, it is important to note that our data on abundance may not be directly comparable with those of other studies using other

methods. Therefore, I mainly focus on the comparison among different transects in each duiker species in the discussion.

In order to determine the number of duiker species and their abundance, dung counts combined with genetic species identification is a useful approach (van Vliet et al. 2008; Bowkett et al. 2009), because species identification of duikers based solely on morphological characteristics of faecal samples is not reliable, particularly for red duikers (van Vliet et al. 2008). Previous studies in Moukalaba could not distinguish red duiker species due to the limitation of direct surveys (Nakashima et al. 2013). However, using dung counts with genetic species identification, I identified two red duiker species (Ogilby's and bay duikers). Camera traps, which have been increasingly used in wildlife studies (Ahumada et al. 2011; Burton 2012; Mugerwa et al. 2012; Msuha et al. 2012; Jennings and Veron 2015), are effective when studying elusive animals such as carnivores and duikers (Rovero and Marshall 2009; Treves et al. 2010). Nevertheless, red duikers cannot always be categorised even using camera traps (Vanthomme et al. 2013). Therefore, dung counts combined with genetic species identification is a powerful tool for monitoring duikers, especially red duiker species.

This is the first extensive study that demonstrates a clear difference in the relative abundance of red duiker species among different types of vegetation. Dung counts combined with genetic species identification suggested a low abundance of bay duikers compared with Ogilby's duikers in Moukalaba. The dung pellets of bay duikers were found only in old secondary forests. Although I may infer the effect of human pressure on their abundance from this result, the abundance of bay duikers may be naturally low considering the low number of their dung pellets even in old secondary forests. Van Vliet et al. (2008) showed a similar result using dung counts with genetic species identification

at Ipassa Reserve, Gabon. There were four species of red duikers (Peter's, black-fronted, white-bellied, and bay duikers) at Ipassa. Approximately 90% (44/50) of the samples were from Peters', a sister species of Ogilby's duiker, while only three samples were from bay duiker. Despite the similarities in morphological and reproductive parameters (Feer 1989; Kingdon and Hoffmann 2013), Peter's or Ogilby's duiker may be naturally more abundant than the other red duiker species. Jimoh et al. (2013) also reported local depletion of bay duiker in the Oban Hills Region, Nigeria. Optimal monitoring plans are needed to ensure that bay duiker is not being driven to local extinction.

At other central African study sites, based on direct surveys, at least three red duiker species were reported to live sympatrically with blue and yellow-backed duikers (White 1994; Muchaal and Ngandjui 1999; Clark et al. 2009; Melletti et al. 2009). Their abundances seem to be at sustainable levels in most central African protected areas (Clark et al. 2009; Remis and Kapnou 2011) and most of the red duiker species were classified as Least Concern in the IUCN Red List. However, in particular area, as is the case of Moukalaba and Ipassa, some species of red duiker may be less abundant than other species. Moreover, red duikers are reported to be hunted (Macdonald et al. 2011; van Vliet et al. 2012). For conservation management, the data on abundance of each red duiker species at multiple study sites, including outside protected areas, is required for updating their conservation status. Dung counts with genetic species identification may be the most appropriate method for clarifying the abundance and conservation status of red duiker species.

2.5.2 Factors affecting duiker abundance

The results of PCA showed that the dung abundance of duikers within the study area is generally influenced by vegetation types (Figure 2–3). However, the constitution of dung abundance was not clearly discriminated between young secondary forest and mountain. Although the scores of first and second factors were similar in these two vegetation types, the relative dung abundance of Ogilby’s duiker was different between these vegetations (Table 2–1). Therefore, vegetation types might be one of the most important factors for determining distribution of duiker species in Moukalaba.

Blue, Ogilby’s, and yellow-backed duikers were found in all Moukalaba habitats, and the dung abundance of blue duikers was positively correlated with that of Ogilby’s and yellow-backed duikers (Figure 2–2a; 2–2b). The positive correlation between the species is most likely because the abundance of all three species was lower in sloped areas. In addition, the dung number of blue and Ogilby’s duikers were lower in transects nearer to the villages; thus, the correlation between the dung abundance of these two duiker species was significant. In contrast, the dung abundance of Ogilby’s and yellow-backed duikers was not correlated (Figure 2–2c). This may be explained by the difference in effect of vegetation types on their abundance as shown below.

The dung abundance of Ogilby’s duikers was lower in young secondary forests. The result was consistent with a previous report that indicated that the abundance of red duikers was higher in old secondary forests than in young secondary forests at Moukalaba using direct observation, but the difference was not significant (Nakashima et al. 2013). Newing (2001) also showed similar results in Taï, Côte d’Ivoire concerning the rare presence of Ogilby’s duiker in secondary forests. Ogilby’s and its sister species, Peter’s

duiker, are the most frugivorous species among duikers (Dubost 1984; Newing 2001); therefore, the distribution of Ogilby's duiker may be highly affected by fruit abundance. The young secondary forests have been regenerated after significant human disturbance and are often poor in fruits (Newing 2001); thus, species that consume more fruits are likely to avoid disturbed forest (Johns 1988).

The dung abundance of yellow-backed duikers was higher in the swamp forests. A previous study in Moukalaba suggested that the encounter rate of yellow-backed duikers did not vary greatly among vegetation types (Nakashima et al. 2013). However, the data were derived from diurnal surveys with one transect per vegetation type, but diurnal surveys may not be accurate for estimating the abundance of yellow-backed duikers, which are cathemeral species (Newing 2001). In central Africa, the black-fronted duiker, whose legs are adapted to waterlogged areas, typically use swamp forests (Dubost 1978). The absence of this typical species in swamp forests may allow yellow-backed duikers to occupy this habitat in Moukalaba. Moreover, because the yellow-backed duiker is the largest duiker species (Newing 2001), its size may facilitate its ability to utilize flooded areas.

Although the dung abundance of blue duikers was higher in old secondary forests than in mountain forests (Figure 2–5), GLMM analyses did not show the effect of vegetation type on its dung abundance. Two out of 3 transects in young secondary forests were close to villages, which might make it difficult to discriminate the effect of human pressure from that of vegetation types. In order to ascertain the effect of distance from villages, I performed GLMM analyses without the data for these two transects (YS1 and YS2). The result was quite similar to that including the two transects: the dung abundance of blue duiker was not affected by vegetation type, but affected by SDV of elevation and

distance from village (first model: SDV of elevation -, Distance from the nearest village +, AIC = 19.6, Akaike weight = 45.8%; second model: SDV of elevation -, AIC = 19.8, Δ AIC = 0.08, Akaike weight = 44.1%). This confirmed the previous result that the density of blue duikers did not differ among vegetation types in Moukalaba (Nakashima et al. 2013). GLMM analyses also indicated that the dung abundance of blue duikers was lower in transects nearer to the villages, and this result may reflect the negative effects of human activity. The negative effect of hunting on the abundance of blue duikers has been also reported in other study sites (Robinson et al. 2011; Nielsen and Treue 2012). Forests in Moukalaba were logged from the 1960s to the 1980s, and logging activity is one of the principal drivers of mammal hunting in Africa (Poulsen et al. 2009; Vermeulen et al. 2009; Breuer and Hockemba 2012). The blue duiker is a major mammal species for bushmeat in central Africa (Fa and Brown 2009; Kümpel et al. 2010), and has been reported to be less abundant where hunting occurs (Topp-Jørgensen et al. 2009; Remis and Kapnou 2011; Nielsen and Treue 2012). Although hunting activity seems to be reduced due to a permanent presence of researchers within the study area, hunting pressure during the last decade was high across Moukalaba forest, especially near the villages (Blaney & Thibault, 2001). The effect of human presence on blue duiker may still remain near the villages.

GLMM analysis showed that the dung abundance of three duiker species was lower in the transects with steeper features (Table 2–2b). This suggests that duikers may not adapt well to sloped areas. Regarding ungulate species such as sika deer (*Cervus nippon*) or muskox (*Ovidos moschatus*), populations inhabiting sloped areas tend to have shorter limbs than populations inhabiting flat areas (Smith et al. 2002; Terada et al. 2012). In African forests, Abbott's and Rwenzori red duikers are reported to use both montane

and sub-montane forests as typical habitats (Treves et al. 2010; Bowkett et al. 2013). However, most duikers, including blue, Ogilby's, and yellow-backed, tend to prefer flat and lowland forests (Kingdon and Hoffmann 2013). The abundance of duikers was low in the mountain forests of Moukalaba probably because their legs may not be adapted to sloped areas. Further studies will be required to assess geographic variations in the morphological traits between duikers typically adapted to montane areas and other duiker species.

2.5.3 Difference in human impacts among duiker species

Human impacts on the abundance of Ogilby's and blue duikers were suggested, but were not apparent in the case of yellow-backed duiker. Ogilby's and blue duikers seem to be affected differently by human activity. The dung abundance of Ogilby's duikers was lower in young secondary forests, which are regenerating after significant human disturbance. In contrast, GLMM analyses showed that the dung abundance of blue duikers was not affected by vegetation type and was affected by the distance from the villages. Although some studies showed low densities of blue duikers within hunting areas (Topp-Jørgensen et al. 2009; Nielsen and Treue 2012), other studies suggest that, despite hunting pressure, the abundance of blue duikers varies only slightly across different zones (Muchaal and Ngandjui 1999; Rist et al. 2009). In this study, I clarified the effect of human pressure on blue duikers using 13 transects and might suggest the vulnerability of blue duikers to hunting pressure near the villages. In blue duikers, adaptation to degraded secondary forests has been reported (Kingdon 1997), unlike in the case of Ogilby's duikers (Newing 2001). This means that the abundance of Ogilby's

duiker is less likely to recover after human disturbance. We should consider the difference in human impacts among duiker species when we set up conservation programs for duikers.

Chapter III

Effect of landscape features on the genetic diversity and structure of duiker species in Moukalaba

3.1 Abstract

Dispersal and gene flow within animal population are influenced by the composition and configuration of the landscape. In this study, I characterized population genetic structures of three coexistent forest duiker species (Cephalophinae) in an approximately 250 km² area of Moukalaba-Doudou National Park, Gabon. I developed original nine microsatellite markers for duikers using next-generation sequencing technology. Population differentiation was analyzed using eleven microsatellite loci, including the markers developed for other bovine species, and mitochondrial DNA control region sequences (~600 bp). Individual identification analyses using microsatellite showed that I obtained 82, 6 and 15 individuals of blue, bay and yellow-backed duiker, respectively. Genetic diversity of bay duiker was lower than the others, which may reflect the low abundance of this species in Moukalaba. In each species, the results of mitochondria and microsatellite showed that there were two genetic groups. In the blue duiker population, two genetically different subpopulations were suggested to have recently contacted. Population history might be one of the reasons to differentiate two subpopulations. In bay duiker, it was difficult to determine a genetic barrier of two genetic groups due to the small sample size. Mountain was suggested to be responsible for observed genetic differentiation in blue duiker, while river might act as a genetic barrier in yellow-backed

duiker. The difference of genetic barriers between two species might be explained by the difference of body size. In this study, using both mitochondrial and microsatellite data, I described the detailed genetic structure of three coexistent species and revealed that different landscape features limited gene flow of different species.

3.2 Development of microsatellite markers for blue duiker (*Philantomba monticola*) using next-generation sequencing and cross-amplification in other duikers

3.2.1 Introduction

Duikers (Cephalophinae) are small ungulates widely distributed in African forests and are important sources of protein and income for local people (Newing 2001; Schenck et al. 2006). Several species of duikers, belonging to the same subfamily, normally live in the same habitat in Central Africa areas (Kingdon 1997). Blue duiker is the smallest current duiker inhabiting all forest types (Dubost, 1980). Across western and central African forests, blue duiker is one of the major mammal species for bushmeat (Wilkie and Carpenter 1999; Newing 2001). It is important to monitor genetic diversity for their conservation, although current hunting pressure on this species was estimated to remain within a sustainable level (Fa et al. 1995; van Vliet and Nasi 2008). Microsatellite markers are useful for describing genetic diversity and genetic structure of wild animals. Some microsatellite markers which were developed in other bovine species were amplified and were polymorphic in duikers (Ntie et al. 2010b). I tried those markers using DNA extracted from fecal samples of duikers, but the amplification of several markers failed probably due to low quality and quantity of DNA. I therefore found it necessary, to develop original microsatellite markers for duikers.

3.2.2 Materials and Methods

I collected 11 muscle tissue samples of blue duiker and two muscle tissue samples each of bay duiker and Ogilby's duiker in Gabonese meat markets. The samples were preserved in 99.5 % ethanol and DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN). I also collected 19 fecal samples of duikers in Moukalaba, and preserved them in Lysis Buffer (0.5 % SDS, 100 mM EDTA, 100 mM Tris-HCl, and 10 mM NaCl), following Hayaishi and Kawamoto (2006). I extracted DNA using QIAamp DNA Stool Kit (QIAGEN) and conducted species identification using mitochondrial control region (Ntie et al. 2010b). I used 11, 1, and 7 fecal samples of blue duiker, bay duiker, and yellow-backed duiker, respectively. To characterize developed microsatellite markers, I used DNA extracted from both tissues and fecal samples.

I used 500 ng of genomic DNA extracted from a tissue of a blue duiker for developing microsatellite markers. Following Adenyo et al. (2012), I prepared DNA library, amplified the library through emulsion PCR, and then sequenced using GS-Junior bench-top sequencer (Roche). In total, I obtained 77,820 sequence reads. The program Msatcommander (Faircloth 2008) was used to screen all sequences for microsatellites. Primers for the sequences including at least 8 di-, 7 tri-, and 7 tetranucleotide repeats were designed using the PRIMER3 software (Rozen and Skaletsky 2000) embedded in Msatcommander. Among 417 primer candidates, I selected nine primers for the sequences which showed high similarity to the registered sequences of cow (*Bos taurus*) through NCBI BLAST. PCR amplification was carried out in 5 µl reaction including DNA (1 µl of DNA extracted from tissue and 2 µl of DNA extracted from faeces), 2.5 µl Multiplex PCR Master Mix, 0.2 µM of fluorescent-labelled forward primer and 0.2 µM of reverse primer. The amplification program included an initial denaturation at 95 °C for

15 min; followed by 45 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s; then 30 min final extension at 60 °C. PCR products size was measured using the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Electropherograms were analyzed using Peakscanner software (Applied Biosystems). The number of alleles, expected and observed heterozygosities (H_O and H_E), Hardy–Weinberg equilibrium (HWE), and cumulative probability of identity (PI) were calculated using GenALex v6.41 (Peakall and Smouse 2006).

3.2.3 Results and Discussion

I successfully amplified nine loci using 22 blue duikers' DNA extracted from tissue and faeces. The number of alleles of each locus ranged from 4 to 14 (mean 9.4), H_O and H_E were 0.333–0.833 (mean 0.716) and 0.625–0.878 (mean 0.817), respectively (Table 3–1). Among all the loci, only CM38 showed a significant deviation from HWE ($P < 0.05$). PI of these nine markers was low (1.5×10^{-12}), which indicates that these markers are enough for individual discrimination. For three other duiker species (bay, Ogilby's and yellow-backed duikers), all nine loci were successfully amplified, and almost all loci were polymorphic (Table 3–2). This result suggests that these markers are useful to analyze genetic diversity of duiker species. Although most duikers are Least Concern species according to the IUCN criteria, the meat serves as an important source of protein and income to people in Western and Central Africa (Newing 2001). The data on genetic diversity of duikers are needed for evaluating and monitoring their status. The developed microsatellite markers presented in this study will be useful to study genetic structure and genetic diversity of duikers. More so, these markers will allow us to monitor

genetic diversity using fecal samples, which are easy to collect in the forest without capturing animals.

3.3 Effect of landscape features on the genetic diversity and structure of duiker species in Moukalaba based on mitochondrial D-loop and microsatellite markers analyses

3.3.1 Introduction

The potential effect of landscape features on dispersal and population differentiation of wild animals has been well documented (Vigilant and Guschanski 2009). Putative barriers of gene flow can be revealed by associating the presence of certain landscape features with observed genetic discontinuities (Yoshio et al. 2008; Gerlach and Musolf 2000; Abebe et al. 2013). For instance, habitat breaks, rivers, roads, vegetation types and mountain are expected to limit gene flow in mammal species (Eriksson et al. 2004; Anthony et al. 2007; Jalit et al. 2008; Pérez-Espona et al. 2008; Vigilant and Guschanski 2009; Inoue et al. 2011). In ungulates, roads, rivers, forest types and mountain have been suggested from landscape genetic studies as dispersal barriers (Hartl et al. 2005; Coulon et al. 2006; Pérez-Espona et al. 2008). However, the majority of landscape genetic studies of ungulates have been conducted in deer species (Coulon et al. 2004; Kuehn et al. 2007; Yoshio et al. 2008; Frantz et al. 2012) and have rarely been conducted in tropical forest. Genetic studies across diverse taxa and regions are needed to fully understand the influence of different landscape types on dispersal pattern of wild animals. Moreover, a few studies analyzed the genetic structure of multiple species which

coexist (Frantz et al. 2012). It is important to investigate multiple species in the same region for clarifying the effect of landscape on genetic structure of wild ungulates.

High diversity of forest ungulates, particularly duikers, is a characteristic of African forest fauna (Gautier-Hion et al. 1980; Flenning et al. 1987). Duikers are territorial species which live solitary or pair (Dubost 1980). Their body size ranges from the 6 kg blue duiker to the 80 kg yellow-backed duiker (Newing 2001; Kingdon and Hoffman 2013), and species with larger body size has larger home ranges (Bowland and Perrin 1995). The effect of landscape on genetic structure may be different in species of different body size even in the same subfamily. The analysis of several duikers will provide insights of the effect of body size on genetic structure.

In west and central African forests, duikers form sets of four to seven sympatric species inhabiting all forest types including mature and secondary forests, gallery forests, swamp forests and mountain environments (Kingdon 1997). I studied the population genetic structure of duiker species in Moukalaba. Moukalaba is comprised of several vegetation types: old secondary forests, swamp forests, gallery forests, young secondary forests and savannahs. Previous studies showed the differences of duiker abundance among vegetation types (Nakashima et al. 2013; Chapter II). These abundance differences may affect gene flow. Moreover, the park has Doudou Mountain and rivers. Therefore, this site is suitable for assessing the effect of landscape on genetic structure. In Moukalaba, at least four duiker species of different body size coexist, namely, blue duiker (4-9 kg in body weight), Ogilby's duiker (14-25.5 kg in body weight), bay duiker (17-28 kg in body weight), and yellow-backed duiker (50-80 kg in body weight) (Chapter II). However, molecular techniques could not discriminate between Ogilby's duiker and Peter's duiker (Johnston et al. 2011; Hassanin et al. 2012; Johnston and Anthony 2012).

In addition, some of their mitochondrial haplotypes are originated from non-functional nuclear translocated copies of mitochondrial DNA (Ntie et al. 2010a). Therefore, I focused on three species of duikers for genetic analyses.

In the present study, I used mitochondrial DNA sequences and autosomal microsatellite loci analysis. Mitochondrial DNA is haploid and maternally inherited (Clyde et al. 1974), whereas autosomal microsatellite loci are diploid and biparentally inherited (Powell et al 1996). Combined analysis of nuclear and mitochondrial markers can provide detailed insight into the genetic structure of duiker species such as the levels of female- and male-mediated gene flow. I used mitochondrial control region (mtDNA D-loop) sequences and eleven autosomal microsatellite markers to examine the population genetic structure of duiker species in Moukalaba. The aim was to assess the genetic diversity and spatial genetic structure of three coexistent duiker species in Moukalaba and discuss the effect of potential landscape features on gene flow in each species.

3.3.2 Materials and Methods

3.3.2.1 Microsatellite genotyping of duiker samples

Among faecal samples I successfully identified species (Table 2–1 in Chapter II), 129, 83, 31 and 6 samples of blue, Ogilby's or Peter's, yellow-backed and bay duikers were selected according the good quality and size (~ 600 bp) of mitochondrial D-loop sequences. To assess nuclear DNA variation of blue, yellow-backed and bay duikers, I used eight microsatellite loci specifically developed in this study (CM01, CM04, CM10, CM14, CM15, CM24, CM26 and CM44; Part 3–2 in this chapter) and three loci which

were developed in other bovine species (BM1225, BM2113 and MM12; Ntie et al. 2010b). I prepared two primer sets using Multiplex Manager 1.0 (Holleley and Geerts 2009): set I (CM01, CM04, CM14, CM24, CM44), and set II (CM10, CM15, BM1225, BM2113, MM12). PCR amplifications were carried out in 5 µl reactions containing 200 nM of each primer and 2.5 µl of QIAGEN Multiplex PCR Master Mix (Qiagen, Tokyo, Japan). After an initial incubation at 95°C for 15 min, PCR amplification was performed for 45 cycles consisting of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 30 s, and a final extension at 60°C for 30 min. The amplification products were separated using capillary electrophoresis, using an ABI3130xl genetic analyzer (Applied Biosystems, CA). Alleles were sized using Peak Scanner Software ver. 1.0 (Applied Biosystems). I run two independent PCR for each sample to confirm the genotype.

3.3.2.2 Individual identification

In order to obtain a list of unique individuals of the whole population of each duiker species, I used the program CERVUS 3.0.3 (Kalinowski et al. 2007). I used only the samples of which genotypes of 8 or more microsatellite loci were obtained. In order to evaluate whether our loci were adequate for individual discrimination between close relatives, I calculated the probability of identity between sibs (PIsibs) (Waits et al. 2001) using GenAlEx ver. 6.41 (Peakall and Smouse 2006).

3.3.2.3 Genetic diversity analyses

I used mitochondrial D-loop sequence data for samples which I confirmed individual identification. I used the data of one sample if I found two or more samples from the same individuals. The analysis of mtDNA variation was based on ~600 bp of control region sequences. For each duiker species, Arlequin 3.5 (Excoffier and Lischer 2010) was used to determine the number of haplotypes, haplotype diversity and nucleotide diversity. For microsatellite data, the number of alleles (A), the observed heterozygosity (H_O), the expected heterozygosity (H_E) and deviations from Hardy–Weinberg equilibrium (HWE) were determined using GenAlEx 6.41 (Peakall and Smouse 2006). Those parameters were calculated across populations for each locus.

To control for differences in sampling size, I used rarefaction for microsatellite allelic richness in the program FSTAT 2.9.3 (Goudet 2002) and for mitochondrial control region haplotypic richness in CONTRIB (Petit et al. 1998).

3.3.2.4 Population differentiation analyses

I constructed median-joining tree of mtDNA haplotypes using NETWORK 4.6.1.0 (www.fluxus-engineering.com), and plotted a frequency distribution of inferred haplogroup found at each sampling location in the map.

To assess population admixture of each duiker species, I analyzed microsatellite data using an individual-based Bayesian clustering method implemented in STRUCTURE software ver. 2.3 (Falush et al. 2003). The program was run after burn-in period of 1,000,000 and Markov Chain Monte Carlo (MCMC) replication of 1,000,000. The number of putative populations K was set to range from 1 to 10 and number of

interactions for each value of K was 10. The program calculates the fractional membership of each individual in each cluster (Q). Each individual was assigned to a cluster based on the highest likelihood of membership. I calculate posterior probability values, $\ln \Pr(X/K)$, using STRUCTURE HARVESTER (Earl and Von Hold 2012). The best value of K or the most likely number of clusters was inferred from ΔK which calculated by the method of Evanno et al. (2005). Results were then combined using the program CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) which determines symmetric similarity coefficient between pairs of runs and average individuals membership proportions. Summary barplots were then generated using DISTRUCT 1.1 (Rosenberg 2004). In STRUCTURE results, potentially admixed individuals are those that did not clearly group in any one cluster, suggesting that these individuals are the products of admixture between subpopulations. Following Lecis et al. (2006), I defined individuals with fractional membership in each cluster (Q) from 0.8 to 0.2 as a potentially admixed.

STRUCTURE clustering analysis showed one cluster for bay duiker samples. This might be due to the small sample size. Thus, patterns of individual differentiation of bay duiker were subsequently examined using principal coordinate analysis (PCA). I determined the genetic distance between individual pairs in GenAlEx. The resulting matrix was utilized to conduct the PCA analysis and plotting the first two principal coordinates using GenAlEx.

3.3.3 Results

3.3.3.1 Individual identification

I successfully obtained genotypes from 100 (78%), 6 (100%) and 26 (84%) samples of blue, bay and yellow-backed duiker, respectively. PIsibs for eight microsatellites was 1.6×10^{-4} in blue duiker, 3.7×10^{-3} in bay duiker and 1.4×10^{-3} in yellow-backed duiker. CERVUS software analysis showed that I obtained 82, 6 and 15 individuals of blue, bay and yellow-backed duiker respectively (Table 3–3). The number of mismatching loci between different individuals was 2 or more. Each individual of duiker was found in a specific transect, except for one individual of yellow-backed duiker which was found in transects S2 and S3. However, in further analyses this individual was attributed to transect S2 because I sampled this individual more frequently in S2 than S3.

3.3.3.2 Mitochondrial DNA diversity

Mitochondrial DNA variation analysis based on ~600 bp of control region sequences, yielded 44, 2 and 7 haplotypes for blue, bay and yellow-backed duiker respectively (Table 3–4). Haplotype diversity was 0.998, 0.757 and 0.813 for blue, bay and yellow-backed duiker respectively. Nucleotide diversity was 0.054, 0.035 and 0.038 for blue, bay and yellow-backed duiker respectively. Haplotypic richness based on minimal sample size of 4 individuals was 14.72, 4.64 and 6.98 for blue, bay and yellow-backed duiker respectively (Table 3–4).

3.3.3.3 Nuclear DNA diversity

All analyzed microsatellite loci were polymorphic (Table 3–5). The mean number of alleles per locus was 14.82, 4.64 and 6.82 for blue, bay and yellow-backed duiker respectively. Average heterozygosity values were 0.710 (H_O) and 0.830 (H_E) in the blue duiker, 0.486 (H_O) and 0.607 (H_E) in the bay duiker, and 0.650 (H_O) and 0.694 (H_E) in the yellow-backed duiker. The mean of allelic richness per locus based on minimal sample size of four individuals was 5.14, 3.82 and 4.00 for blue, bay and yellow-backed duiker (Table 3–5).

3.3.3.4 Population differentiation for each duiker species

In bay duiker, there were two different haplotypes, which differed by 33 mutations. One haplotype was shared by individuals found in transect OS1, OS2, S2 and YS1, while the other haplotype was shared by individual found in transect OS3 and OS4 (Figure 3–2a).

Median-joining network revealed that mitochondrial haplotypes of yellow-backed duiker included four haplogroups denoted as A, B, C and D (Figure 3–1a). Haplogroup A was shared by individuals found in transects YS1 and YS2; haplogroup B in transects S1, S2 and YS2; haplogroup C in transects M2, M3, OS3 and YS3, and haplogroup D in transects M1, OS1, OS2, S1, S2 and S3 (Figure 3–3a).

Regarding blue duiker, mitochondrial haplotypes included three haplogroups denoted as A1, A2 and B (Figure 3–1b). Haplogroup A1 was found in almost all the transects except for OS2; haplogroup A2 was mainly found in transects OS3, OS4, M2

and M3; while haplogroup B was mainly found in transect OS1, OS2, S1, S2, S3, M1 and YS3 (Figure 3–4a).

STRUCTURE clustering analysis showed detailed structure of blue and yellow-backed duiker individuals (Figure 3–5). The most probable structure clustering of each species population was at $K = 2$. Regarding yellow-backed duiker, there were no admixture individuals, and all the samples in M1, OS1, OS2, S1, S2, S3, YS1 and YS2 had cluster I type and other individuals found in OS3, M2, M3 and YS3 had cluster II type (Figure 3–3b).

In the case of blue duiker, there were three admixture individuals (Table 3–6). Almost all the individuals found in M1, OS1, OS2, S1, S2, S3, YS1, YS2 and YS3 had cluster II types, and cluster I types were found only in OS3, OS4, M2 and M3 (Figure 3–4b). Individuals with cluster II type were also found in OS3 and OS4, and admixture individuals were found in OS2, M2 and M3 (Table 3–6). All the individuals with mitochondrial haplogroup A2 had nucleic cluster I type and almost all the individuals with mitochondrial haplogroup B except for one individual had nucleic cluster II type (Table 3–6). Individuals with mitochondrial haplogroup A1 have diverse nucleic cluster types in M2, M3, and OS3.

PCA analysis based on microsatellite data set of bay duiker were shown in Figure 3–2b. The first and second coordinates respectively explained 36.02% and 24.76% of the total of genetic variation. The PCA plot showed a clear distinction between individuals collected in transects OS3 and OS4 and those collected in transects OS1, OS2, S2 and YS1 (Figure 3–2b).

3.3.4 Discussion

3.3.4.1 Individual identification and genetic diversity

Individual identification of wild mammals is essential for determine genetic diversity indices. In this study, microsatellite genotypes attributed 6, 15 and 82 individuals of bay, yellow-backed and blue duikers respectively. Samples of most individuals collected twice or more were found in the same transect and one individual of yellow-backed duiker was found in two transects. These results were reasonable when thinking their territory size. The size of territories varies from 2 to 4 ha for the small blue duiker, 20-80 ha for medium-size bay duiker and around 200 ha for the larger yellow-backed duiker (Dubost 1980; Feer 1989; Bowland and Perrin 1995). The distances between transects in our study was at least 1 km, and therefore it was reasonable that only one of yellow-backed duiker was found in two transects.

Genetic diversity values in both mitochondrial and microsatellite data were low for bay duiker compared to blue and yellow-backed duiker in the Moukalaba forest (Table 3–4 and 3–5). The abundance of bay duiker has been reported to be low compared to other duiker species within the study site (Chapter II). This low genetic diversity of bay duiker may be due to its small population size. However, mitochondrial diversity values of bay duiker in Moukalaba appear to be even high when compared with those of the endangered Abbott's duiker in the Udzungwa Mountains, Tanzania (Table 3–4). Genetic diversity of bay duiker in Moukalaba might not be critical as an endangered species.

Moreover, overall genetic diversity values were high for blue duiker within the study site (Table 3–4 and 3–5). For instance, haplotype and nucleotide diversities were 0.998 and 0.054 respectively. Previous report on the genetic diversity of blue duiker

indicated 0.902 for haplotype diversity, 0.026 for nucleotide diversity and 12 for haplotypic richness in the Udzungwa Mountains (Bowkett et al. 2014). This might reflect high abundance of blue duikers compared with other duiker species (Chapter II). When I compare the genetic diversity of blue duiker between two study sites, it appears to be higher in Moukalaba than in Udzungwa (Table 3–4). Nakashima et al. (2013) reported that the density of blue duiker in Moukalaba is the highest in the African forest. This high genetic diversity of blue duiker may reflect the high abundance of this species in Moukalaba.

3.3.4.2 Population genetic differentiation of duiker species in Moukalaba

In this study, I found substantial differentiation of duiker population at the small geographical scale for all the 3 duiker species. For bay duiker, I found two mitochondrial haplotypes which differed by many mutations, therefore I can consider that there exists two subpopulations (Figure 3–2a) (subpopulation I: OS1, OS2, S2, YS1; subpopulation II: OS3, OS4). The PCA analyses based on autosomal microsatellite data showed that the four individuals in subpopulation I was clustered (Figure 3–2b). However, it was difficult to expect the genetic barriers because of small sample size.

For yellow-backed duiker, autosomal microsatellite data clearly showed two subpopulations (subpopulation I: M1, OS1, OS2, S1, S2, S3, YS1, YS2; subpopulation II: M2, M3, OS3, YS3). Individuals in subpopulation II had only mitochondrial haplogroup C. On the other hand, individuals in subpopulation I had three mitochondrial haplotypes. Male-mediated gene flow was expected to homogenize nucleic regions in subpopulation I. Both mitochondrial and microsatellite analyses showed the genetic gap

between subpopulation I and II. This genetic gap may be partially due to the Doughetsi River (Figure 2–1). River has been identified as a barrier for dispersal in other mammals (Cullingham et al. 2009; Vigilant and Guschanski 2009; Hartmann et al. 2013; Mitchell et al. 2015). In order to avoid predation, duikers are reported to be rarely seen on the border of rivers (Dubost 1980). Moreover, in Ituri forest Republic Democratic of Congo, duikers are reported as prey of crocodiles and African rock pythons (Hart 2000). African rock pythons and crocodiles are observed in the rivers of Moukalaba-Doudou National Park (Akomo-Okoue personal observation). The risk of predation may limit movements across the rivers and explain its gene flow barrier.

In the case of blue duiker, when I focus on the biased distribution of haplogroup A2 and B (Figure 3–4a), I can consider that there exists two subpopulations (subpopulation I: M1, OS1, OS2, S1, S2, S3, YS1, YS2, YS3; subpopulation II: M2, M3, OS3, OS4). Autosomal microsatellite analyses showed two distinct clusters in our samples and these two cluster types were differentially distributed approximately according to subpopulations. This genetic gap may be partially due to the mountain. The mountain areas around M3 and M2 were steep (Figure 2–1) and the abundance of blue duikers were lower in steeper areas, especially in M2 and M3 (Chapter II). Mountain has been reported to be one of genetic barriers in other mammals including red deer (Ernest et al. 2003; Rueness et al. 2003; Pérez-Espona et al. 2008; Zalewski et al. 2009). Furthermore, Alexander and Waters (2000) have also suggested that optimal areas for movement of another ungulate species, wapiti *Martes Americana*, was low topographic areas. The mountain might limit gene flow between blue duiker populations probably due to the difficulties of the movements along steep areas.

In blue duikers, a little gene flow was observed between subpopulations. Almost all the individuals in subpopulation I had nucleic cluster II type and all the individuals with mitochondrial haplogroup A2 in subpopulation II had nucleic cluster I types. However, eight individuals with mitochondrial haplogroup A1 in subpopulation II had diverse nucleic types (Table 3–6). Among them, one individual had nucleic cluster I type which dominated in subpopulation II, while five individuals had nucleic cluster II type which dominated in subpopulation I. This suggests that some individuals with mitochondrial haplogroup A1 and nucleic cluster II types recently migrated from subpopulation I to II. There was only one admixture individual in subpopulation I, and therefore gene flow from subpopulation II to I may be limited, if any. Although some minor difference exists, the place of genetic gap in yellow-backed and bay duiker was quite similar. The gap might reflect population history such as the place of refugia. More data in and around Moukalaba will clarify the exact history of duikers in this region.

I suggested that the expected genetic barriers were different between yellow-backed and blue duikers. River and mountain might be a genetic barrier for yellow-backed and blue duiker, respectively. Body size may explain the difference. I analyzed the effect of landscape features on the abundance of duikers (Chapter II). They showed that the abundance of blue duikers was negatively affected by steeper features, while the abundance of yellow-backed duikers was affected by vegetation type (Table 2–2a in Chapter II). This result suggests that the effect of mountain may be higher in blue duiker, the smaller species. For the predation risk in the river, yellow-backed duiker, the larger species, may be higher. In the rodent species, the predation risk increased with rodent body size (Kotler 1984). Similarly, predation risk of yellow-backed duiker around the river may be higher than that of blue duiker, which makes yellow-backed duikers to avoid

taking a risk of crossing the river. Although more data are needed to confirm the fact, the difference in body size may differentiate the genetic barriers of duikers.

In this study, using both mitochondrial and microsatellite data, I described the detailed genetic structure of three coexistent duiker species. Although duikers were continuously distributed in Moukalaba, I found clear genetic structure at the small geographic scale. This suggests that population history of duikers may be complex. Duikers are widely distributed in tropical forest of Africa. The analyses of detailed genetic structure of duikers and their population history may be useful for understanding the place of refugia or history of duikers in the forests.

Chapter VI

General discussion

4.1 Noninvasive genetic sampling tool for wildlife studies

Noninvasive genetic techniques have recently received much interest. The use of DNA extracted from faeces can provide the information of species, individual and sex, which were useful for understanding the ecology of wild mammals (Waits and Paetkau 2005; Beja-Pereira et al. 2009; Lampa et al. 2013). In this study, I collected fresh faeces from duikers in different habitat types and conducted species identification using genetic method. I successfully identified duiker species, some of which are difficult to discriminate using other methods. Dung count combined with genetic species identification was able to estimate the difference of abundance in different habitat types of duiker species. Although camera-traps have advantages for assessing distribution of cautious terrestrial mammals (Rovero and Marshall 2009; Treves et al. 2010; Vanthomme et al. 2013), dung count combined with genetic species identification may be regarded as another candidate for investigating abundance of mammals, because dung piles are relatively easy to observe and collect, particularly for terrestrial mammals. In addition, an advantage of molecular censuses is to discriminate the similar looking species which were difficult to discriminate even when using camera traps. For instance, three red duikers (bay, Peters' and Ogilby's duikers) cannot be always distinguished by camera traps (Vanthomme et al. 2013). Moreover, noninvasive genetic sampling can, of course, allow genetic studies on free-ranging animals without capturing or even observing them, and can address the questions that are difficult to answer using other methods. Contrary to

traditional census techniques, dung count combined with genetic species identification can allow to understand dispersal within animal populations in variable environments and to determine potential barriers on gene flow of wild animals.

In this study, individual identification from fecal samples was conducted, using original microsatellite markers developed for duikers. I successfully obtained genotypes from 78%, 100% and 84% samples of blue, bay and yellow-backed duikers, respectively. All microsatellite loci were polymorphic with substantial allelic richness across all duiker species. Before I developed original markers, I tried some microsatellite markers which were developed for other bovine species and reported to be useful in duikers (Ntie et al. 2010b) using DNA extracted from faecal samples of duikers. However, the amplification of several markers failed probably due to low quality and quantity of DNA. High PCR success rate of genotyping with DNA from faeces in this study may suggest that non-invasive samples can provide more useful data when using original microsatellite markers developed for target species.

4.2 Landscape and distribution patterns of duiker species in Moukalaba forest

In this study, genetic species identification showed that four duiker species coexisted and three of them were found in all the examined habitats. Moreover, our result showed the positive correlation between blue duiker abundance with that of Ogilby's and yellow-backed duikers in almost habitat types. Habitat separation of these three major species may be not clear in the study area. At Makokou, Gabon, diets of these three species were overlapped (Gauthier-Hion et al. 1980, Dubost 1984). Although the larger duiker species consumed the larger fruits, the diversity of fruit species eaten was

overlapped (Dubost 1984). Duikers are frugivorous species; at least 68% of diet of some species (blue, Ogilby's/Peters', bay, black-fronted and white bellied duikers) is made up of fruits and seeds. The vegetative part (leaves, petioles and stems) is secondly important and represent 16 to 31% of their diet (Gauthier-Hion et al. 1980, Dubost 1984, Feer 1989). Contrasting activity (diurnal and nocturnal) patterns may reduce direct competition at food patches through temporal separation. Of the three abundant duiker species, two (blue and Ogilby's duiker) are diurnal and one (the larger yellow-backed duiker) is cathemeral species (Dubost 1980, Newing 2001). From the foregoing arguments, there may exist feeding competition among duiker species at least to some extent. However, in Moukalaba, Takenoshita et al. (2008) showed that fallen fruits were available throughout the year although the abundance varied according to seasons. Annual fruit census was also conducted in the transects which I used in this study and fallen fruits were abundant (Akomo-Okoue personal observation). High abundance of fallen fruits may mitigate the competition for food resources between duikers. Further analyses of feeding behaviour and ranging patterns of duikers are needed to ascertain the reason why duikers coexist in the same habitat.

Effect of landscape features on their abundance and genetic structure was different among duiker species. In particular, the abundance of Ogilby's duikers was low in young secondary forests and that of yellow-backed duikers was high in swamp forests. In contrast, the abundance of blue duiker was not affected by vegetation type. Blue duiker is the most abundant duiker species in the communities where it occurs in central African forest (White 1994; Muchall and Ngandji 1999; Hart 2000; van Vliet 2008; Jimoh et al. 2013, Viquerat et al. 2012; Nakashima et al. 2013) and has been suggested to adapt in a wide range of forested and wooded habitats including mature and secondary forests,

gallery forests, dry forest patches and regenerating forests (Dubost 1980; Kingdon 1997; Muchall and Ngandji 1999). The ecological success of blue duikers is made possible by their small body size which allows them to occupy smaller patches of habitat and exploit dispersed food sources (Dubost 1980).

Genetic structure analyses suggested that there exist two subpopulations in all the three species. Mountain was suggested to be responsible for observed genetic differentiation in blue duiker. Considering that the dung abundance of blue duikers was low in mountain area, utilization and movement in mountains may be limited in blue duikers. In contrast, river might act as a genetic barrier in yellow-backed duiker. Predation risk of yellow-backed duiker, the largest species, around the river may be high, which makes yellow-backed duikers to avoid taking a risk of crossing the river. The different effect of landscape among coexisting duiker species may reflect the difference of their ecology such as ranging behaviour and dispersal patterns.

Not only the landscape but also demographic history of duiker species may have affected their distribution and genetic structure. In this study, using both mitochondrial and microsatellite data, I described the detailed genetic structure of three coexistent duiker species. Although duikers were continuously distributed in Moukalaba, I found clear genetic structure at the small geographic scale. The place of genetic gap in three duiker species was quite similar, although some minor difference exists. The gap might reflect population history such as the place of refugia. In addition, two genetically different subpopulations of blue duiker were suggested to have recently contacted. These results suggest that population history of duikers may not be simple. Duikers are widely distributed in tropical forest of Africa. The analyses of detailed genetic structure of

duikers and their population history may be useful for understanding the place of refugia or history of fauna in the forests.

4.3 Diversity of duiker species in Moukalaba forest

Good knowledge of species diversity in a study area provides basic and fundamental information for ecological studies, and also for planning and evaluating conservation strategies for the region (Bowkett et al. 2014). Duikers form an important group including three genera and twenty species widely distributed throughout African forests and all weighing less than 80 kg (Kingdon 1997). In central Africa, at least six duiker species coexist: blue duiker, Ogilby's/Peter's duiker, white-bellied duiker, black-fronted duiker, bay duiker and yellow-backed duiker (Kingdon 1997; Christy et al. 2008; Kingdon and Hoffmann 2013). However, the results of my present study suggest that four duiker species (blue duiker, Ogilby's/Peter's duiker, bay duiker and yellow-backed duiker) coexist in Moukalaba forest. Duiker species are less diverse in Moukalaba than in two other protected areas in Gabon. White-bellied duiker is present in Ipassa Man, Biosphere Reserve Makokou, and in Lopé (Dubost 1979; White 1994; van Vliet 2008), while the species is absent in Moukalaba. Black-fronted duiker, typical swamp forest dwelling species, is present in Ipassa Man and Biosphere Reserve Makokou (Dubost 1979; van Vliet 2008), while the species is absent in Moukalaba. Similarly, Nakashima (2015) conducted camera-trapping survey in the study site and found that total number of forest dwelling species is less diverse in Moukalaba (39 species) than in Lopé (44 species) or in Ipassa Makokou (48 species).

As suggested also by Nakashima (2015), the lower number of duiker species in the present study site may be caused by separation from large-continuous Pleistocene refuges by a natural boundary. It has been recognized that places where species persist during glaciations were very limited, and current distribution of species is shaped according to their origin areas (Provan and Bennett 2008). The mountainous area in the study site is thought to be one of the Pleistocene refuges (Sosef et al. 2004); however, this area would be too small to sustain large number of forest-dwelling mammals including duikers throughout the glaciation period. The nearest large refuge is the massif du Chaillu (Maley 2001) which is located approximatively 150 km from the study area, but is separated by the Ngounié River (a branch of Ogooué River) and by savannah from Moukalaba (Figure 4–1). My study suggests that river might be gene flow barrier for duiker species at least yellow-backed duiker. Furthermore, duikers preliminary live in the forest (Dubost 1979; Kingdon 1997). Natural boundaries such as savannah and Ngounié River may have limited the opportunity for dispersal of forest duiker from the refuge. History of fauna may explain the low number of duiker species found in Moukalaba-Doudou National Park.

Moreover, ecological factors such as vegetation types may influence diversity of duiker species. The black-fronted duiker which typically use swamp forests (Dubost 1979), is present at Ipassa Makokou, but absent in Moukalaba. Vegetation type in Moukalaba and Ipassa Makokou is considerably different, with Moukalaba being forest savannah mosaic (Takenoshita et al. 2008; Nakashima et al. 2013), whereas Ipassa is a typical dense forest with wide swampy clearings namely *bais* (Caballé 1978). *Bais* are comprised of streams, pools and saturated soils supporting aquatic and semi-aquatic vegetation; and are usually frequented by several species (Magliocca and Gauthier-Hion

2002). Although there are small extended swamps forests in Moukalaba, I have not yet observed forest clearings known as bais. The abundance of yellow-backed duikers was high in swamp forest in Moukalaba, instead of black-fronted duiker. The absence of bais in Moukalaba may contribute to explain the low diversity of duiker species.

4.4 Implications for conservation

Across large parts of the world, wildlife has to coexist with human activity in different types of landscape. This coexistence usually led to the decline of biodiversity. In Africa, biodiversity in tropical forests is of great concern, as these forests face serious threats, mainly due to human activities (Poulsen et al. 2009; Vermeulen et al. 2009; Breuer and Hockemba 2012). For sustainability, declines in biodiversity highlight the need to develop strategies for species monitoring particularly in developing countries. In Moukalaba, at least 4 duiker species coexisted, namely blue duiker, Ogilby's duiker, bay duiker and yellow-backed duiker. Blue, Ogilby's, and yellow-backed duikers were found in all the studied transects. Furthermore, I found that dung pellets of blue duiker were more abundant and its abundance was positively correlated with that of Ogilby's and yellow-backed duiker. This positive correlation between the abundance of blue duikers with that of two other major duikers (Ogilby's and yellow-backed) suggest that blue duikers can be considered as an indicator of duiker abundance within study site. When we need the information on only the total abundance of duikers, monitoring only blue duikers may be enough. However, in some situations, the information on the abundance of each species is also important for their conservation. It is better to choose the appropriate method for monitoring small ungulates depending on the purpose and the

budget. If the detailed information on species diversity is needed, dung counts with genetic species identification may be one of the most appropriate methods.

The first problem in conservation management is the lack of wildlife information especially in developing countries, so the estimation of abundance and genetic diversity of hunted species has a high priority. In addition, genetic diversity is one of the levels of biodiversity that the World Conservation Union (IUCN) has recommended for conservation (McNelly et al. 1990). Genetic variation is a central concept of evolutionary biology that has been linked to ecosystem recovery and species ability to respond to environmental changes (Hedrick and Miller 1992). Lack of diversity is typically considered as evidence for a small or declining population (Garza and Williamson 2001). Thirty to forty years prior to our study, most of the Moukalaba forest was selectively logged, and hunting pressure was high. Before becoming a national park in 2002, Moukalaba was classified as a wildlife reserve for the fauna and flora that were being exploited in the 1990s (Blaney and Thibault 2001). Since 1999, a research project of Kyoto University focused on great apes has been conducted (Ando et al. 2008), and, consequently, hunting activities seem to be reduced around the study area. Nonetheless, the level of genetic diversity of three coexistent duikers was high. The effect of human impacts on genetic diversity was difficult because data on the abundance and genetic diversity of those species are not available before logging. Continuous monitoring are needed to accumulate the information for conservation.

Recent increase of African population caused higher human pressure on duikers. I found that the dung abundance of Ogilby's duikers and blue duikers was affected by human impacts, while that of yellow-backed duikers was not affected. Habitat disturbance was suggested to affect the distribution of Ogilby's duikers, and that of blue duiker was

suggested to be affected by hunting activity. My results might suggest the vulnerability of duikers to human pressures. Consequently, additional efforts for the implementation of conservation programs are required especially near the villages in protected areas.

4.5 Future perspectives

This study serves as a first step in long term study on duikers which will clarify how those species belonging to the same subfamily (Cephalophinae) coexist. To clarify the unclear habitat separation of duiker species within the study site, further works are required to adequately assess their diet composition and their ranging patterns.

Diet analysis is one of the important approaches for clarifying how several duiker species coexist in same habitats inferring food preference by each species. Stomach content analyses of blue, Peter's (sister species of Ogilby's duiker) and yellow-backed duiker species were previously conducted (Dubost 1984) and the study clarified that these duikers ate mostly fruits. The stomach analysis is invasive method and requires the dead of duikers. Therefore, the sample size is not enough for analyzing the seasonal changes of diet and the difference in diet among vegetation types. DNA based plant species identification from animal faeces has been recently conducted (Bradley et al. 2009). This method will clarify seasonal diet change of duikers without capturing animals. These data can provide new insights on the coexistence of duiker species.

In addition, home range is important factor to interpret animal behaviour. Home range is an area traversed by the individual in its normal activities of feeding, mating and caring for young (Dubost 1980; Feer 1989). Study on ranging patterns including home range characteristics (size, fluctuations, core areas and overlap within and between

species) of different species may be also investigate to understand more on their sympatry. Forest duikers are, by their cryptic behaviour, difficult animals to study with regard ranging patterns. Little information on movement patterns can be gathered from noninvasive genetic method; consequently home-range characteristics of forest will be conducted by radio-tracking. The aim will be to correlate forest duiker home-range characteristics with environmental factors.

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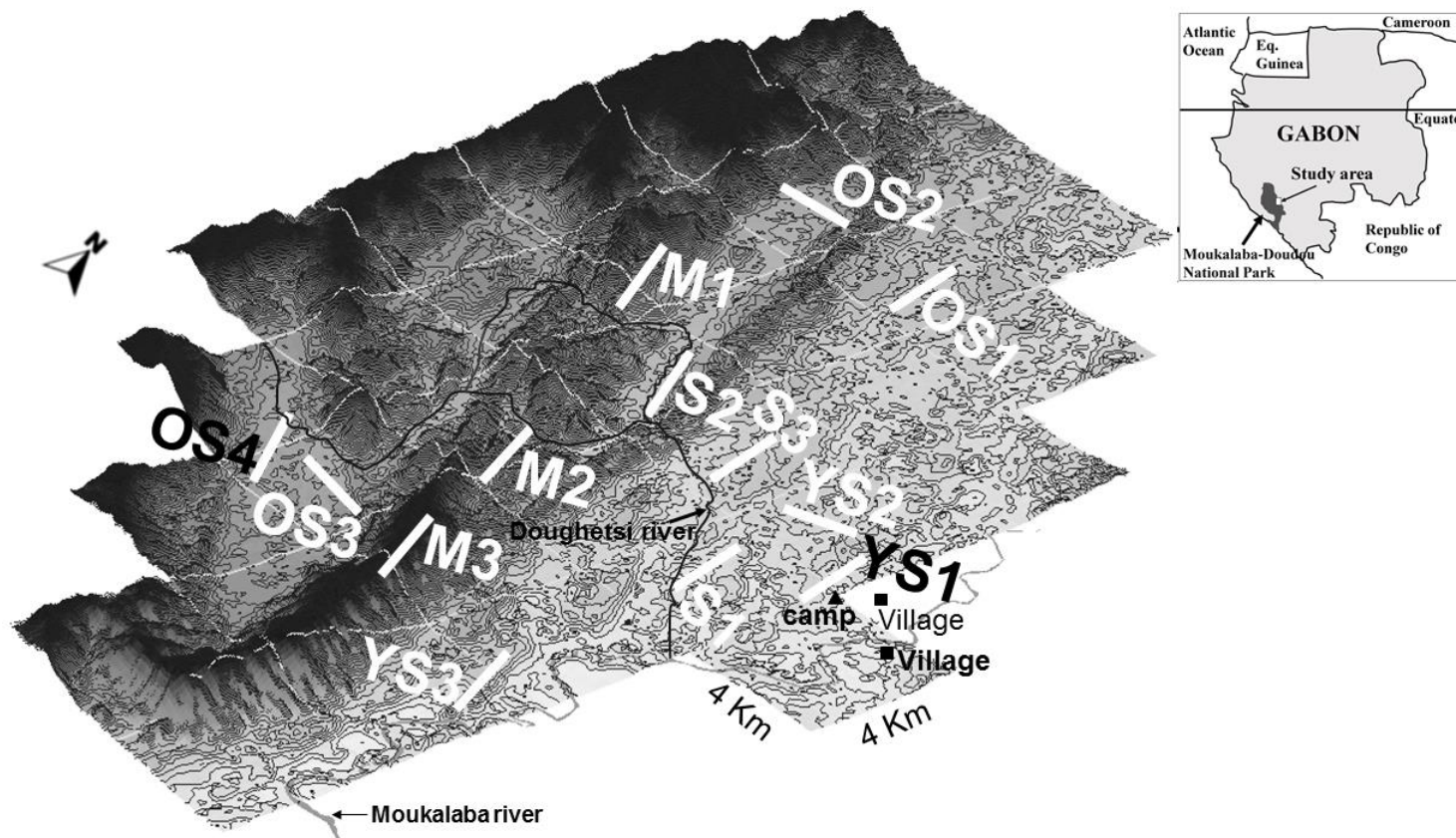


Figure 2–1: Map of sampling area showing natural landscape features that could influence duiker gene flow and distribution. Solid white line on the map correspond to transect. Dark areas are areas with mountain and slope, while light areas are flat areas. YS young secondary forest; OS old secondary forest; S swamp forest and M mountain forest.

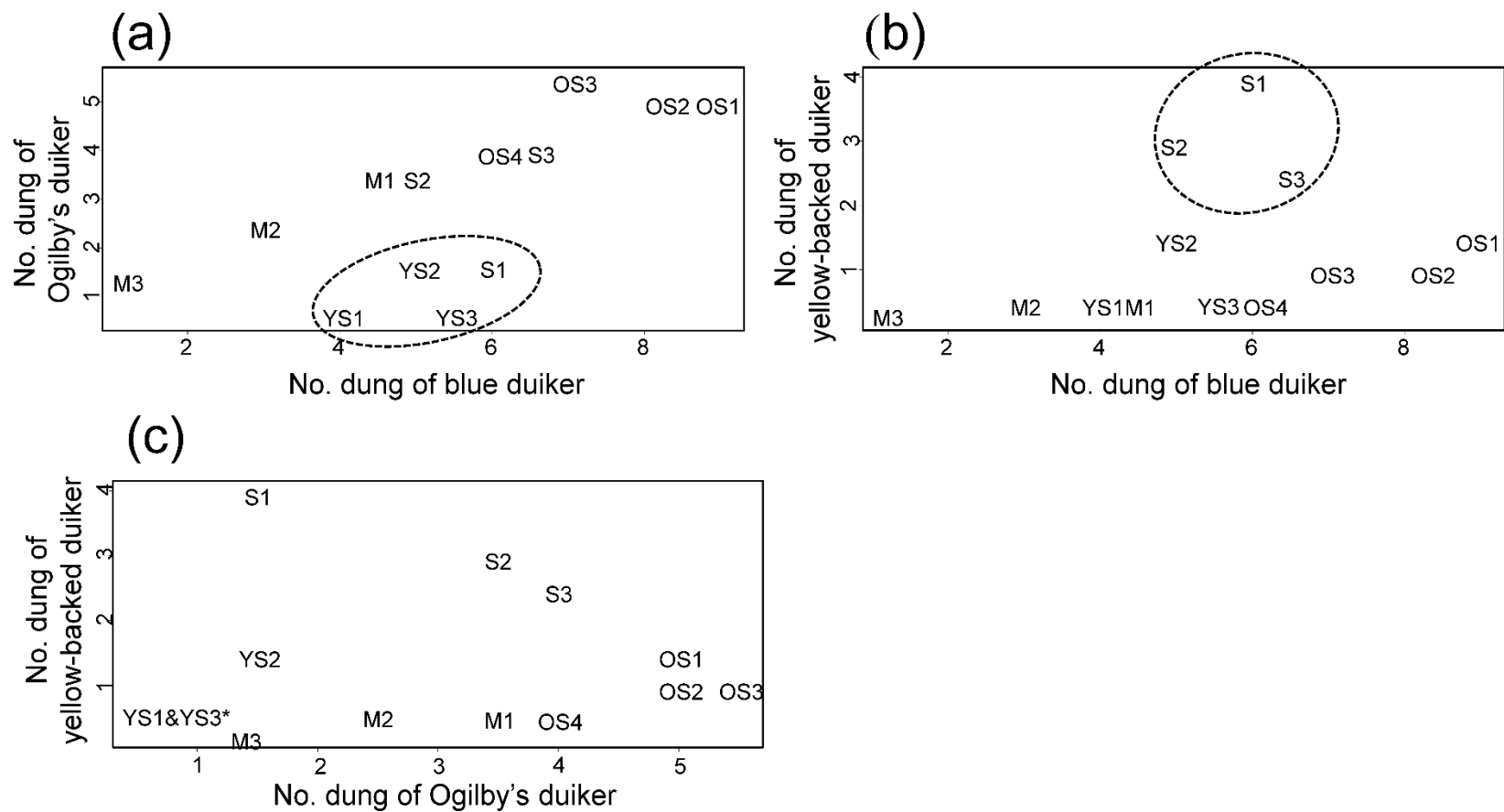


Figure 2–2: Correlation of dung numbers per transect per visit: **a** blue and Ogilby's duikers; **b** blue and yellow-backed duikers; **c** Ogilby's and yellow-backed duikers; *asterisk*, YS1 and YS3 share the same ratio. YS young secondary forest, OS old secondary forest, S swamp forest and M mountain forest.

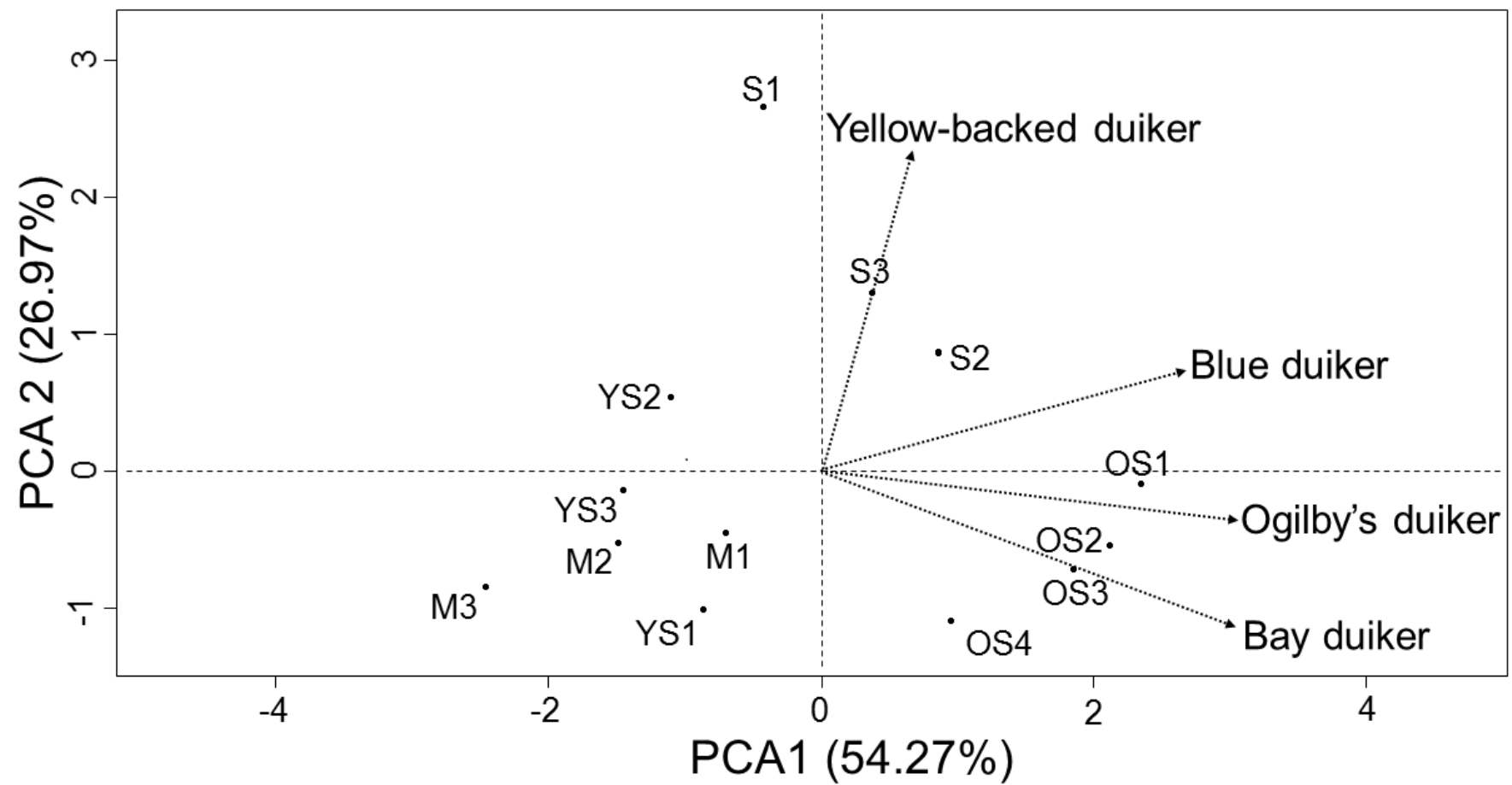


Figure 2–3: Principal component analysis (PCA) of transect characteristics. YS young secondary forest, OS old secondary forest, S swamp forest and M mountain forest.

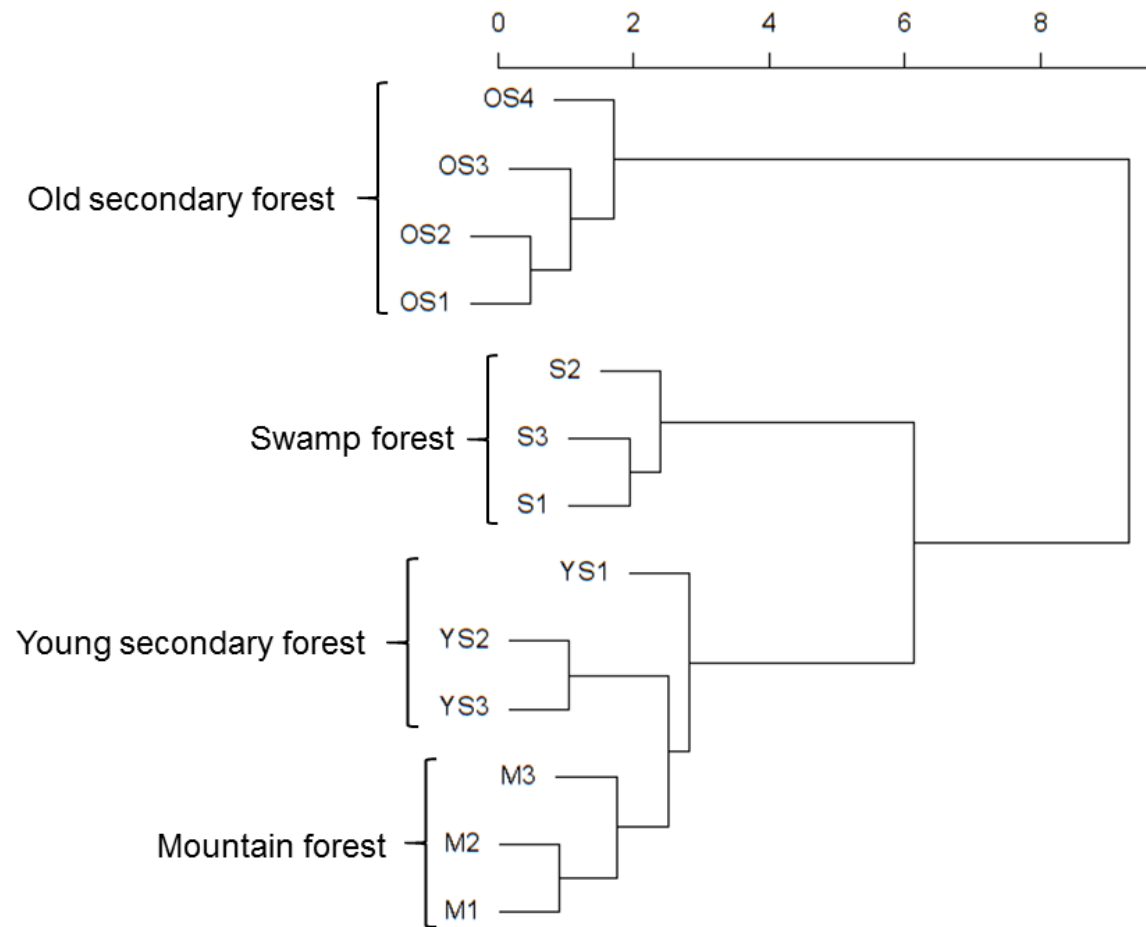


Figure 2–4: Cluster analysis of transect characteristics. *YS* young secondary forest, *OS* old secondary forest, *S* swamp forest and *M* mountain forest.

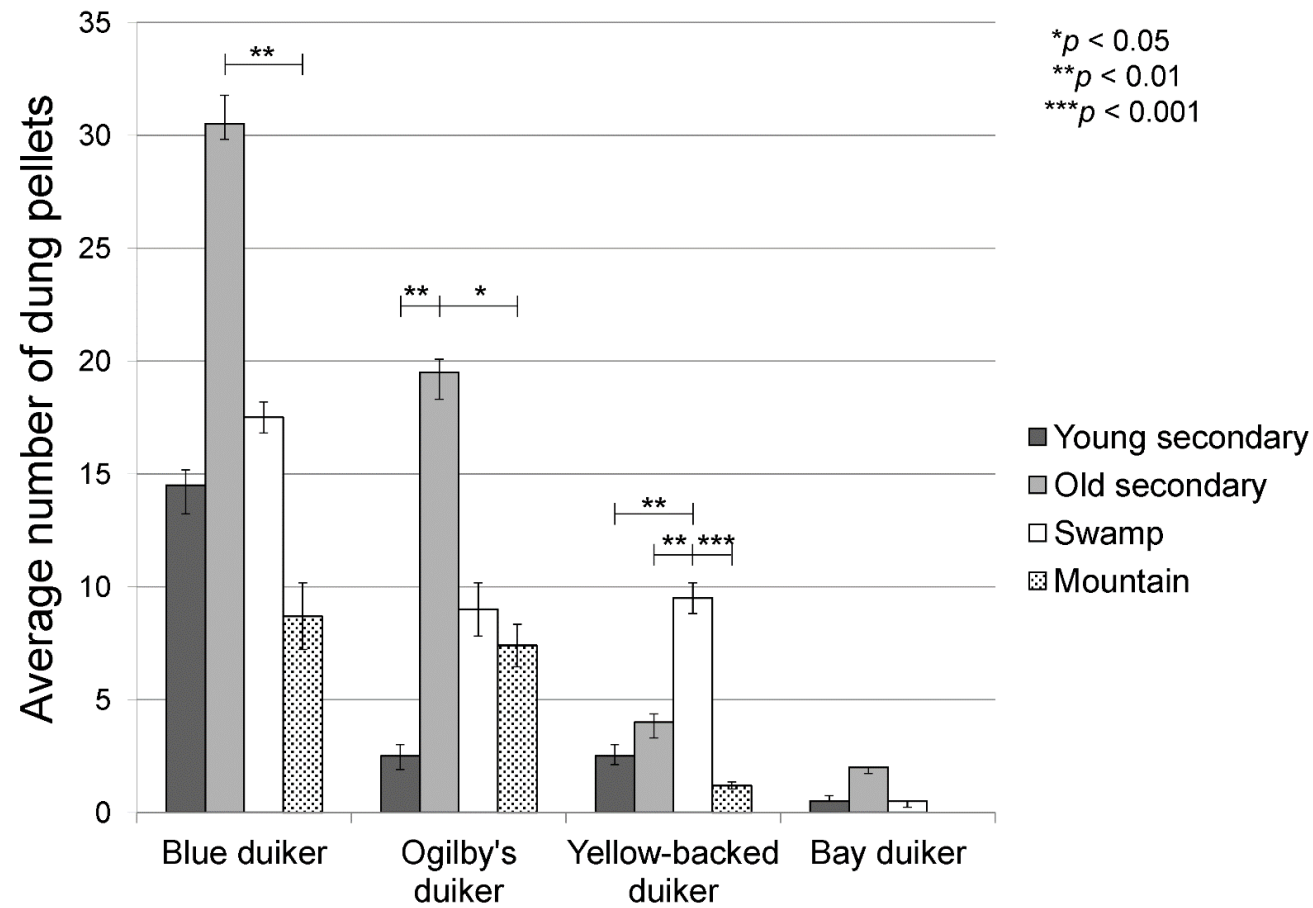


Figure 2–5: Differences in the dung abundance of duiker species among vegetation types.

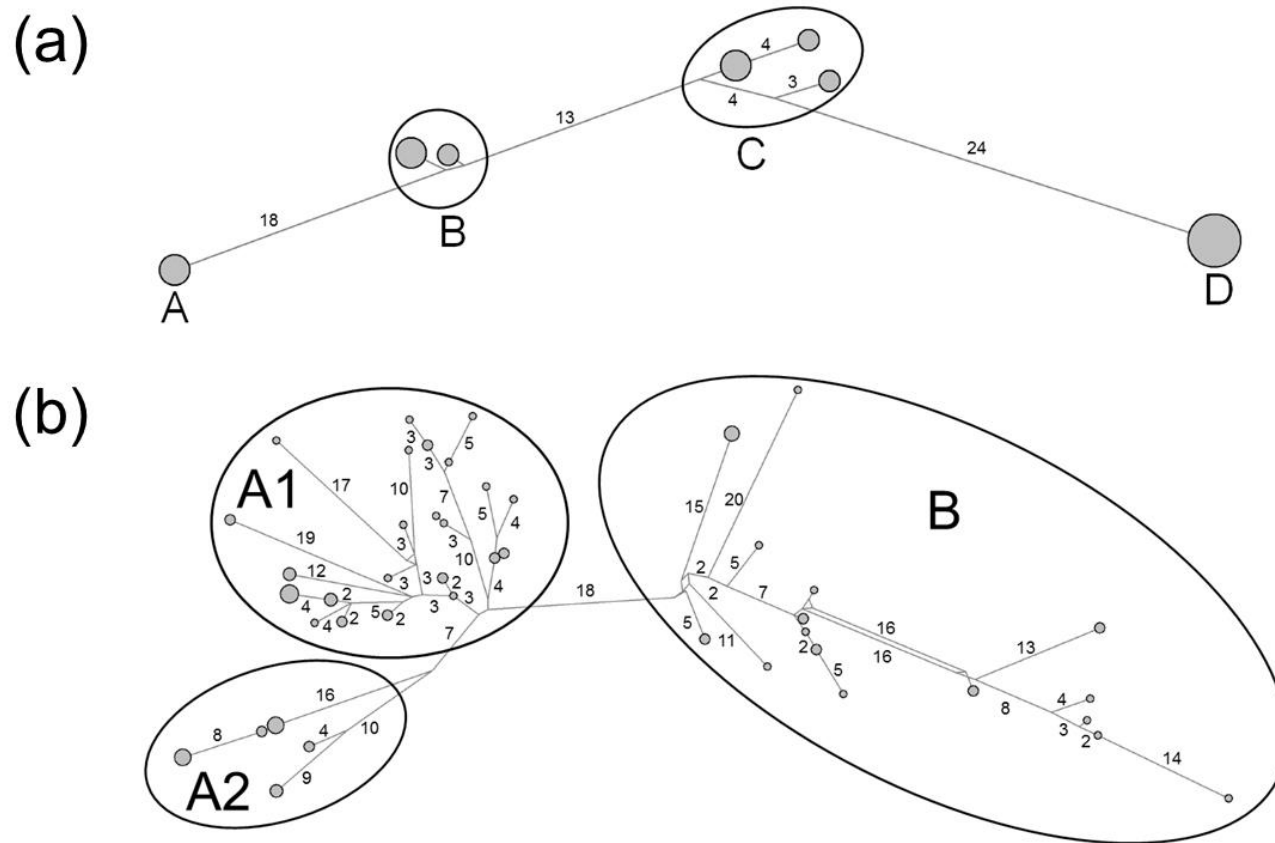
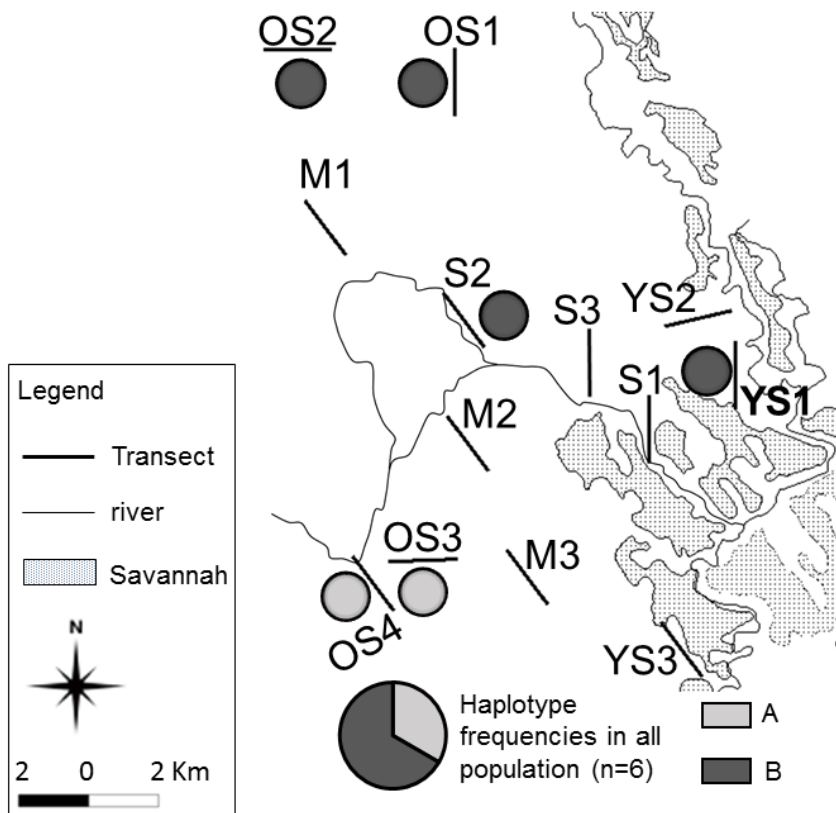


Figure 3-1: Median-joining tree of mtDNA haplotypes of yellow-backed duiker (a) and blue duiker (b) in Moukalaba-Doudou National Park, Gabon. Circle size is proportional to the total number of individuals of that haplotype in each species. Number of substitutions (>1) are showed between haplotypes.

(a) Mitochondrial DNA



(b) Nuclear DNA

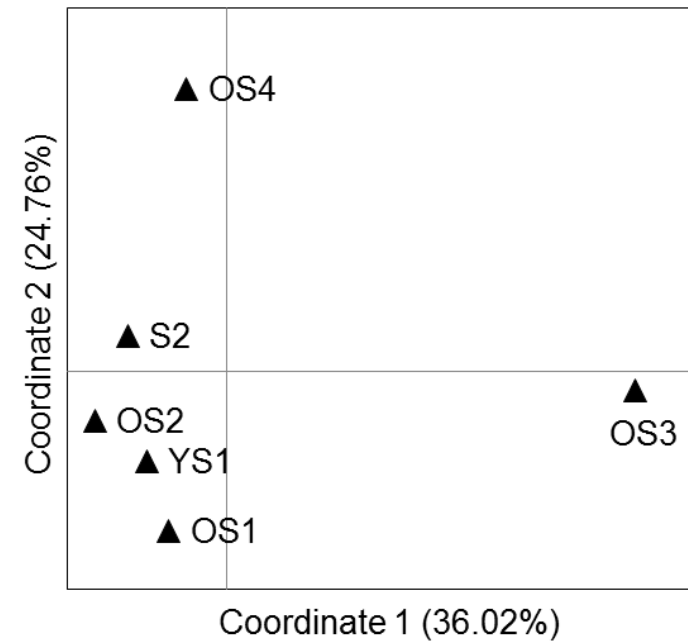


Figure 3–2: (a) Mitochondrial DNA haplotype constitutions of bay duiker in Moukalaba habitats. The size of circle is proportional to the number of individuals in each transect. (b) Principal coordinate analysis (PCA) of bay duiker individuals from 6 transects (OS1, OS2, OS3, OS4, S2 and YS1) based on microsatellite data.

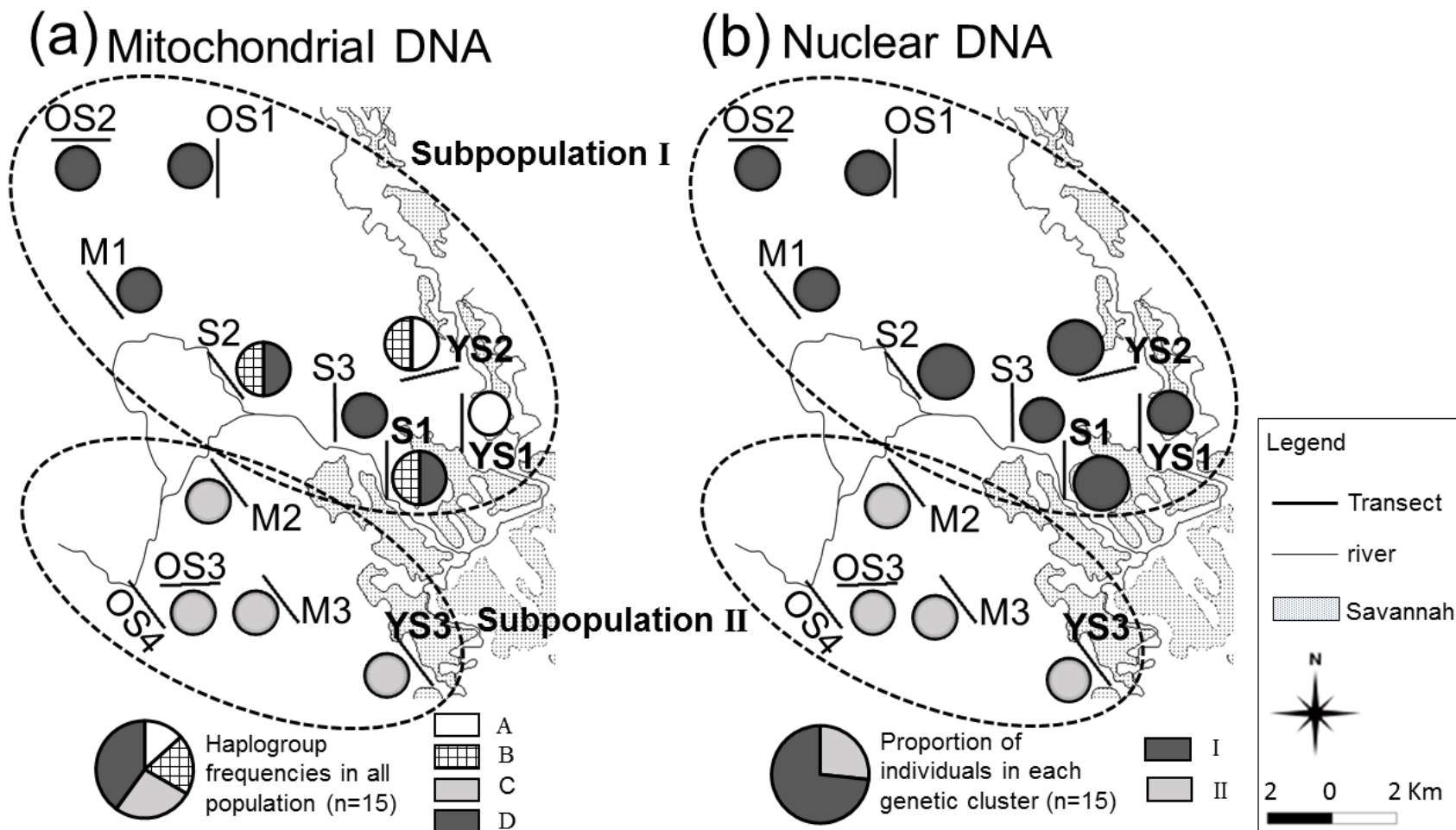


Figure 3–3: Constitutions of haplogroup of mitochondria (a) and genetic cluster type of microsatellite of yellow-backed duiker in Moukalaba habitats. The size of circle is proportional to the number of individuals in each transect.

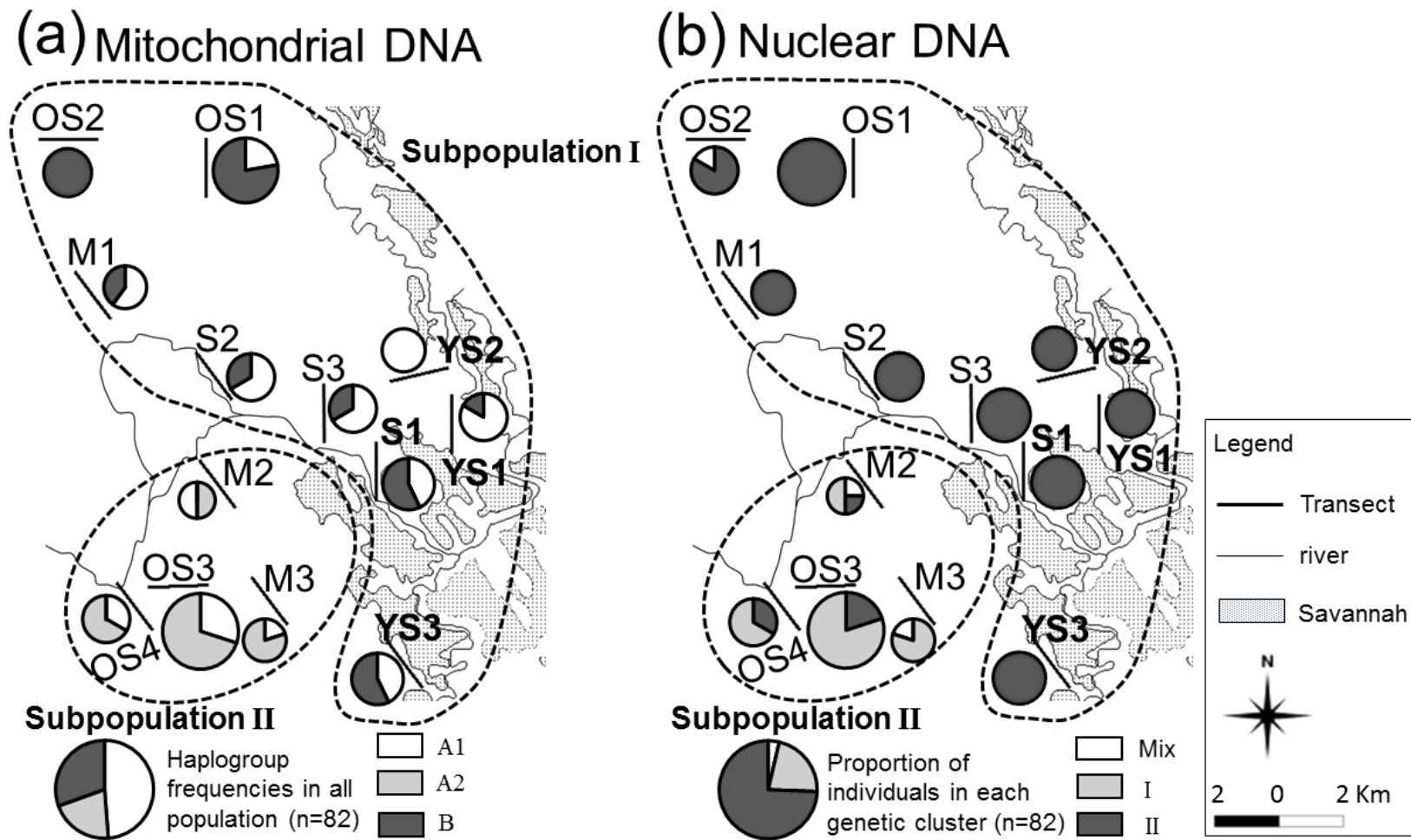


Figure 3–4: Constitutions of haplogroup of mitochondria (a) and genetic cluster type of microsatellite of blue duiker in Moukalaba habitats.

The size of circle is proportional to the number of individuals in each transect.

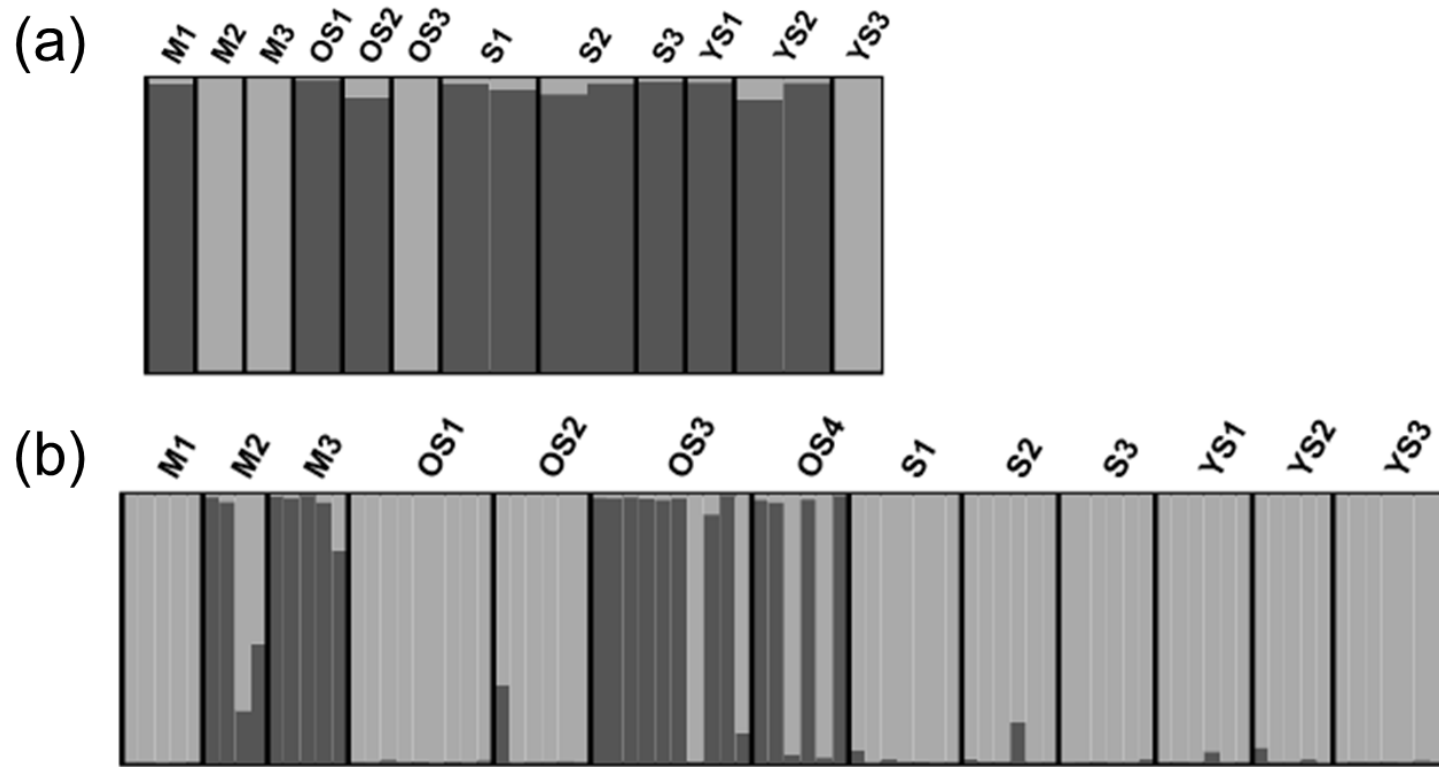


Figure 3–5: Visualisation of the Bayesian clustering analysis implemented in STRUCTURE for K=2, where each K is shown as a different greyscale shade. (a) For yellow-backed individuals (n = 15). (b) For blue duiker individuals (n = 82). Sampling sites are shown as single vertical bars. Note that upper the plot I indicated sampling sites. Location of sampling sites see Figure 2–1.

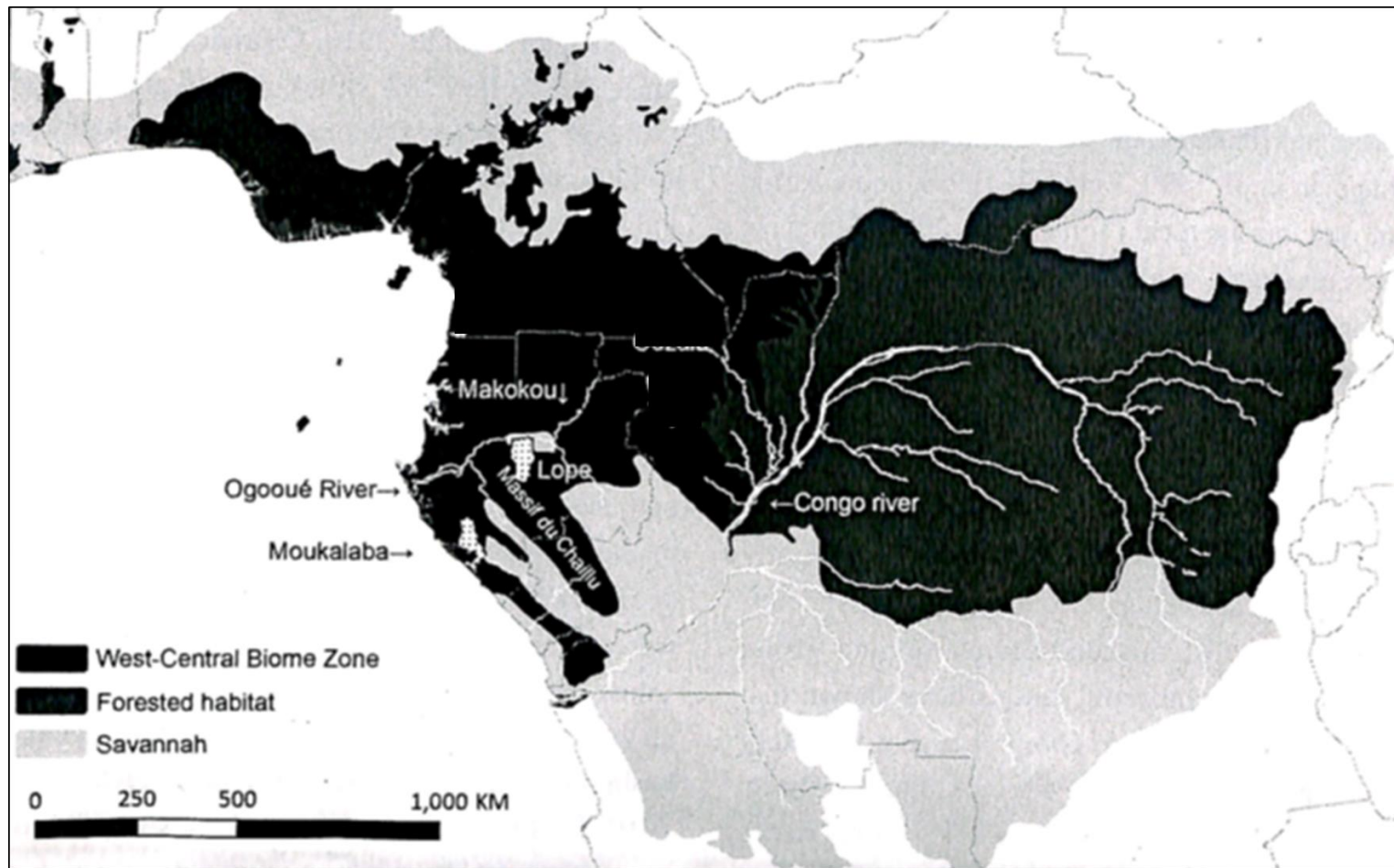


Figure 4–1: Geographical location of Moukalaba-Doudou National Park. Locations of National Parks (Lopé and Ipassa Makokou) and Massif du Chaillu were also shown on the map. Figure from Nakashima 2015.

Table 2–1: Number of faecal samples from each duiker species in each transect

Transect	Vegetation type	No. visits	PCR success (%)	Number of faecal samples from each duiker species				No. species
				Blue	Ogilby's ^a	Yellow-backed	Bay	
YS1	Young secondary	2	85	8	1	1	1	4
YS2	Young secondary	2	76	10	3	3	0	3
YS3	Young secondary	2	81	11	1	1	0	3
OS1	Old secondary	2	82	18	10	3	1	4
OS2	Old secondary	2	77	17	10	2	1	4
OS3	Old secondary	2	80	14	11	2	1	4
OS4	Old secondary	2	92	12	10	1	1	4
S1	Swamp	2	84	12	3	8	0	3
S2	Swamp	2	75	10	7	6	1	4
S3	Swamp	2	84	13	8	5	0	3
M1	Mountain	2	77	9	7	1	0	3
M2	Mountain	2	85	6	5	1	0	3
M3	Mountain	5	78	6	7	1	0	3
Mean		-	81	11.2	6.4	2.7	0.5	3

^aThis might include Peter's duiker (see text)

Table 2–2: Summary of the model selection for blue, Ogilby’s, and yellow-backed duikers with the following explanatory variables: (a) vegetation types, distance from the nearest village and standard deviation (SDV) of elevation and (b) distance from the nearest village and SDV of elevation. All the models within 2 AIC units of the best model are shown

Adopted factors	AIC	ΔAIC	Akaike weight (%)
(a) Blue duiker			
(i) SDV of elevation-, Distance from the nearest village +	22.3	0.00	77.0
Ogilby’s duiker			
(i) Vegetation+	29.2	0.00	40.5
(ii) SDV of elevation-, Distance from the nearest village +	30.5	1.32	20.9
Yellow-backed duiker			
(i) Vegetation+	29.6	0.00	61.1
(b) Blue duiker			
(i) SDV of elevation-, Distance from the nearest village +	22.3	0.00	81.2
Ogilby’s duiker			
(i) SDV of elevation-, Distance from the nearest village +	30.5	0.00	66.3
(ii) Distance from the nearest village +	31.9	1.45	32.1
Yellow-backed duiker			
(i) SDV of elevation-	35.1	0.00	58.0

Table 3–1: Characteristics of microsatellite markers of blue duiker

Locus	Accession no.	Primer sequence (5'-3')	Repeat motif	T _a	Size range (bp)	N _A	H _O	H _E
CM01	AB775345	F: HEX-CCTCCTTCCCAACTCCCAG R: AGAGCCCACATTCCTAGCC	(AC) ₁₀	57	154-178	8	0.833	0.832
CM04	AB775346	F: FAM-GGTCATTCTTCTGGCTTGAG R: GCTTCCTTCCCAATTTGCTAAAC	(GT) ₁₀	57	296-344	14	0.722	0.886
CM10	AB775347	F: HEX-AGGGAGTGGTTGGCAGATG R: CGCTCACTGTAGGGAAAGG	(AC) ₁₁	57	244-274	12	0.778	0.886
CM14	AB7753458	F: NED-CTTACCCGCCAGAGTTTGC R: TGGCCCTGACTCTGTTGAC	(GT) ₁₁	57	247-274	11	0.833	0.886
CM15	AB775349	F: HEX-CAGGGTGGTATGCCGGAAG R: CCTCACAGCTGCTTAGTGC	(AC) ₁₁	57	317-349	11	0.833	0.869
CM24	AB775350	F: HEX-GTGGACTTGTTGCAGTGGC R: ACAGTCGGGACTTCCTTGG	(AGC) ₈	57	223-231	4	0.833	0.625
CM26	AB775351	F: FAM-CAACGTCACCTCAGAAGCC R: TGACCCTCAAACCAGCCTC	(AG) ₁₁	57	220-234	6	0.556	0.722
CM38	AB775352	F: FAM-TGCAGGAGATGTGGGTTCC R: GCAAGGAGAGCTTACCATGAG	(AC) ₁₁	57	237-251	7	0.333*	0.779
CM44	AB775353	F: HEX-AGATGCTGAGAACTCTGTGG R: CGAGGCCAGTACATGGTTAATG	(AC) ₁₀	57	279-309	12	0.722	0.830

T_a, optimal annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity

* Significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction ($p < 0.05$)

Table 3–2: Cross-amplification of nine developed microsatellite loci in bay, Ogilby’s, and yellow-backed duikers

Locus	Bay duiker			Ogilby’s duiker			Yellow-backed duiker		
	<i>N</i>	<i>N_A</i>	Size range	<i>N</i>	<i>N_A</i>	Size range	<i>N</i>	<i>N_A</i>	Size range
CM01	3	3	165-169	2	3	162-170	7	5	161-176
CM04	3	3	292-314	2	2	289-292	7	8	287-323
CM10	3	4	264-277	2	2	247-264	7	6	252-276
CM14	3	4	246-269	2	3	240-263	7	4	240-262
CM15	3	5	340-354	2	3	336-342	7	7	332-350
CM24	3	2	221-223	2	2	225-231	7	4	221-228
CM26	3	4	216-225	2	1	221	7	5	219-230
CM38	3	5	208-237	2	2	221-223	7	4	222-228
CM44	3	4	263-278	2	2	263-265	7	4	260-272

N, number of samples; *N_A*, number of alleles

Table 3–3: Number of individuals of each duiker species in each transect. The number of faecal samples of which genotype at least 8 microsatellite loci was obtained is shown in brackets.

Transect	Number of individuals from each duiker species		
	Blue duiker	Yellow-backed duiker	Bay duiker
YS1	6 (6)	1 (1)	1 (1)
YS2	5 (7)	2 (2)	0
YS3	7 (9)	1 (1)	0
OS1	9 (10)	1 (2)	1 (1)
OS2	6 (9)	1 (1)	1 (1)
OS3	10 (10)	1 (1)	1 (1)
OS4	6 (6)	0	1 (1)
S1	7 (10)	2 (6)	0
S2	6 (6)	2 (6)*	1 (1)
S3	6 (10)	2 (3)*	0
M1	5 (8)	1 (1)	0
M2	4 (4)	1 (1)	0
M3	5 (5)	1 (1)	0
Total	82 (100)	15 (26)	6 (6)

*same individual of yellow-backed duiker was found in those transects

Table 3–4: Mitochondrial D-loop diversity in forest duikers in African forest

Species	No of individuals	No. of haplotype	Haplotype diversity	Nucleotide diversity	Haplotypic Richness	Study site	Source
Blue duiker	82	44	0.998	0.054	14.72	Moukalaba, Gabon	This study
Yellow-backed duiker	15	7	0.813	0.038	6.98		
Bay duiker	6	2	0.757	0.035	4.64		
Blue duiker	48	13	0.905	0.026	12.00	Udzungwa, Tanzania	Bowkett et al. 2014
Abbott's duiker	73	6	0.381	0.006	4.27		

Table 3–5: Microsatellite diversity indices for each of 11 loci in blue duiker (n=82), yellow-backed duiker (n=15) and bay duiker (n=6). N , number of samples successfully analyzed; N_A , number of alleles; H_O , observed heterozygosity; H_E expected heterozygosity; A_R allelic richness. Mean number of alleles (allelic richness) rarefied based on minimal sample size of four individuals

Locus	Blue duiker					Yellow-backed duiker					Bay duiker				
	N	N_A	H_O	H_E	A_R	N	N_A	H_O	H_E	A_R	N	N_A	H_O	H_E	A_R
CM01	75	15	0.79	0.89	5.68	13	5	0.77	0.63	3.14	5	5	0.60	0.72	4.40
CM04	72	26	0.90	0.93	6.52	15	9	0.53	0.62	3.90	6	7	0.83	0.82	5.54
CM10	67	18	0.64	0.90	5.95	12	8	0.58	0.77	4.50	6	6	0.66	0.75	4.82
CM14	77	19	0.77	0.90	5.92	14	6	0.64	0.62	3.57	6	7	0.67	0.79	5.31
CM15	69	13	0.76	0.86	5.25	13	11	0.92	0.88	6.08	4	3	0.25	0.60	3.00
CM24	81	8	0.51	0.54	3.07	14	4	0.36	0.50	2.84	6	2	0.00	0.28	1.91
CM26	79	10	0.72	0.81	4.57	15	6	0.80	0.74	4.00	6	3	0.50	0.49	2.65
CM44	72	17	0.66	0.89	5.69	15	6	0.73	0.70	3.80	6	3	0.17	0.29	2.33
BM1225	79	13	0.67	0.76	4.26	13	9	0.85	0.80	4.90	6	5	0.50	0.68	4.15
BM2113	78	16	0.91	0.92	6.09	12	5	0.42	0.66	3.38	6	6	0.67	0.75	4.66
MM12	82	8	0.43	0.69	3.50	11	6	0.55	0.71	3.86	6	4	0.50	0.52	3.24
Average	75.4	14.8	0.71	0.83	5.14	13.3	6.8	0.65	0.69	4.00	5.7	4.6	0.49	0.61	3.82

Table 3–6: Relationships between nucleic types and mitochondrial haplogroups in blue duikers

Nucleic types	Number of individuals	Mitochondrial haplogroups	Transect
I	17	A2	M2, M3, OS3, OS4
I	1	A1	OS3
II	35	A1	M1, M2, OS1, OS3, OS4, S1, S2, S3, YS1, YS2, YS3
II	26	B	M1, OS1, OS2, S1, S2, S3, YS1, YS3
Admixture (I: 0.79, II: 0.21)	1	A1	M3
Admixture (I: 0.45, II: 0.55)	1	A1	M2
Admixture (I: 0.29, II: 0.71)	1	B	OS2