Socio-genetics and population structure of two African colobus monkeys in Cantanhez National Park, Guinea Bissau

By

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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“O que dá o verdadeiro sentido ao encontro é a busca,
e é preciso andar muito para se alcançar o que está perto.”

“What gives true meaning to the achievement is the act of seeking, and
we need to walk far to get what is close”

José Saramago

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SUMMARY

This thesis tested hypotheses related to the dispersal, behaviour patterns and response to forest fragmentation of two endangered colobus monkey species living in sympatry in Cantanhez National Park, Guinea Bissau. Western black-and-white colobus (BWC: *Colobus polykomos*) and Temminck's red colobus (TRC: *Procolobus badius temminckii*) are two forest dwelling primates that share most of their ecological requirements but exhibit contrasting social systems, namely in dispersal, group size and social organization. By combining behavioural data obtained for one social group of each species and non-invasive genetic data (15 microsatellite loci and a fragment of the mitochondrial control region) from eight black and white colobus and six red colobus social groups, I examined: i) historical and current dispersal patterns; ii) the within-group distribution of social interactions among males and females, and iii) the effect of forest fragmentation on genetic structure. I found evidence for historical and/or long-range dispersal via males in BWC and via females in TRC. However, a change in the current dispersal pattern was detected for BWC, as both sexes seem to be dispersing. Behavioural analysis showed that TRC females exhibit stronger social bonding than BWC females. More interestingly, and contrary to what was described for the species, TRC females seemed to prefer to engage in grooming other females rather than males and males only rarely groomed other males. Finally, analysis of genetic structure indicates the existence of only one genetic unit for each species, although some fine-scale spatial genetic structure was found for TRC. Whereas BWC seemed to be able to use the available forest corridors to disperse between forest patches, TRC females tend to disperse to immediately adjacent groups showing some constraint in the ability to disperse throughout the park. I hypothesise that the detected changes in dispersal mode in BWC and social dynamics in TRC may constitute behavioural local responses to habitat degradation. Constraints in dispersal found for TRC support the evidence that forest fragmentation should be playing an important role shaping these colobus monkey social systems.

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CHAPTER 1

General Introduction

1.1 Studying primate societies

A number of primate societies have been subject of observational studies for many decades. These studies have focused on the behaviour, ecology and social organization of primates in their natural environment and have transformed our understanding of their social systems and evolution. However, even the most complete long-term studies were not able to fully elucidate certain features of social systems. For many primate species, long-term studies on mating systems, dispersal patterns and within-group relatedness are very difficult to conduct, especially for long-lived, slow reproducing species and for those arboreal species, which are not easy to follow (Di Fiore et al., 2009; Goossens & Bruford, 2009). Consequently, observational studies have been deficient in explaining the effect of kinship on shaping patterns of social behaviour or to examine the link between individual behaviour (e. g. dominance interactions, alternative mating tactics) and reproductive success. These aspects of primate societies are extremely important since some of the fundamental models that aim to explain the evolution of primate sociality make assumptions of male-male and/or female-female kinship as a starting point to understand the evolutionary consequences of cooperative and competitive behaviours (Di Fiore, 2003).

Testing these sociobiological hypotheses has taken years of painstaking fieldwork but the relationship between observed social interactions and genetic data has rarely been established. This lack of knowledge in primate studies started to change in the 1990s with development of the non-invasive genotyping. From this point, hypotheses based on decades of field observations were now possible to test within months (Woodruff, 2004).

Before non-invasive DNA sampling, genetic studies of wild non-human primates were very rare compared with other groups of animals. This was mainly due to the fact that genetic material collected invasively (blood, tissue) was, logistically and ethically, almost impossible for many populations. Regarding this, non-invasive genetic analysis using new, high precision, molecular tools is therefore a great development in primatology, allowing the exploitation of previously inaccessible information (e.g. kinship, reproductive success, dispersal patterns) in wild populations (e.g. Goossens et al., 2003; Hammond et al., 2006; Blair & Melnick, 2011). It is now possible to combine long-term observational data with molecular information that enable primatologists to understand the links between behaviour and social structure on the one hand, and population genetic structure on the other, thus allowing the inference of features of primate social systems such as dispersal patterns, reproductive

skew and patterns of kinship (e.g. Ross, 2001). Primatologists can now use genetic data to infer information about individual behaviours and the features of primate social organization that may have given rise to them. Behaviours such as dispersal and mating strategies that are very difficult to observe and of great interest for behavioural ecologists, can now be accessed by non-invasive molecular analysis (Di Fiore, 2003). Besides parameters related to behavioural patterns, genetic data also provide primatologists with information about effective population size, detection of changes in this parameter and dating of such changes, sex ratio, population structure, inbreeding and outbreeding coefficients, home ranges of individuals, gene flow and population assignment (Woodruff, 2004). Gathering all this information, primatologists are able to design effective conservation programmes to manage and conserve endangered primate populations (Di Fiore, 2003; Goossens et al., 2006).

Nevertheless, working with these kinds of samples remains extremely challenging, time-consuming, expensive and prone to error. The DNA present in faeces or hair is in much lower quantity and quality than in other fresh tissue, leading very often to genotyping errors (e.g. "allelic dropout", false alleles) (e.g. Taberlet et al., 1996; Gagnaux et al., 1997; Goossens et al., 1998; Goossens et al., 2000). In addition, because this DNA is highly fragmented, only small fragments can be analysed, e.g. microsatellites and short mitochondrial DNA sequences: the most widely used molecular markers in molecular primatology. The polymerase chain reaction (PCR), the procedure used to amplify such poor sources of DNA, may be inhibited by co-extracted compounds present in faecal material. As a result, non-invasive DNA analysis requires the application of the rigorous standards for replication of results (Taberlet et al., 1996) with the consequent disadvantage of becoming very costly and time consuming.

1.2 Molecular markers

1.2.1. Mitochondrial DNA

Mitochondrial DNA is found in the form of a small, circular plasmid genome and exists in many copies (up to hundreds) per cell (reviewed by Goossens et al., 2003). The latter characteristic of this marker makes it one of the most used in non-invasive genotyping, since it is present in much higher quantities than nuclear DNA, thus being relatively easy to obtain even in degraded samples. It is present in all individuals but is maternally transmitted, with lack of recombination, is haploid

and features a relatively high evolutionary rate. These characteristics make this molecular maker suitable for studies of phylogenetics, phylogeography and for behavioural studies of matrineality, as patterns of maternal relatedness and sex-specific population structure (e.g. Avise et al., 1987; Avise, 1989; Hashimoto et al., 1996). Its effective population size is approximately ¼ of that of nuclear genes and thus mtDNA haplotypes are more sensitive to genetic drift and fixation than the nuclear ones (Avise et al., 1987).

Mammalian mtDNA comprises 35 genes and the control region. It is the genetic marker of choice to resolve higher taxonomic level relationships (e.g. systematics). The control region is the most variable portion of the mtDNA, thus is the most suitable for the study of individuals and groups below the species level (Morin & Goldberg, 2004).

The mitochondrial genome has been the most widely used molecular marker in nonhuman primate studies and the entire molecule is now available for more than 30 species, including one *Colobus* and one *Procolobus* species (Raaum et al., 2005; Sterner et al., 2006).

Despite of all the above advantages of mtDNA, this molecule has also some limitations to its use. The fact that the entire molecule is inherited intact from the mother does not provide any information about paternity. Another limitation is that in most species, fragments of the mitochondrial genome have been incorporated into the nuclear genome. When nuclear mitochondrial inserts (also known as numts) are amplified instead of the actual mtDNA, incorrect relationships can be inferred. Cloning of PCR products and subsequent sequencing of multiple clones for each product can sometimes reveal the presence of both true mtDNA sequences and numts in a sample and thus facilitates the identification of the latter (Morin & Goldberg, 2004). Typically, mtDNA sequences used in non-invasive genotyping are between 300 and 400bp long. Consequently, even sequencing errors of less than 1% could lead to false exclusions of matrilineality. Methods that take such errors into account have not yet been developed (Goldberg & Wrangham, 1997).

1.2.2. Microsatellites

Microsatellites (also called simple sequence repeats [SSR] and short tandem repeats [STR]) are mainly present in nuclear chromosomes in thousands of copies scattered throughout the genome (Goossens et al., 2003). They are short 70 to 200bp sequences of tandem repeats of a repetitive motif of 2 to 6bp and their variation is due to changes in the number of repeats. STRs are highly variable with

each *locus* containing five to ten or more alleles segregating in a single population. This extraordinary variability makes microsatellites ideal Mendelian markers (i.e. co-dominant) for analysis at a species and population level, such as sociobiological studies (Woodruff, 2004).

The discovery of this type of molecular marker constituted one of the major steps forward in the analysis of the individual genetic relationships in a variety of animal species (Queller et al., 1993). In addition to the aforementioned characteristics, they are also randomly distributed throughout the genome, commonly occurring in noncoding regions and are typically selectively neutral, making them ideal markers for population level studies and the marker of choice in the analysis of population structure and dispersal patterns, assessment of parentage and individual identity, and estimation of degree of relatedness between populations or pairs of individuals (Weber & Wong, 1993).

There are thousands of known microsatellites in the human genome. For the majority of studies of nonhuman primates it is more practical to screen previously characterized human markers than to generate species' specific microsatellites using genomic libraries (Morin et al., 1998). This approach has resulted in a discovery of a considerable number of highly variable microsatellites in a variety of Old World Monkey species (e.g. Morin et al., 1994; Coote & Bruford, 1996; Goossens et al., 2000; Zhang et al., 2001).

Despite of all the cited advantages, the use of these molecular markers can raise some significant problems, which researchers should be aware of. "Null alleles" (non-amplification of some alleles in the population) can occur when there are nucleotide mismatches in the primer biding sites causing PCR failure for one allele. This phenomenon can cause incorrect assignment of homozygous genotypes to heterozygous individuals (Callen et al., 1993), but it is possible to detect this error by using appropriate software (e.g. Micro-Checker). Statistical tests for Hardy-Weinberg equilibrium (HWE) or verification of Mendelian heritability in known families can also help to identify the presence of null alleles within a data set (Brookfield, 1996).

"Allelic dropout" produces the same effect as null alleles (false homozygous) but for a different reason. It results from the failure of the PCR to amplify one or more alleles as a consequence of the poor quality and/or quantity of the template DNA. This phenomenon is very frequent with non-invasive samples, such as faeces, hair and food wadges (partly eaten food left by the animal and which contains its saliva). In order to avoid incorrect genotyping, prior evaluation of the DNA concentration and integrity and subsequent replication of the PCRs should be made (Taberlet et al., 1996; Goossens et al., 1998; Goossens et al., 2000, Morin et al., 2001).

PCR artefacts, such as "false alleles" are also possible in studies using non-invasive sampling, resulting in the false assignment of heterozygous genotypes to homozygous individuals. Falsely generated alleles due to PCR artefacts are a consequence of heterospecific DNA present in the sample that is extracted along with the DNA of the target species (Morin & Goldberg, 2004).

Nevertheless, applying appropriate measures to control for such genotyping errors allows researchers to obtain very reliable results when using non-invasive samples (Taberlet et al., 1996; Goossens et al., 2000; Morin et al., 2001; Roon et al., 2005; Arandjelovic et al., 2009).

The high evolutionary rates of STRs occasionally result in new mutations arising between generations. In a study of kinship this can result in a false exclusion of an ancestor (e.g parent). Statistical analysis in maximum likelihood statistics (e.g. Goodnight & Queller, 1999) can correct for both mutation and user errors in the genotype assignment (Balloux & Lugon-Moulin, 2002).

1.3 Molecular studies of primate societies

Non-invasive genetic analysis was a big step forward in the study of primate societies, having today a major role when applied to long-term observational studies of nonhuman primates (Morin et al., 1993; Altmann et al., 1996; Constable et al., 2001; Goossens et al., 2006). Below, I will focus on some of the most remarkable studies that used a molecular approach to investigate features of the social systems, such as a) within-group relatedness, b) dispersal patterns and c) effect of habitat fragmentation on genetic structure.

1.3.1 Within-group relatedness

The assumption that individuals should preferentially address their affiliative and cooperative behaviour to more closely related counterparts constitutes a starting point for many of the models that try to explain the evolution of primate social systems, since cooperating with kin increases the inclusive fitness of the individual (e.g. Hamilton, 1964a, b; Gouzoules & Gouzoules, 1987). Nevertheless, testing the hypothesis that greater indices of affiliative and cooperative interactions reflect closer kin between individuals was only possible for the maternal side and restricted to long-term behavioural studies. The development of molecular techniques for quantifying relatedness accurately allowed to extent such studies to a wider range of

primate populations (Queller & Goodnight, 1989). Highly variable molecular markers such as microsatellites provide the possibility for researchers to estimate pairwise relatedness in wild populations between individuals of unknown relationship (Blouin et al., 1996; Csilléry et al., 2006). For example, long-term behavioural studies on chimpanzee communities have demonstrated the existence of strong affiliative and cooperative bonds among males (Goodall, 1986; Boesch, 1996). The most plausible explanation for these interactions was the fact that these behaviours evolved as a consequence of kin selection, and the fact that males were more closely related as a consequence of male philopatry (Di Fiore, 2003). Two studies using a molecular approach to evaluate within-community relatedness of chimpanzees at Gombe (e.g. Gagneux et al., 1999; Vigilant et al., 2001) have found no significant difference between male-male and female-female relatedness. These results suggest that within-community male chimpanzees are not more closely related to each other than are females. This same trend was also found for chimpanzee communities in Tai National Park (Ivory Coast) and Budongo Forest Reserve (Uganda) (Lukas et al., 2005). Thus, the affiliative and cooperative behaviours observed among males do not seem to have arisen as a direct result of kin selection, suggesting the existence of other evolutionary mechanism (e.g. mutualism, reciprocal altruism) that may be responsible for the observed behaviours in these chimpanzees. Many observational studies with cercopithecine species have documented higher rates of affiliative and cooperative behaviours among females belonging to the same matriline. Altmann et al. (1996), using a molecular approach supported this assumption for savanna baboons from Amboseli, Kenya, finding that females were more closely related to each other than males.

The coefficient of relatedness can be defined as the probability that two individuals share an allele that is identical by descent (IBD) (Kalinowski et al., 2006). There are several coefficients that estimate the IBD probabilities using either linear regression (e.g. Queller & Goodnight, 1989; Lynch & Ritland, 1999; Wang 2002) or maximum-likelihood (e.g. Kalinowski et al., 2006) based methods. Most these estimators range from -1 to 1 where positive values indicate that individuals are more related than expected by chance and negative values indicate that the dyad is less related than expected by chance. Half-siblings are expected to share 0.25 of their genome, a probability that rises to 0.5 for full-siblings and parent-offspring dyads and to 1 for completely identical individuals (identical twins) (Queller & Goodnight, 1989). Recently, Ducan and collaborators (2010) estimated pairwise relatedness coefficients in a population of the spider *Anelosimus studiosus* to investigate whether cooperating individuals were more related to each other than by chance. They found

that females sharing the same webs were related on average at the levels of half-siblings (0.25), a value significantly higher than random pairs from the population. Regardless of the above and some other examples, studies addressing this problem are still scarce, even though kin selection is still one of the major assumptions underlying sociobiological theories of primate social relationships. The recent development and constant improvement of non-invasive genotyping techniques provide primatologists with the possibility of combining genetic analysis with classic long-term observational data. This will allow researchers to address key sociological questions in several primate taxa, which social systems are still not understood.

1.3.2 Dispersal patterns

Some of the first molecular studies on wild nonhuman primates focused on the effect of sex-biased dispersal on the distribution of genetic diversity within and between groups. There is a contrasting pattern of mitochondrial *vs* nuclear genetic markers when females are philopatric. Species where males mediate dispersal are expected to exhibit a population genetic substructuring to their mitochondrial genes. In contrast, little or no genetic substructuring is expected for autosomal and Y-linked genes since males are providing gene flow when leaving their natal groups to breed in other social units. Conversely, species characterized mainly by female dispersal, are expected to show similar low mitochondrial and autosomal genetic substructuring, since females homogenize both these genomes when dispersing across social groups. In contrast, there is an expected population genetic structuring in the Y-linked genes as a consequence of male philopatry. When both sexes disperse, no pattern of genetic substructuring is expected for either genome (Avise, 1995, 2000; Lawson Handley & Perrin, 2007). When analysing dispersal patterns, one needs to be aware of the fact that pre- (juveniles) and post-dispersing individuals (adults) should be analysed separately. Whereas analysing both age cohorts can give us valuable information on the dispersal rates through the contrast between adults and juveniles, the inclusion of pre-dispersers in the sample can mask the sex bias in the dispersal (Lawson Handley & Perrin, 2007). Genetic methods to access the direction of the sex-bias in dispersal have been classified in those that measure sex-biased gene-flow, by using sex-specific markers such as the maternally inherited mtDNA or a region of the paternally inherited Y chromosome, and the ones that measure instantaneous dispersal, that uses recombining biparental markers such as the autosomal microsatellites (Prugnolle & de Meeûs, 2002). Historical patterns of sex-biased dispersal can be measured through the distribution of haplotypes in

and among populations or by comparing genetic differentiation (F_{st}) in males and females for the maternal and paternal inherited markers (Lawson Handley & Perrin, 2007). For example, Melnick and Hoelzer (1993, 1996) have studied several species of macaques (rhesus macaques: *Macaca mulatta*; japanese macaques: *M. fuscata*; long-tailed macaques: *M. fascicularis*; pigtailed macaques: *M. nemestrina* and toque macaques: *M. sinica*) and found the pattern of genetic structuring typical of female philopatry. Using both allozymes and RFLP they found a very high within population diversity for nuclear genes. For all these species, local populations contained between 50 to 90% of the total genetic diversity present within the species. On the contrary, mtDNA showed very little variation within social groups and local populations. For example, in rhesus macaques, only 9% of the mtDNA variation was attributed to variation within local populations, while the remaining 91% was attributed to differences between local populations, highlighting the strong genetic substructuring in this genome supporting the strong female philopatry in these species. Long-term observational studies focusing on wild populations of common chimpanzees (*Pan troglodytes*), have documented male philopatry and female dispersal in this species (e.g. Goodall, 1986; Boesch & Boesch-Achermann, 2000). Morin et al. (1994) confirmed these observations when in his study of the Kasakela community at Gombe he found 15 mitochondrial haplotypes from just 19 individuals. Goldberg and Ruvolo (1997) sampled multiple populations of eastern chimpanzees (*Pan troglodytes schweinfurthii*) across its geographic range and found that about 80% of the mtDNA variation of the subspecies was also present within local populations. These patterns of mitochondrial diversity found within chimpanzee communities contrasts with the ones described above for the *Macaca spp.* indicating the adoption of different dispersal strategies. A study on wild hamadryas baboons (*Papio hamadryas hamadryas*) (Hammond et al., 2006) using mtDNA, autosomal and Y-linked markers provided evidence for female-biased dispersal in this species, since genetic substructuring (F_{st}) was much higher for the Y chromosome than for either the autosomal or the maternally inherited mtDNA. These genetic data suggest a stronger female dispersal, helping to solve the conflict raised by observational data that suggested dispersal by both males and females.

The direction of instantaneous sex-biased dispersal can be accessed using microsatellite loci and by the population levels of genetic structure of females and males, such as F_{st}, F_{is} and relatedness (r) (Weir & Cockerham 1984; Whitlock & McCauley 1999; Goudet et al., 2002), or through the probability of an individuals to be natal from the population where it was sampled by calculating the mean (mAIc) and variance (vAIc) of the corrected assignment index (Paetkau et al., 1995; Favre et

al., 1997; Waser & Strobeck 1998; Goudet et al., 2002). The dispersing sex is expected to exhibit negative values of mAIc, as immigrants have lower probability of belonging to the population, and higher value of vAIc, as the dispersing sex will include natal and dispersing individuals (Goudet et al., 2002). Blair and Melnick (2011), using microsatellite data from a population of Central American squirrel monkey (*Saimiri oerstedii citrinellus*) in Costa Rica, found evidence that dispersal is mediated by both sexes as no differences were found in population differentiation (F_{st}) and population assignment (mAIc) between males and females. Radespiel et al (2003) used seven microsatellite *loci* to estimate relatedness coefficients among grey mouse lemur (*Microcebus murinus*) and showed dispersal biased towards males in this species. Males were found at greater geographic distances from other closely related males and their potential mothers, than females. Harris and colleagues (2009) conducted the only study so far where dispersal patterns were analysed for African colobus monkeys. By using multilocus genotypes of *Colobus guereza*, within- and between-group relatedness was calculated for both sexes, suggesting a more extensive male dispersal, although some highly related female dyads were also found among neighbouring social groups. However, the ability of these methods to detect the sex bias in dispersal is highly dependent on the strength of the bias and the rate of the dispersal (Goudet et al., 2002). In cases where only one sex disperses, all measures perform well detecting the pattern. However, when the bias becomes less strong towards one sex, all measures loose power, although F_{st} and mAIc still perform well when the bias drops to 80:20 (Lawson Handley & Perrin, 2007). Also, it is important to keep in mind that the ability for these measures to perform well is highly dependent on the number of individuals *per* population, number of populations and number of *loci*. Goudet and colleagues (2002) conducted simulation experiments to evaluate how the power of these measures detecting sex-biased dispersal is affected and found that it is better to increase the number of individuals for each population than the number of populations and that results are not significantly improved when using more than 20 polymorphic microsatellites.

Individual-based assignment tests based on likelihood (e.g. Waser & Strobeck, 1998) or Bayesian methods (e.g. Pritchard et al., 2000) offer an alternative to the above mentioned summary statistics and are proving to be a powerful approach to identify immigrants and therefore to test for sex-biased dispersal if males and females are considered separately (Lawson Handley & Perrin 2007). Bayesian (as implemented in STRUCTURE software, Pritchard et al., 2000) and partial Bayesian methods (e.g. implemented in GENECLASS, Cornuet et al., 1999) have some advantages over F-statistics, relatedness and population-based assignment tests as

they do not average over the population, allow to immediately identify the immigrant individuals and can incorporate geographical information (Manel et al., 2005). Berry and colleagues (2004) tested the power of individual-based assignment tests by comparing Bayesian and partial Bayesian analysis of microsatellite data with long-term data on mark-recapture of grand skink. They found that dispersal estimates were very similar using the two approaches, therefore illustrating the power of such methods to elucidate dispersal patterns.

1.3.3 Effect of habitat fragmentation on population structure

Habitat fragmentation can be defined as the process that leads to transformation of a previously large continuous area of habitat into a number of smaller patches isolated from each other by habitats different from the initial state (Wilcove et al., 1986). Such modification can have negative effects on populations that, due to this transformation, become themselves fragmented, split into several units, isolated from each other and usually of smaller size than the original (Frankham et al., 2002; Frankham, 2006). Although natural processes can cause population fragmentation and structure, habitat modification resulting from human activities occurs at such rapid pace that most natural populations are likely to be negatively affected (Gerlach & Musolf, 2000; Epps et al., 2005). However, whether habitat fragmentation affects a certain species is highly dependent on the species biology, the degree of habitat fragmentation and the matrix of habitat between patches. Species that are capable of maintaining high dispersal rates despite habitat fragmentation (e.g. several terrestrial large bodied mammals) are most likely not to be affected (Kareiva, 1987; Debinski & Holt, 2000; Villard, 2002). On the other hand, less mobile species or the ones highly dependent of their habitat (e.g. arboreal, forest-dependent species), most likely will experience fragmentation of their populations as a result of the suppression of dispersal between patches (e.g. Goossens et al., 2006; Craul et al., 2009; Liu et al., 2009).

The partitioning of genetic diversity among populations within a species is known as population structure and is a consequence of the mating system, genetic drift and gene flow. While genetic drift causes stochastic loss of genetic diversity in local populations and consequently increases the genetic differentiation among populations, gene flow is responsible for the introduction of novel genetic information and diminishes the differentiation among populations (Templeton et al., 2001). Habitat loss and habitat fragmentation are two processes of habitat transformation that have different but reinforcing effects on the population genetic structure (de

Jong et al., 1994): habitat loss lowers the effective population size (Ne), resulting in loss of heterozygosity and genetic diversity due to genetic drift and inbreeding; habitat fragmentation reduces gene flow among sub-populations, increasing genetic drift and local adaptation (Frankham et al., 2002; Frankham, 2006). The amount of genetic diversity that is lost in a fragmented population is a result of the degree of isolation between fragments that depends on: the spatial distribution of fragments in the landscape, habitat type between fragments, capacity of the species to disperse and consequent dispersal rates, time since modification, distance between fragments, number of fragments, the distribution of population sizes among fragments and historical genetic structure resultant from natural barriers (Frankham et al., 2002; Frankham, 2006).

Population genetic structure can be accessed trough classical population genetic methods such as F-statistics (Wright, 1931; Wright, 1943) or *via* landscape genetics where the individual is the study unit (Manel *et al.* 2003).

1.3.3.1 Landscape Genetics

Studies that wish to analyze population structure due to habitat modification should take the spatial features of the habitat into account (Epperson & Li, 1996). Such approach is called landscape genetics, which combines molecular population genetics with landscape ecology in order to explain the distribution of genetic diversity accordingly with the landscape features (Manel et al., 2003; Storfer et al., 2007). Unlike classical population genetics, landscape genetics does not require previous knowledge of discrete populations and the sample unit is the individual and not the population (Manel et al., 2003). The great interest of applying such approach to understand the effect of habitat modification on the genetic structure is that landscape genetics allow to identify discontinuities in the habitat that are responsible for disruptions in the genetic patterns (Manel et al., 2003). The identification of such disruptions allow conservation managers to understand the species connectivity and make decisions on the most important habitat to preserve the species genetic diversity (Safner et al., 2011). I will focus on three of the most widely used tools to identify genetic patterns in landscape genetics: a) isolation-by-distance and isolation-by-barrier, b) spatial autocorrelation and c) bayesian clustering approaches.

Isolation-by-distance (IBD) is the process where the genetic differentiation between individuals increases solely due to the geographic distance as a result of geographically restricted dispersal (Chesser, 2003). This analysis measures the

correlation between a matrix containing genetic distances between individuals and a matrix containing the dyadic geographic distances, usually using Mantel tests (Mantel, 1967; Smouse et al., 1986; Epperson, 2003). Under a scenario where there is equilibrium between genetic drift and gene flow, a positive correlation between the two matrices is expected, indicating IBD. However, the presence of barriers to gene flow in populations that otherwise would be panmictic can also give indication of a positive correlation that in this case is not due to the geographical distance (Guillot et al., 2009). Nevertheless, when the genetic drift is main factor shaping the population structure, as it should be the case in small or isolated populations, this correlation will be weak (with very large residuals) as it will be when gene flow is has a stronger influence (with small residuals) (Hutchison & Templeton, 1999). For example, the larger-bodied lemur *Lepilemur edwardsi*, was found to be negatively affected by forest fragmentation in northwestern Madagascar (Craul et al., 2009). This was evident from the fact that populations from forest fragments showed lower genetic diversity than the ones from more preserved areas. In addition, the distribution of genetic patterns could not be explained by isolation by distance model but instead, by stochastic genetic drift (Craul et al., 2009).

In some cases, more important than linear geographical distances, is the presence of suitable habitat gaps that can act as barriers to migration. To estimate the isolation-by-barrier is necessary to have information on the species' habitat preferences and ecological requirements as well as on its ability to disperse. Because the presence of habitat gaps might not be independent from the geographic distance, a partial Mantel test that controls for the effect of geographic distance is the method of choice for this type of analysis (Cushman & Landguth, 2010). Controlling for discontinuities in the habitat can be very important, especially for those arboreal primates highly dependent on the presence of continuous forest to disperse. In the case of the Yunnan snub-nosed monkey (*Rhinopithecus bieti*), a forest-dependent primate endemic to the Tibetan Plateau, a partial Mantel test showed that 36.23% of the genetic distance was explained by gaps of suitable habitat in the landscape and only 4.92% of the genetic distance was a result of the geographic distance (Liu et al., 2009).

Spatial autocorrelation methods have been widely used to compare relatedness of pairs of individuals with their geographical distances. It tests wether the genotype of an individual is dependent on the genotype of a second individuals in a neighbouring location (Manel et al., 2003). Any deviation of these relationships from zero means that individuals at that distance class are more (positive values) or less (negative values) related than expected at random, suggesting spatial genetic

structure. This analysis has proven to be robust as long as the spatial scale of the sampling is smaller than the spatial scale of the autocorrelation (Slatkin & Arter, 1991; Epperson & Li, 1996). In the spatial autocorrelation, the researcher defines classes of distances and as a consequence, the detection of the specific location of a genetic discontinuity (e.g. mountain, river) is not possible (Manel et al., 2003).

Bayesian clustering approaches use multilocus genotype to cluster individuals into populations that minimize Hardy-Weinberg (HW) and linkage disequilibrium (LD). They use a Markov chain Monte Carlo (MCMC) algorithm to assign an individual to a cluster where its posterior probability is highest. Any departure from a random matting system leads to the subdivision of the dataset into sub-populations that maximize the equilibrium (Dawson & Belkhir, 2001; Pritchard et al., 2000; Manel et al., 2003; Beaumont & Rannala, 2004). Since individuals are assigned to populations according to their genetic information, knowing the spatial coordinates of the samples allows identifying migrants and the distance of dispersal (Manel et al., 2003). Although extremely robust, these methods can lead to erroneous clustering of individuals when other factors besides population structure (e.g. small population sizes, bottleneck, inbreeding and admixture) cause disruption in HW and LD (Manel et al., 2003). There are several softwares that use the Bayesian clustering approach. Spatially explicit packages, such as BAPS (Corander et al., 2008), Geneland (Guillot et al., 2005) or TESS (Francois et al., 2006; Chen et al., 2007), allow the incorporation of geographical coordinates of individual samples and therefore have proven to be very useful in landscape genetics, as they allow the detection of genetic discontinuities in space. Recently, Safner et al. (2011) using both simulated and empirical data compared these three softwares concluding that all are very robust detecting genetic discontinuities. However, all fail to detect true genetic boundaries when there is a strong pattern of isolation-by-distance. Frantz et al. (2009) using both explicit and non-explicit spatial analysis (e.g. STRUCTURE, Pritchard et al., 2000) also found that the number of clusters identified in a population of European wild boar (*Sus scrofa*) was overestimated in the presence of high IBD levels.

1.4 The Cercopithecidae

The Old World Monkeys comprise the Cercopithecidae family which can be divided in two ecological and morphological distinct subfamilies: *Cercopithecinae* (cheek-pouched monkeys) and *Colobinae* (leaf-eating monkeys) (Delson, 1992; Groves, 1993, 2001). Fossil records provide evidence that the two subfamilies diverged from a common ancestor in the Miocene and from that period, each group

experienced a complex evolutionary history, involving several distinct radiations (Oates & Davies, 1994).

The colobines can be distinguished from their cercopithecine relatives by several derived morphological traits, such as a multi-chambered ruminant-like stomach, that are adaptations to the more folivorous diet (Strasser & Delson, 1987).

1.5 Phylogeny and Phylogeography of the *Colobinae* Subfamily

Taxonomically, the *Colobinae* subfamily comprises, in most classifications, 30 species. The extant colobines have been grouped into four to nine genera and based on both morphology and geographical distribution and have been subdivided into an African clade and an Asian clade. These two clades are thought to have diverged in the Miocene (about 12 Ma) (Delson, 1975). The Asian clade occurs throughout Southeast Asia, including China and the Indian subcontinent, occupying a variety of ecological niches ranging from arboreal niches in tropical rainforests to the harsh semi-terrestrial habitats in the Himalayan foothills (Bennet & Davies, 1994). It includes the "odd-nosed" group and the "langur and leaf monkey" group comprising four and three genera, respectively (Oates & Davies, 1994; Ting, 2008). They can be distinguished from their African relatives by a number of shared, derived features, including a shorter face and the presence of a suborbital fossa in the skull (Groves, 1989).

The first record of an African colobine fossil is in deposits dating to the late Miocene (9-8.5 Ma; Kingston et al., 2002). This group seems to be scarce in Africa until the Pliocene, at which time there was a radiation of these animals, most of which were large-bodied, adapted to terrestriality, and are classified as distinct genera. It is in the early Pleistocene deposits that forms morphologically similar to the extant colobine species appear for the first time (reviewed in Jablonski, 2002; Frost & Alemseged, 2007). Some authors have suggested that the early colobines were partly terrestrial, with the predominant arboreal condition of these primates having a recent origin (Leakey et al., 2003). However, a recent molecular study using two African colobine samples suggests that the radiation of the African clade began much earlier (Sterner et al., 2006). The details of the African colobines diversification remain unclear and very few extinct forms can be connected to the living ones (Ting, 2008).

The African colobines – the colobus monkeys, are represented by three distinct groups distributed across the African rainforest belt: the black-and-white colobus (genus *Colobus*), the red colobus (genus *Procolobus*, subgenus *Piliocolobus*) and olive

colobus (genus *Procolobus*, subgenus *Procolobus*) (Delson, 1975; Strasser & Delson, 1987). The extensive diversification within the genus level could be a consequence of both past arid climate conditions (glacial periods) and their great adaptation and dependence on forest trees. During glacial periods they would confine their distribution into glacial refuges, promoting their divergence and when climate conditions become more favourable they would radiate and adapt in different forest patches.

The red and olive colobus are both grouped in the genus *Procolobus* and share a set of anatomical characteristics that distinguish them from the black-and-white colobus. Female *Procolobus* have sexual swellings, male *Procolobus* have separate ischial callosities and a sagittal crest, and young males have a perineal organ; *Procolobus* have a four-chambered stomach while *Colobus* have a three-chambered arrangement; *Colobus* have a sub-hyoid sac (absent in *Procolobus*) and a large rather than small larynx (Napier, 1985; Strasser & Delson, 1987). In contrast to *Procolobus*, adult male *Colobus* make resonant, low-pitched loud calls and *Colobus* mothers allow other females of the group to handle their infants (Oates and Davies, 1994). A study by Ting (2008) using mtDNA sequences to explore the relationships of African colobines provides the first molecular data that supports the close relationship between red and olive colobus. He estimated that the African radiation started by the late Miocene with black-and-white colobus diverging from the other colobus monkeys by 7.5 Ma. The red and olive colobus share a sister taxon relationship and diverged from one another by 6.4 Ma (late Miocene) (Fig 1.1).

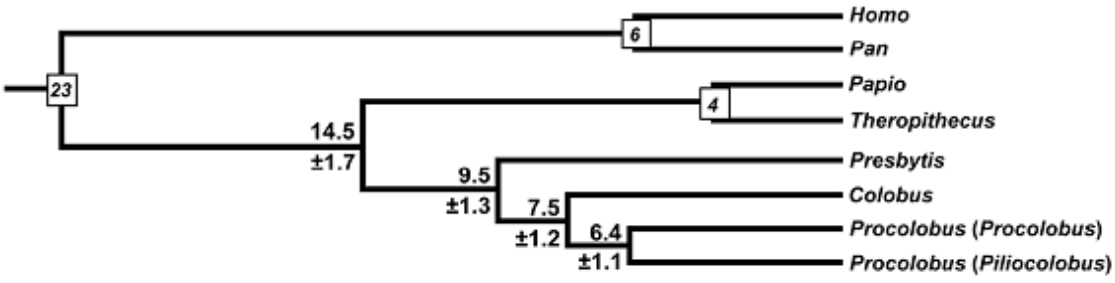

Figure 1.1 Catarrhine mitochondrial likelihood and Bayesian tree based on NADH3, NADH4, NADH4L, and NADH5 genes (3,831 base pairs). All nodes supported by bootstrap values _85 and posterior probabilities _0.90. Divergence date estimates (Ma) from penalized likelihood shown with two standard deviations. Calibration points are boxed and italicized. *Cebus* was the outgroup taxon. Classification follows Grubb et al. (2003). (Ting, 2008)

Many African colobines are among the world's most endangered primates. They are very well adapted to the forest canopy but this adaptive strategy has made them vulnerable to changes in their habitat. The main treats to their survival are human

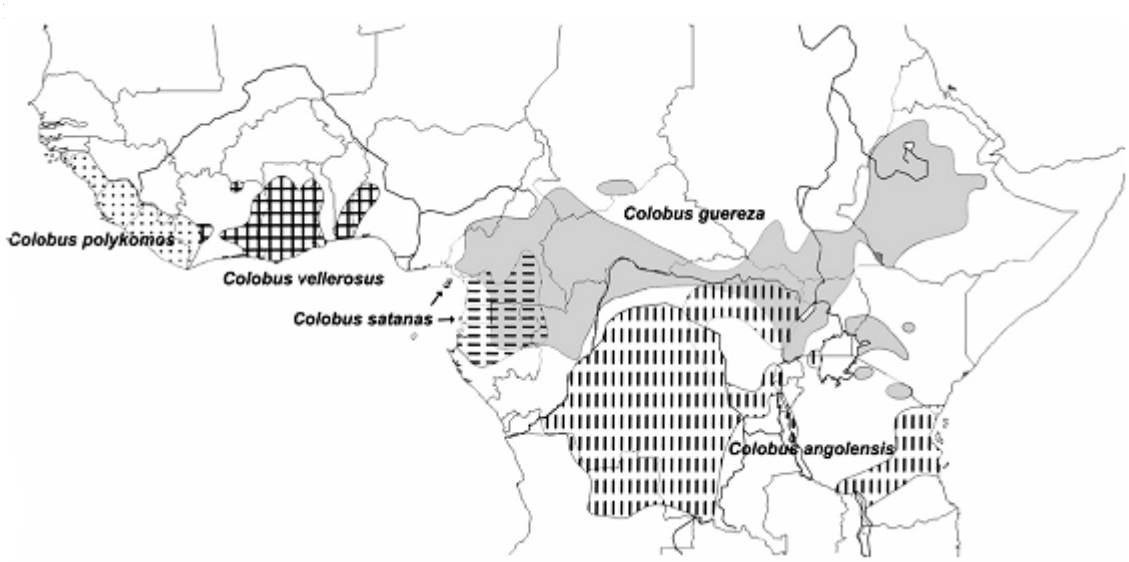

Figure 1.2 Distribution of black-and white colobus (*colobus*) species. Classification follows Grubb *et al.* (2003). Adapted from Oates and Trocco (1983) and Oates et al. (1994). (Ting, 2008).

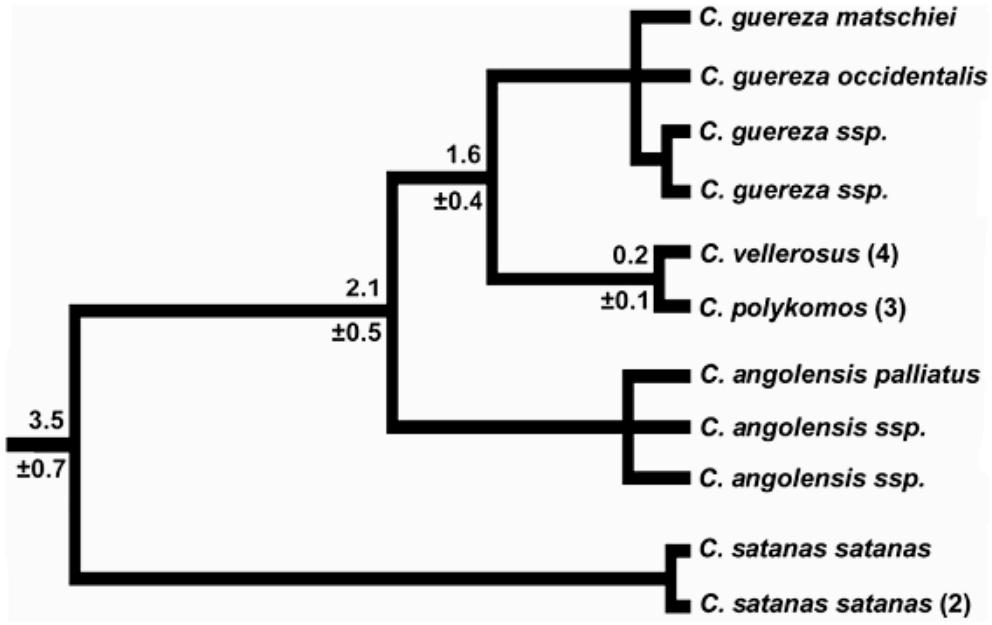

Figure 1.3 Black-and-white colobus (*Colobus*) mitochondrial likelihood and Bayesian tree. All nodes supported by bootstrap values _85 and posterior probabilities _0.90. Divergence date estimates (Ma) from penalized likelihood shown with two standard deviations. Numbers in parentheses indicate individuals with identical sequence that were not included in the analysis. Classification follows Grubb et al. (2003). (Ting, 2008)

1.6 Genus *Colobus*, black-and-white colobus

The *Colobus* genus has five recognized species with a continuous distribution throughout equatorial Africa (Fig 1.2). These species are *C. satanas*, *C. polykomos*, *C. vellerosus*, *C. guereza* and *C. angolensis* (Oates & Trocco, 1983; Grubb et al., 2003).

Each species exhibits a different coat pattern: *C. satanas* is completely black, *C. guereza* has a characteristic white peridorsal mantle and the other three species have different combinations of white or grey markings on the tail, thighs, shoulders and/or head (Oates & Davies, 1994). All newborns of this genus exhibits white pelage while the neonates of *C. satanas* are brown (Oates & Davies, 1994).

The analysis of mtDNA (Ting, 2008) showed that black-and-white colobus lineages started to diversify by the end of the Pliocene and the beginning of the Pleistocene with *C. satanas* being the first to diverge (3.5 Ma), followed by *C. angolensis* (2.1 Ma) and then by *C. guereza* (1.6 Ma), leaving *C. polykomos* and *C. vellerosus* as sister taxa that only diverged from each other by 200,000 years ago (Fig 1.3).

All black-and-white colobus groups studied so far seem to have young leaves and/or seeds as preferred food items. This diet is the most likely explanation for these animals being noted for their inactivity. At times of the year when these items are scarce, mature leaves substitute them as the main dietary item. Although this is the general pattern observed between the species, some seem to be more truly folivorous (as *C. guereza*) than others (*C. satanas*) which rely more on seeds (Oates & Davies, 1994).

In terms of social structure, these animals are known to typically have small group sizes, never exceeding 20 individuals. Most species appear to show a multi-male multi-female social organization, however in the *C. guereza* and *C. polykomos* single adult male groups seem to predominate (Oates & Davies, 1994).

1.7 Subgenus *Piliocolobus*, red colobus

As for the black-and white colobus, red colobus monkeys are distributed across Equatorial Africa from The Gambia to Zanzibar but in a more fragmented manner, being completely absent from large areas of the western equatorial forest, such as Gabon and mainland Equatorial Guinea (Fig 1.4) (Ting, 2008). This patchy distribution could be a consequence of both natural barriers (e.g. rivers) and great levels of forest destruction in Central Africa due to human activities.

The different forms of red colobus display different patterns of coat colour varying from black, brown, red and white hair (Oates & Davies, 1994).

Their classification is unstable with little consensus on the number of species that should be recognized. There are at least 18 forms within the genus but the relationships among them are one of the unsolved problems of African primate taxonomy. Classification at the subspecies level is relatively stable but the diversity that they exhibit exceeds what usually is observed within a single species (pelage,

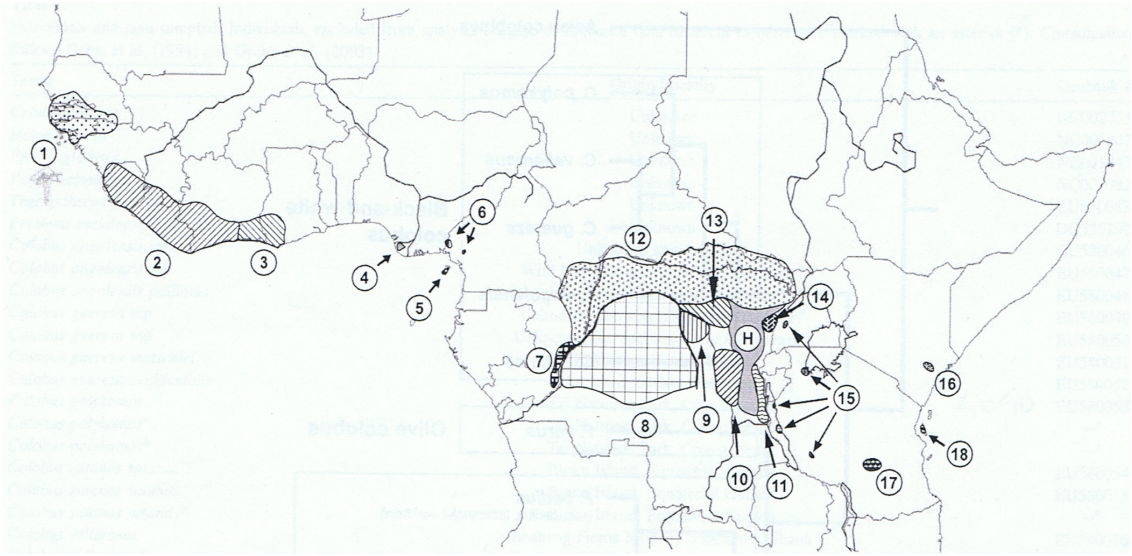

Figure 1.4 Distribution of red colobus [*Procolobus (Piliocolobus)*] taxa. Subspecies are shown due to the uncertainty in red colobus species level classification. Area marked with an H refers to a putative zone of hybridization between adjacent taxa. Shading indicates the range of the species referred to by the adjacent number. 1: *P. badius temminckii*; 2: *P. b. badius*; 3: *P. b. waldroni*; 4: *P. b. epieni*; 5: *P. b. pennantii*; 6: *P. b. preussi*; 7: *P. b. bouvieri*; 8: *P. b. tholloni*; 9: *P. b. parmentieri*; 10: *P. b. lulindicus*; 11: *P. b. foai*; 12: *P. b. oustaleti*; 13: *P. b. langi*; 14: *P. b. ellioti*; 15: *P. b. tephrosceles*; 16: *P. b. rufomitratus*; 17: *P. b. gordonorum*; and 18: *P. b. kirkii*. Classification follows Oates *et al*. (1994). Distributions from Colyn (1991, 1993), Oates et al. (1994) and author's own notes. (Ting, 2008)

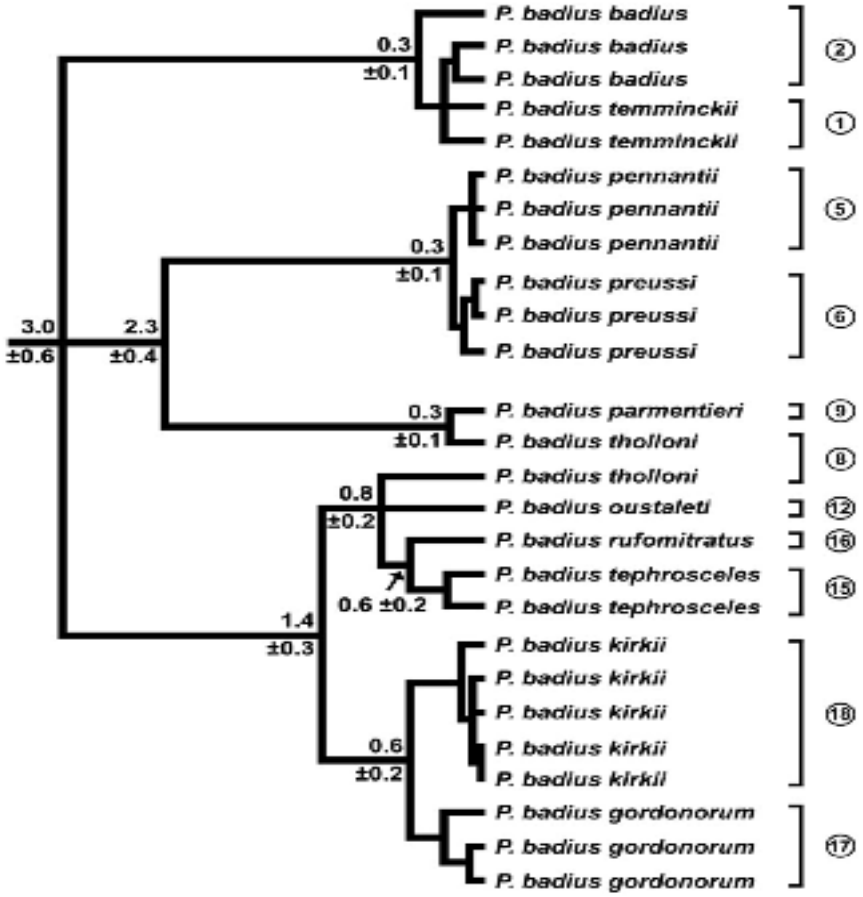

Figure 1.5 Red colobus [*Procolobus (Piliocolobus)*] mitochondrial likelihood and Bayesian tree. All nodes supported by bootstrap values _85 and posterior probabilities_0.90. Divergence date estimates (Ma) from penalized likelihood shown with two standard deviations. Numbers correspond to range distributions from Fig. 4. Classification follows Oates et al. (1994). (Ting, 2008)

vocalizations and cranial morphology). However the complexity of the variation pattern obscures the evolutionary relationships, thus limiting the recognition of specific units (Rahm, 1970; Napier, 1985; Groves, 2001, 2007). Because of the lack of consensus regarding their classification, several authors chose to consider only one species: *Procolobus* (*Piliocolobus*) *badius* (Oates & Davies, 1994; Ting, 2008).

Phylogenetic study using mtDNA sequences (Ting, 2008) has revealed three major clades in the red colobus group. One contains the West African subspecies *P. b. badius* and *P. b. temmincki*. The second contains the western equatorial group (*P. b. pennantii* and *P. b. preussi*) and two individuals from taxa that reside in the Congo Basin (*P. b. tholloni* and *P. b. parmentieri*). A second individual from one of these Congo Basin forms (*P. b. tholloni*) appears in the third clade together with *P. b. oustaleti*, *P. b. rufomitratus* and *P. b. tephrosceles*. This clade also contains a sister taxon relationship between *P. b. kirkii* and *P. b. gordonorum*. The three main clades estimated to have diverged by 3.0 Ma. The clade containing *P. b. pennantii* and *P. b. preussi* separated from *P. b. tholloni* and *P. b. parmentieri* by 2.3 Ma. *P. b. kirkii* and *P. b. gordonorum* diverged from the other East and Central African taxa by the early Pleistocene (1.4 Ma) and the remaining red colobus mitochondrial lineages diverged by the mid-late Pleistocene (Ting, 2008) (Fig 1.5).

In contrast to the black-and-white colobus, red colobus live in large social groups ranging from 12 to > 80 individuals, with typical groups between 25 and 40 individuals. These monkeys usually live in multi-male multi-female groups although some populations (e.g. Tana River) have only one fully adult male. As cited above, these colobines are one of the few primate species where dispersal is mainly female biased, so that the female bonding within social groups is predicted to be weak (Oates & Davies, 1994).

As in the *Colobus* genus, the preferred food items are young foliage and fruit items. Mature leaves function as a standby item that becomes most important when their preferred items are scarce (e.g. Struhsaker, 1975).

These monkeys are heavily predated by chimpanzees in some areas of their distribution (e.g. Tai National Park, Ivory Coast; Gombe Stream National Park, Tanzania) (Boesch & Boesch, 1989). The high predation pressure that they are subject to is a possible explanation for the large group sizes and the existence of interspecific associations with other primates (e. g. Diana monkeys (*Cercopithecus diana***)**, vervets (*Cercopithecus aethiops*)) (Holenweg et al., 1996; Galat-Luong & Galat, 2005). Bshary and Noë (1997), demonstrated that when in association with Diana monkeys, chimpanzees avoided hunting red colobus.

1.8 Study Species

1.8.1 Western black-and-white colobus (Colobus polykomos, Zimmerman, 1780)

This species occurs from Southern Senegal to the Ivory Coast (Fig 1.2) (Gippoliti & Dell'Omo, 2003), inhabiting moist lowland forest and moist forest zone in gallery forests (Oates & Davies, 1994). The Sassandra River marks the boundary between this species and *C. vellerosus*, east of which it is believed to occur in a hybrid population ("*dollmani*") (Grooves, 2007). The western black-and white colobus is classified as Vulnerable by the IUCN Red List, which means that it could be facing a high risk of extinction in the wild (IUCN, 2008).

These animals (Fig 1.6) live in one-male multi-female units with relatively small group sizes, never exceeding 20 individuals, comprising 1-3 adult males and 4-6 adult females. They have very well defined home-ranges but these can overlap extensively with other conspecific groups as well as with other primate species (Galat & Galat-Luong, 1985; Korstjens, 2001; Korstjens et al., 2005). Within social units, females maintain closer relationships with one another than they do with males, while no pronounced dominance hierarchy is observed. If there is more than one adult male in the group the affiliative interactions with one another are almost non-existent and they display a clear dominance hierarchy (Dasilva, 1989). As in all other *Colobus* species, dispersal is mainly male mediated, with individuals dispersing at around 2-4 years old or as adults when secondary dispersal occurs. However, some episodes of female migration have been reported (Korstjens et al., 2005), as in black colobus and *C. guereza* (Harris et al., 2009). The extent of female dispersal in this species still not very well understood, needing further investigation. However it is believed that western black-and-white females only disperse when the costs of staying in their natal group are high (e.g. inbreeding avoidance) (Korstjens et al., 2005). Since the females are the more philopatric sex, social groups are composed by matrilines, explaining the more extensive social interactions within this sex. Korstjens et al. (2002) reported that agonistic interactions were relatively common among *C. polykomos* females, with the most aggressive interactions taking place in a foraging context. The aggressive behaviour was displayed towards females from their own social group and during intergroup encounters, where they joined the adult male in defending the group's home range. The study group in Tai National Park showed a weak bonding among females with no preference of association or grooming with each other. These results contrast with other *Colobus spp.*, where there is limited involvement of females during intergroup encounters and social bonds are stronger among females of the same social group (Oates, 1977; Struhsaker & Leland, 1979).

Figure 1.6 *Colobus polykomos* in Iemberén forest, Cantanhez National Park.

As referred above for the *Colobus* genus, western black-and-white colobus feed mainly on young leaves and seeds, in liane tangles. At times of the year when these food items are scarce (wet season) mature foliage is the most consumed item and as a consequence of changing feeding strategies, daily journeys become shorter (Dasilva, 1989, 1992, 1994). The trees that these monkeys' select to feed in are usually the least common in the forest and patchily distributed (Korstjens et al., 2002). They have been reported to feed mainly in the lower layers of the forest canopy, being sometimes observed feeding on or near the ground. The upper and middle canopy are usually used for resting and travelling activities (Oates & Davies, 1994); Booth, 1956; Dasilva, 1989). As a way to economize energy, *C. polykomos* individuals spend a considerable part of their time resting. The percentage of time dedicated to this activity increases even more during the wet season, when mostly mature foliage (less digestible) is eaten (Dasilva, 1989, 1992).

Another striking characteristic of black-and-white colobus is the distinctive high-volume, low-frequency, resonating loud call of adult males, termed "roar". In *C. polykomos*, when more than one adult male is present in the social group, they roar together. This loud call is believed to have the function of adverting to other neighbouring males of the roarer's strength and condition, keeping them away from

their territory and "his" females (Dasilva, 1989).

C. polykomos females do not exhibit pronounced perineal swellings. Dasilva (1989) reported all births to occur during the dry season (December-February), probably strongly related with the seasonality of the study site (Sierra Leone, Tiwai). The few data from wild populations concerning this issue suggest interbirth intervals of around 24 months (Struhsaker & Leland, 1987; Dasilva, 1989). Infants are born with completely white pelage and pink skin, thus being very conspicuous and apparently very "attractive" to other females of the group that will hold the infant for considerable amounts of time. This "allomothering" behaviour is very common in this species, as it is in other colobine monkeys, and its evolutionary adaptative function is still under discussion (Oates & Davies, 1994).

1.8.2 Temminck's red colobus, (Piliocolobus badius temmincki, Kuhl, 1820)

This subspecies occurs in Senegal, The Gambia, northern Guinea and Guinea-Bissau (Fig 1.4) and is classified as Endangered, which means that it is facing a very high risk of extinction in the wild (IUCN, 2008).

These animals (Fig. 1.7) live in large multimale-multifemale groups with numbers ranging from 12 to 65, comprising several adult males and females. In contrast to the loud calls of black-and-white colobus, the western red colobus use a graded vocalization system for intragroup communication since they live in much larger and more complex social units (Marler, 1970). As in all other red colobus subspecies, *P. b. temminckii* dispersal is mainly female mediated, thus leading to a description of their society as patrilineal. Social interactions among females are very rare and "allogrooming" is more frequent among males and different sexes. Within groups, males often cooperate with each other in aggressive interactions with other groups (Struhsaker & Leland, 1979). However, in a population at Abuko Nature Reserve, The Gambia, Starin (1991) found evidence for active female participation in aggressive intergroup encounters. In this population, both males and females took part in these events, each sex directing its aggression towards males in opposing groups. The author advanced some possible explanations for the phenomena: a) lack of body size dimorphism; b) female dispersing with other females of the same natal troop, resulting in a high degree of relatedness among females within troops; c) small number of troops in the area resulting in high degree of previous relationships among females of opposing troops. In this case, genetic analysis of the relatedness between females could help to understand this uncommon behavioural pattern. If there are few groups in the area (few groups where to disperse to) and females transfer in peers this would lead to closely related adult females within social groups. The greater bonding between related females in addition to coalition formation

Figure 1.7 *Procolobus badius temminckii* in Iemberén forest, Cantanhez National Park.

in resource defence would give evidence to the strong effect of kinship in driving social behaviour in this species. As opposed to other red colobus groups, in the Abuko groups there was no inter-male grooming, proximity or coalitionary support. Males only cooperated when either an alien male or a neighbouring troop was in proximity. The differences exhibited by this population relative with other western red colobus give support to the importance of environmental factors shaping the social systems. In this subspecies, as in all other red colobus so far studied, "allomothering" behaviour appeared to be very rare, probably as a consequence of the weak female bonding (Struhsaker & Oates, 1975).

Western red colobus rely for their diet mainly on young leaves and fruit items. Mature foliage becomes more important in their diet at times of the year when the preferred items are scarce. These animals also explore other food resources such as flowers, floral buds and leaf buds. Much of their food is taken from the largest, most common trees in their habitat, usually with clumped distributions, both spatially and temporally, and often widely dispersed in the forest. This pattern of food distribution may explain the large group sizes. It is frequent for these monkeys to travel on the ground between clumps of trees (Oates & Davies, 1994). Temmincks' red colobus spend a greater proportion of their time resting (> 50%), dedicating approximately 25% of their time to feeding activities (Starin, 1991).

Dominant males perform most copulations, as a result of both male-male

competition and female choice. Females are described as giving conspicuous courtship displays and "quaver" calls during copulation (Struhsaker, 1975; Starin, 1991). *Temminckii* populations in strongly seasonal environments had the great majority of their births concentrated in the dry season with an interbirth interval of 27 to 32 months (Starin, 1991).

1.8.3 Differences between western black-and-white and western red colobus

Despite being closely related species from the same subfamily, sharing several traits and found very often living in sympatry (e.g. Tiwai, Sierra Leone; Tai, Ivory Coast; Cantanhez, Guinea-Bissau), these two colobines differ in some important life history traits that are summarized in table 1.1.

Table 1.1 Traits that differ among the two study species. Information assembled from Oates & Davies (1994).

Trait	*C. polykomos*	*P. b. temminckii*
Group size	11- 20	12 - 65
Social structure	1 – 3 males, multifemale	Multimale - multifemale
Dispersal	Mainly male mediated	Female mediated
Social bonding	Among females	Among males
"Allomothering"	Present	Absent
Sexual swelling	Absent	Present
Preferred food items	Young leaves and seeds	Young leaves and seeds
Preferred trees	Rare species	Common species
Food distribution	Clumped	Clumped patches widely distributed
Home range (ha)	24[a, b]	34[c, d]
"Roar"	Present	Absent
Main predators	Humans, crowned hawk-eagle	Humans, chimpanzees
Interspecific associations	Absent	Diana monkeys, Vervets

[a] data from Tiwai study site, Sierra Lione (Dasilva, 1989); [b] home range for a group size of 9-11 individuals; [c] data from Abuko study site, The Gambia (Starin, 1991); [d] home range for a goup size of 24-30 individuals.

Although red colobus share the same preferred food items with the black-and-white colobus species, they seem to have a more varied diet. Such diversity may in part be result from the greater emphasis on ephemerally available food items (e.g. flowers, bulbs). The difference in their diet is reflected in their stomach morphology. Red colobus have a distinct four-chambered stomach while in black-and-white colobus it is hard to distinguish between the first two chambers (Kuhn, 1964). The single large chamber in black-and-white colobus may be an adaptation to a relatively uniform, high fibre diet, while the separate chambers in red colobus could be adaptations to a more varied and less digestible diet. In addition to exploiting a wider variety of food items, red colobus also obtain their food from different sites in the forest canopy from those exploited by the black-and-white colobus (Korstjens et al., 2002).

Although these different ecological and behavioural patterns are widely recognized, their adaptive functions remain unknown.

The western black-and white colobus is classified as Vulnerable by the IUCN Red List, which means that it could be facing a high risk of extinction in the wild.

1.9 Study Site

1.9.1 Republic of Guinea-Bissau

Guinea-Bissau is one of the smallest countries in coastal West Africa (36 125 km^2), located between latitude 10º55'N and 12º40'N and longitude 13º38'O and 16º43'O, being bordered by Senegal to the north and Guinea-Conakry to the south and east (Fig. 1.8). It includes a number of small offshore islands, the Bijagós archipelago, almost connected to the mainland by wide intertidal mud flats. Mangroves and savannah are the predominant habitats, but it is still possible to find patches of primary forest in the southwest (administrative regions of Tombali and Quinara) and in the northwest (Cacheu administrative region) (Gippoliti & Dell'Omo, 2003). Mangroves are predominant along the coast, originally covering 11% of the country, including tracks extending inland along six major estuaries.

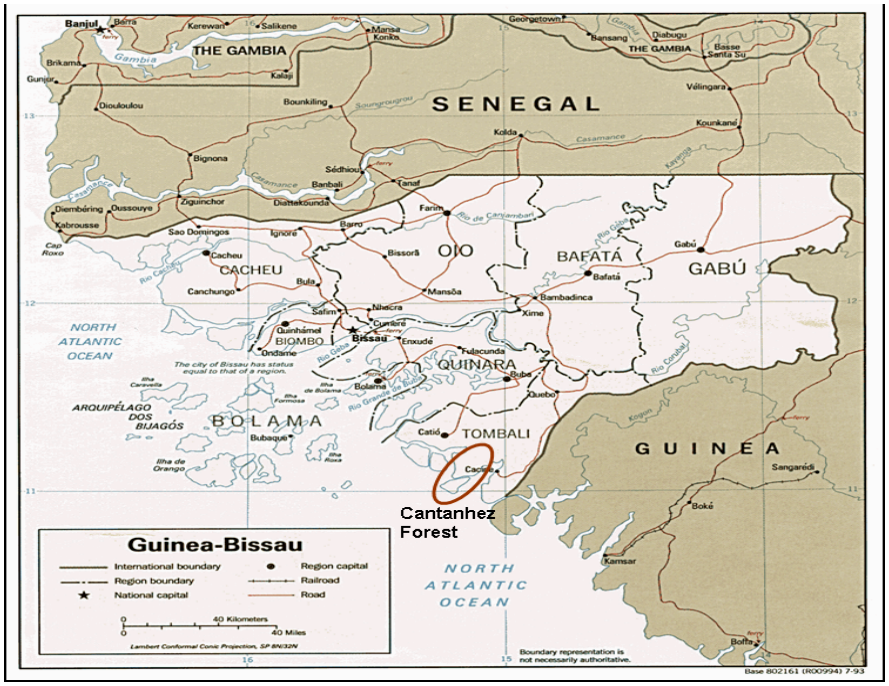

Figure 1.8 Map of the administrative regions of Republic of Guinea-Bissau.

With a population of 2.4 million in 2004, encompassing about 20 ethnolinguistic groups, the country has experienced a population growth of about 2% per year (Sousa, et al., 2005). As a consequence of several years of war, the population suffers from severe poverty and is highly dependent on the country's natural resources.

Fishing and farming are the main economic activities in the country. Rice is the major crop, being the staple food of the Guinean people. It can be cultivated in mangrove or forest soils. Cashew nut production is increasing and it's exportation along with fish and seafood represents the main foreign exchange (Guimarães, 2007). As a result of extensive agriculture and cutting of trees for commercial purposes, destruction of forest is the major threat for Guinea-Bissau biodiversity (Gippoliti & Dell'Omo, 2003). In addition, hunting of large and medium-sized terrestrial fauna has had a great impact on animal populations (IBAP, 2007)

There is a great lack of scientific information on the vertebrate fauna of the country. Besides some intermittent expeditions made between end of the 19th century and middle of the 20th century, it was only in 1989 that a more comprehensive wildlife inventory was carried out by the Direction Office of Forestry and Hunting of Guinea-Bissau (DGFG) and the Canadian Co-operation (Limoges, 1989). Oates (1986), listed 11 primate species in the country. Gippoliti and Dell'Omo (1996) investigated the primates of the Cantanhez forest and the Cacine basin where seven primate species were listed and in 2007 they evaluated the distribution and status of primates in different areas of the country. In the latter study they confirmed the existence of ten primate species in Guinea-Bissau: Senegal bushbaby (*Galago senegalensis*), Western black-and-white colobus (*Colobus polykomos*), Western red colobus (*Piliocolobus badius temminckii*), Green monkey (*Cercopithecus sabaeus*), Campbell's guenon (*Cercopithecus campbelli campbelli*), Lesser white-nosed guenon (*Cercopithecus nictitans stampflii*), Patas monkey (*Erythrocebus patas*), Sooty mangabey (*Cercocebus aethiops*), Guinea baboon (*Papio papio*) and Western chimpanzee (*Pan troglodytes verus*). The black-and-white colobus is reported to exist in the extreme north-western part of the country, southeast and southwest. The red colobus used to be widely distributed through the country. However, Limoges (1989) reported a disjunct distribution, with an extensive range in the south and apparently isolated populations in the northeast.

Great efforts have been made to study and conserve the coastal ecosystems of Guinea-Bissau (Altenburgh et al., 1992). Nevertheless, terrestrial ecosystems have been relatively neglected. As a consequence, little is known about distribution and population status of many taxa (Gippoliti & Dell'Omo, 1996). Gippoliti and Dell'Omo (2003) suggested that Temminck's red colobus should serve as an umbrella species for forest conservation in the country due to their dependence on a wide diversity of forest trees and that a management plan should be implemented before its status become critical.

Nowadays, the country has 6 protected areas under IBAP (Institute for

Biodiversity and Protected Areas) management, covering 470,000 ha in which one third corresponds to terrestrial areas. Guinea-Bissau has signed seven conventions aiming the protection of its habitats and biodiversity: Convention on Biological Diversity (1995), Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (1990), Ramsar Convention (1991), the Convention on Migratory Species (1995) (Sousa, et al., 2005), the convention on Climate Change, the Convention Against Desertification, and African-Eurasian Waterbird Agreement (IBAP, 2007).

Since the Guinean human population is highly dependent on forest resources for subsistence, all conservation plans should consider the rural communities and assure them the legal right to manage at least part of the forest resources (Sousa, et al., 2005). It is important that national and international conservation authorities take into consideration and try to reconcile the needs of both human communities and wildlife populations.

1.9.2 Cantanhez Forest

The Cantanhez Forest can be described as a savannah-forest mosaic, with mangroves growing along the rivers. Natural ecosystems include sub-humid forest, woodland and grassland savannah, mangroves and palm trees. With an area of 1.067 ha it is located in the Tombali and Quinara administrative regions, in the southwest of the country (Fig 1.9). The annual temperature ranges from 28ºC to 31ºC and the annual rainfall from 2000 to 2500mm, being the highest value in Guinea-Bissau (Simão, 1997). It is a highly human populated area with around 20,000 habitants distributed among 13 villages. Consequently, deforestation in order to create crops and rice fields is an increasing problem and the forest is becoming heavily degraded (IBAP, 2007).

The most south-western region of the forest is a peninsula that encompasses 14 forest patches (Fig 1.9), most of them connected to each other. These southern forest patches have been considered critical sites for biodiversity conservation in Guinea-Bissau (e.g. IUCN, 1993), being preserved by the local people for several years (Temudo 1998, 2009). Each forest patch has a communitarian guard (local) that is responsible to monitor all the authorized subsistence hunting (by local people) and to prevent illegal hunting (including confiscation of weapons) and illegal activities such as deforestation.

The entire Cantanhez area was declared a National Park in early 2008 (Fig 1.9). In addition, activities such as poaching and forest destruction are now forbidden under

the national laws. However, the national authority (IBAP), which is responsible for the national parks management, is not operating in the park yet. Consequently, the situation is still the same as it was before the park was declared.

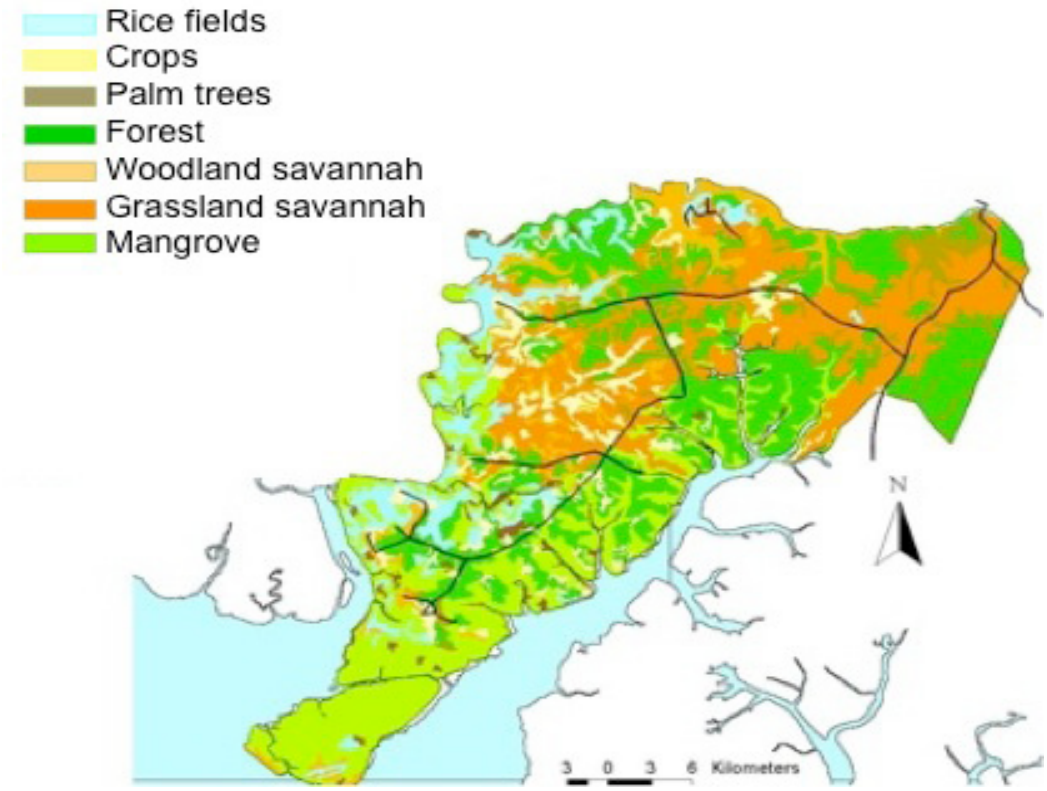

Figure 1.9 Map of the land use in the Cantanhez National Park.

It is possible to find six primate species living there, including, chimpanzees, guinean baboons, western red colobus, western black-and-white colobus, campbell's guenon and green monkey. It has the distinction of comprising the most western population of the endangered *Pan troglodytes verus* (Sousa et al., 2011; Hockings & Sousa, 2011) and it is also the only site where a inter-specific association between red and black-and-white colobus has been reported (Gippoliti & Dell'Omo, 1996). As in other regions of the country, some of the primate species and other big and medium sized mammals have their survival threatened by intense forest destruction and poaching.

1.10 Objectives

The present study focuses on the social organization and population structure of two taxa of colobine monkeys: *Colobus polykomos* and *Procolobus badius temminckii* present in Guinea-Bissau, through analysing the genetic structure within

and between social groups at a local scale. Moreover, behavioural data is added to the study in order to address socio-genetic questions. By doing so, I provide novel information at the level of social group and the population, for both genera. By combining behavioural with genetic data in these two African colobine monkeys exhibiting different social systems I aimed to analyse: a) dispersal patterns exhibited by each species, b) social behavioural patterns among males and females and c) effects of habitat fragmentation on the population genetic structure.

The overall conclusions anticipated from this project also aims to provide information to enable adequate conservation measures that national authorities can apply to primate communities of Guinea-Bissau. During this study I worked in close proximity with IBAP and community guards from Cantanhez National Park, participating in a series of workshops and talks concerning primate conservation in Guinea Bissau. Following the same principle, at the end of this study the final results will be communicated at a conservation workshop to be held, in Guinea-Bissau, with the aim of suggesting conservation measures to national authorities and local communities.

1.11 Hypotheses

African colobine monkeys have been subject of ecological and behavioural studies across their distribution range. However, very little is known about their genetic structure and, if it is concordant with observational data, concerning dispersal patterns, mating systems and patterns of kinship and behaviour. Since observational data in arboreal primates is very difficult to obtain, new information can be expected from genetic data that cannot be obtained by direct observation of individuals. Regarding what is known for the western red colobus and western black-and-white colobus social systems I hypothesised the following:

1. *Genetic evidence will support observations of female-biased dispersal in red colobus and male-biased dispersal in black-and-white colobus*.

   Predictions:

   a) Maternally inherited DNA will be more diverse within red colobus than within black-and-white colobus groups.
   b) Maternally inherited DNA will be more structured among black-and-

white colobus than among red colobus groups.

c) Similar low genetic structuring for both bi-parental and maternally inherited DNA will be found among red colobus groups.
d) Stronger genetic structure is expected for maternally inherited DNA than for bi-parentally inherited DNA among black-and-white colobus groups.
e) Higher levels of relatedness are expected between adult males than between adult females within red colobus social units.
f) Higher levels of relatedness are expected between adult females than between adult males within black-and-white colobus social units.

2. *Behavioural patterns, such as affiliative interactions and coalitions formation, are strongly affected by kinship*

Predictions:

a) Affiliative interactions will be more frequent between females of black-and-white colobus than those of red colobus.
b) Within black-and-white colobus groups, affiliative interactions will be more frequent between females than between males and different sexes.
c) Within red colobus groups, affiliative interactions will be more frequent between males and/or different sexes than between females.

3. *Habitat fragmentation will play a role shaping the genetic structure of both Colobine species, as a consequence of the decrease of dispersal capacity between more isolated forest fragments*

Predictions:

a) There will be an absence of isolation-by-distance as a result of the dispersal throughout the area being determined not by geographical distance but by presence of forest corridors between fragments;
b) Dyads of highly related individuals belonging to different social groups will not necessarily be geographically closer but will reflect habitat connectivity between the forest patches that they occupy.

1.12 Thesis Structure

In order to accomplish the aforementioned goals and test the hypotheses, I non-invasively sampled social groups for each species throughout Cantanhez National Park. Each sample was genotyped for 15 microsatellite *loci* and a fragment of the mitochondrial DNA d-loop region. The data was explored as follows:

Chapter 2 explores whether molecular data supports the evidence for male-biased dispersal in black-and-white colobus and female dispersal in red colobus. The patterns obtained from both types of molecular data, by using a series of population and individual-based methods, allowed us to explore the spatio-temporal changes in the mode of dispersal. This chapter has been accepted in *American Journal of Physical Anthropology* as:

Tania Minhós, Elizabeth Nixon, Claudia Sousa, Luis M. Vicente, Maria Ferreira da Silva, Rui Sá and Michael W. Bruford (*accepted*). Genetic evidence for spatio-temporal changes in the dispersal patterns of two sympatric African Colobine monkeys.

In Chapter 3 we aimed to understand if intra-group social behaviour was affected kinship. By analysing the social behaviour of one social group of each species, we investigated if social affiliative interactions were more frequent among individuals of the sex that had more related dyads. This chapter is currently in preparation to be submitted as:

Tânia Minhós, Cláudia Sousa, Luís Vicente, Michael W. Bruford (*in prep*). How important is kinship shaping the intra-group social dynamics of two sympatric African colobus species?

In Chapter 4 we investigated whereas the two sympatric social systems were responding differently to the habitat fragmentation. We combined Bayesian genetic clustering analysis and fine-scale spatial methods in order to identify current genetic discontinuities in the park. This chapter is currently in preparation to be submitted to *Molecular Ecology* as:

Tania Minhós, Claudia Sousa, Luis M. Vicente, Maria Ferreira da Silva, Rui Sá and Michael W. Bruford (*in prep*). The interaction of social system, genetic structure and habitat fragmentation in two threatened primate species.

Finally, in Chapter 5, I summarize the most important findings of this study and its limitations, directions of future research and discuss the implications for the colobus conservation in Guinea Bissau.

The data chapters of this thesis (Chapter 2-4) were written as separate manuscripts to be submitted to scientific journals. Each one of these chapters includes an abstract, introduction, specific questions and hypotheses, methods, results and discussion. As

a consequence, some content repetition may occur especially in the introduction and methods sections. Additionally, formatting is not concordant over different chapters as it follows the rules of the journal for submission (except for Chapter 4).

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Chapter 2

Genetic evidence for spatio-temporal changes in the dispersal patterns of two sympatric African Colobine monkeys

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2.1 Abstract

Western black-and-white colobus and Temmink's red colobus are two forest-dependent African primates with similar ecological requirements, often found in sympatry. Their most striking difference lies in their social system: black-and-white colobus living in small groups with mainly male-mediated dispersal but where females can also disperse, whereas red colobus live in larger groups with males described as philopatric. To investigate whether genetic evidence supports the reported patterns of dispersal based on observational data, we examined eight black-and-white and six red colobus social groups from Cantanhez National Park, Guinea-Bissau. Microsatellite markers revealed a lack of sex-biased dispersal for black-and-white colobus. Gene flow, mainly mediated by females, better explained the genetic patterns found in red colobus, with some evidence for less extensive male dispersal. In contrast to the microsatellite data, low mitochondrial diversity for the black-and-white colobus suggest that historical and/or long-range male-mediated gene flow might have been favored. In red colobus, the co-existence of three divergent mitochondrial haplogroups suggests that the Cantanhez population contains a secondary contact zone between divergent lineages that evolved in allopatry. Female-biased dispersal in this species may be a major factor contributing to the colonization by such differentiated mitochondrial lineages in the region. Overall, we find evidence for a spatio-temporal change in the dispersal patterns of the colobus monkeys of Cantanhez, with mitochondrial DNA indicating dispersal by mainly a single sex and microsatellite data suggesting that recently both sexes appear to be dispersing within the population.

2.2 Introduction

Sex-biased dispersal has evolved in many group-living animals and is an almost ubiquitous feature of primate life-histories. Understanding a species' dispersal system is necessary for the study of its socio-ecology, population dynamics and genetic structure (reviewed in Lawson Handley and Perrin, 2007). An individual may be induced to disperse by various proximate causes such as pressure from within the group or attraction to extra-group individuals (Jones, 2003). Evolutionary theories attempt to explain the ultimate causes of dispersal and view it as a mechanism for the avoidance of negative consequences of group-living (e.g. Hamilton, 1967; Dobson 1982; Pusey, 1987; Pusey and Parker, 1987; Clutton-Brock, 1989). Dispersal can, for instance, reduce inbreeding, competition between kin for local resources and competition between mates (Lawson Handley

and Perrin, 2007). Dispersal, however incurs costs to the individual. For example, lack of familiarity with a territory renders an individual vulnerable and may reduce its ability to find resources. There is also a risk of aggression from groups that the dispersing individual may encounter (Jack and Isbell, 2009). Interaction between forces that promote and oppose dispersal may explain the imbalance in dispersal rates and distances between the sexes.

Aspects of a species' biology such as sex-biased dispersal, are expected to leave traces in an organism's genome (Avise, 1994; Sunnucks, 2000; Di Fiore, 2003) and can be seen through variance in allele frequencies among social groups (Altmann et al., 1996; Dobson et al., 1998; Gompper et al., 1998, Hammond et al., 2006). The clonal maternal inheritance of mtDNA means that for female philopatry, haplotype diversity is expected to be low within groups, due the lack of arrival of new haplotypes, genetic drift and lineage sorting (Avise, 1994; Di Fiore, 2003). For species in which females disperse, mtDNA diversity is expected to be high, due to the introduction of novel haplotypes by immigrating females. However, not only is group diversity expected to differ among dispersal modes, but also substructure patterns within populations. While both mitochondrial haplotype and microsatellite allele frequencies in female-dispersing species are expected to be homogeneous (e.g. Melnick and Hoelzer, 1992; Morin et al., 1994; Di Fiore, 2003), female-philopatry is expected to lead to high mitochondrial differentiation between groups in contrast to autosomal markers, as male dispersal will homogenize this genome throughout the population (Avise, 1994; Di Fiore, 2003). This pattern was recently found for the squirrel monkey (*Saimiri oerstedii citrinellus*) (see Blair and Melnick, 2012) where previous observational data suggested female dispersal but genetic data indicated that males might be the main dispersers, at least over longer distances. Dispersal mechanisms are not always easy to predict based on systematics alone (e.g. Faulkes et al., 1997) and can vary among closely related species as in, for example, squirrel monkeys (Boinski et al. 2005) and can even vary among populations of the same species as in the white-bellied spider monkeys (Di Fiori, 2009). The African colobines also illustrate this variation very well; besides the fact that black-and-white colobus and red colobus are known to exhibit different modes of dispersal, variation in dispersal patterns have also been reported among species of the two genera (Harris et al 2009; Struhsaker 2010).

Western black-and-white colobus (*Colobus polykomos*) and Temmink's red colobus (*Procolobus badius temminckii*) belong to the Old-World sub-family Colobinae. African colobus monkeys share many aspects of their ecology such as predominantly arboreal lifestyles within the woodlands of the tropics (Oates, 1994) and dietary similarities – with most species favoring young foliage including some

seeds, mature leaves, flower and fruits (Oates et al., 1994). Nevertheless, group sizes are consistently different, with *P. b. temminckii* living in large groups, averaging 25-40, in comparison to *C. polykomos* groups with 16 or less individuals (Oates, 1994). Group composition between the two species also differs: red colobus groups are usually multi-male, multi-female, with a minimum of three adult males and at least twice as many adult females; black-and-white colobus groups on the other hand, consist of multiple females with often only one adult male (Oates, 1994). A major difference between the two species' social systems is their pattern of dispersal. In black-and-white colobus, although dispersal is reported to be more biased towards males, both sexes have already been described to disperse (e.g. *C. polykomos*: Korstjens et al*.* 2002; *C. santanas*: Fleury and Gautier-Hion, 1999; *C. guereza*: Harris et al., 2009; *C. vellerosus*: Teichroeb et al*.* 2009), while in red colobus, females are the main dispersers (Marsh, 1979; Starin, 1991, 1994; Struhsaker, 2010). Female dispersal in black-and-white colobus has been explained as either a consequence of intra-group competition for resources or inbreeding avoidance (Korstjens et al., 2002, 2005; Harris 2005). As they feed on patchily distributed species within their home range, territory expansion is energetically costly. Consequently, young females may be forced to disperse in order to avoid increasing group size (Korstjens et al., 2005). These two related species (Ting, 2008) share similar ecological requirements, exhibit contrasting social systems and often live in sympatry, and are therefore good models for understanding the determinants of dispersal behavior and its impact on the genome.

Western black-and-white colobus occurs from southern Senegal to the Ivory Coast (Gippoliti and Dell'Omo, 2003), while *P. b. temminckii* occurs in Senegal, Gambia, northern Guinea and Guinea-Bissau (Oates et al., 1994). The socio-ecology of these two primates has been studied previously (*C. polykomos*: Galat and Galat-Luong, 1985; Dasilva, 1989 and 1992; Korstjens, 2001; Korstjens et al., 2005 and *P. b. temminckii*: Marler, 1970; Struhsaker, 1975; Struhsaker and Leland, 1979; Starin, 1991 and 1994), although not in Guinea-Bissau. Little is known about African colobine population genetic diversity and structure. The studies conducted so far have focused only on few colobus populations from eastern Africa (Harris et al., 2009; McDonald and Hamilton, 2010; Mbora and McPeek, 2010). Harris et al*.* (2009) used both observational and genetic data to describe the complex dispersal system of a *Colobus guereza* population in Uganda, where the genetic structure of social groups is shaped by male-mediated gene flow together with less common episodes of female dispersal. McDonald and Hamilton (2010) examined the genetic diversity and phylogenetic relationships among Kenyan and Tanzanian *Colobus angolensis palliatus* populations. Only one study on red colobus (*Piliocolobus badius*

rufomitratus) has been reported from the Tana River in Kenya, which evaluated mitochondrial diversity within this population (Mbora and McPeek, 2010).

Here we intensively sampled several social groups for both colobus species in Cantanhez National Park, Guinea-Bissau (Fig. 1), to evaluate the effect of dispersal patterns on genetic diversity, within-population structure and historical demographic processes. Given that female dispersal is expected for both species, we predicted the two species to exhibit similar patterns of mitochondrial diversity and structure: high genetic diversity and homogenized haplotypes throughout the population. For nuclear DNA, we did not expect to find differences between sexes for *C. polykomos* as a consequence of dispersal by both males and females. However, if *P. b. temminckii* males are philopatric we would predict them to show higher genetic differentiation between social groups, higher probability of assignment to source population and higher levels of within group relatedness (Di Fiore, 2003; Hammond et al., 2006, Lawson Handley and Perrin, 2007).

2.3 Material and Methods

2.3.1. Study site and sampling

Surveys were conducted throughout Cantanhez National Park, in south-western Guinea-Bissau (NE limit: 11°22'58''N, 14°46'12''E; SW limit: 11°2'18''S. 15°15'58''W; Fig. 1), which comprises peninsular and fragmented coastal forest. Faecal samples from one social group per fragment (ranging from 47,5 to 2500 ha; Simão 1997) were collected per species. Approximately 10 samples were collected per *C. polykomos* social group and 30 for each group of *P. b. temminckii*, in order to ensure that more than half of the group was sampled in most cases. The size and age-sex composition of each group could not be determined for non-habituated groups and we did not directly observe animals defecating but collected only fresh faecal material. To minimize multiple sampling of individuals, we only collected samples that were 2m or more apart. Samples were stored using the 'two-step' approach (Roeder et al., 2004). Samples from eight black-and-white colobus and six red colobus social groups were analyzed (Fig. 2.1).

2.3.2. DNA extraction and amplification

DNA from 380 faecal samples was extracted using the QIAampDNA Stool Kit (Qiagen, Valencia, CA) following the manufacturer's instructions and stored at -20 ºC. All samples were genotyped for 15 human-derived microsatellite loci (Table 2.1), first successfully used in *Colobus guereza* by Harris *et al.* (2009), multiplexed in

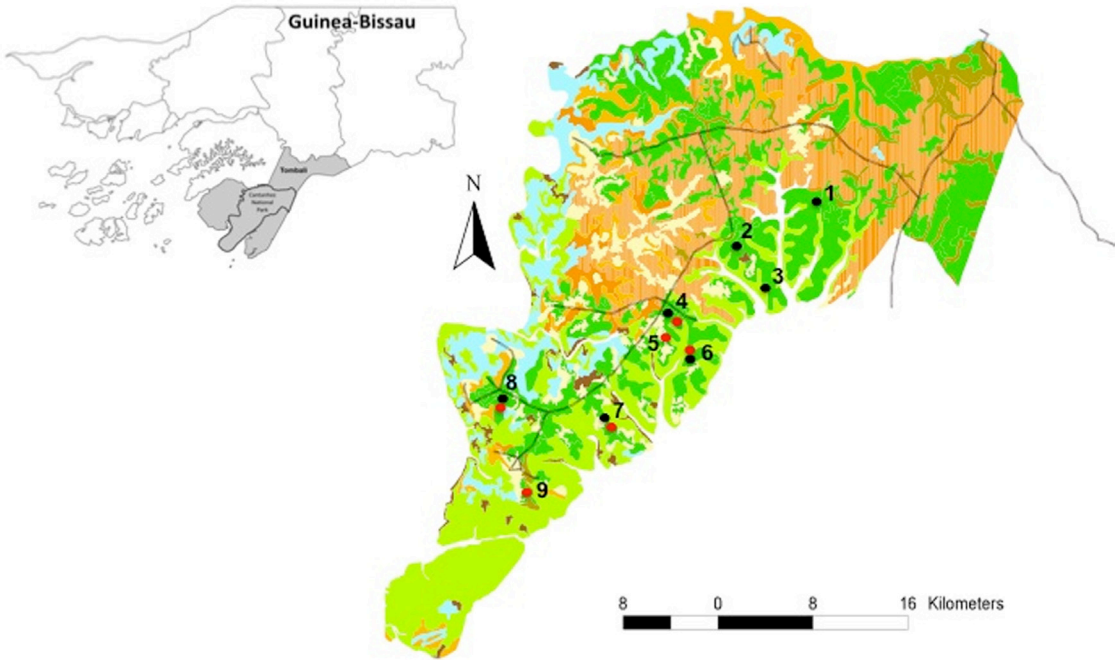

Figure 2.1 – Map of the land cover for Cantanhez National Park (provided by INEP): dark green – forest; light green – mangrove; yellow – savanna; blue – rice fields; beige – crops; brown – tannes. Black circles: black-and-white colobus sampled groups; red circles: red colobus sampled groups. 1 – Cancira; 2 – Amidara; 3 – Deep Amidara; 4 – Focal and Neighbor; 5 - Madina; 6 – Cambeque; 7 - Cangode; 8 – Muna; 9 – Cungha.

three panels of five loci. Molecular sex identification of each sample was carried out following the protocol developed by Villesen and Fredsted (2006) and multiplexed together with the microsatellites. PCRs contained 7.5µL Multiplex PCR Master Mix (Qiagen), 0.1µM of each primer, 0.004mg/ µL of BSA (Bovine Serum Albumin, NEB) and 4.35µL of ultrapure water, and 1.5µL template DNA in a final volume of 15µL. Reactions, including negative controls, were performed in a VeritiTM 9902 Thermal Cycler (Applied Biosystems) as follows: 30 min at 95 °C; 40 cycles of 0.5 minutes at 94 °C, 1.5 minutes at 55-57 °C, 1.5 minutes at 72 °C followed by 30 minutes at 72 °C. PCR products were analyzed by Macrogen (Korea) Inc. An internal size standard (ROX labeled HD400) was added and alleles were scored using GeneMapper® v3.2 (Applied Biosystems). To account for allelic dropout, we used the simulation software GEMINI v.1.4.1 (Valière et al., 2002) that uses the allele frequencies, allelic dropout and false allele rate observed for a subset of samples, and estimates the number of PCR repeats and number of times an allele would need to be scored in order to produce genotypes with 95% confidence. As a result, each sample was positively amplified for a minimum of four independent reactions. The locus D2s442 was excluded from the dataset as genotypes were missing for 25% of *C. polykomos* and 20% of *P. b. temminckii* samples respectively. D12s372 was monomorphic for *P. b. temminckii* and was also excluded for this species. PI_{sib} values (the probability of identity (PI) that accounts for the presence of related individuals in the sample; Taberlet and

Luikart, 1999; Waits et al., 2001) were calculated using GenAlEx 6.41 (Peakall and Smouse, 2006) yielding values of 1.9 x 10^{-3} for C. polykomos and 1.2 x 10^{-3} for P. b. temminckii. Low quality DNA samples, i.e. Quality Index (QI, Miquel et al., 2006) below 0.5 or more than three missing loci, and samples that could be replicates from the same individual were excluded. A total of 52 *C. polykomos* individuals from eight social groups were genotyped for 11-14 loci with 97.2% complete genotypes and a mean QI of 0.84. In addiction, 72 *P. b. temminckii* individuals from six social groups were genotyped for 10-13 loci with 96.5% complete genotypes and a mean QI of 0.77. We used Micro-Checker (van Oosterhout et al., 2004) to test for null alleles and FSTAT (Goudet, 2001) to conduct exact tests for Hardy-Weinberg and linkage disequilibrium. Highly related individuals, resulting from sampling social groups, had a strong effect in inducing apparent population substructure (not shown) and were subsequently removed from the dataset. After this correction, none of the loci showed evidence for null alleles, linkage disequilibrium or deviating from Hardy-Weinberg equilibrium.

We sequenced up to 478bp of the hypervariable domain (HVI) of the mitochondrial control region for 56 *C. polykomos* and 79 *P. b. temminckii*. Primers were designed using conserved sequences analyzed from GenBank for *P. b. badius* (DQ355301; Sterner et al., 2006) and *C. guereza* (AY863427; Raaum et al., 2005). Primers amplified for both species (L15449Clbs: 5' CCRCCAATACCCAAAACTGG 3', H15973Clb: 5' AGGAGAGTAGCACTCTTGTGC 3'). PCR conditions were the same as for the microsatellite except for primer concentration (2µM of each primer) and annealing temperature (63ºC). Each 13µL PCR product was purified using 4µL (1:2 ratio) of Exonuclease I (10 U/µL) (USB Corp.) and Shrimp Alkaline Phosphatase 1 U/µL (USB Corp.) by incubation at 37°C for 30 min, followed by 15 min at 80°C and finally for 5 min at 12ºC. Sequences were run by Macrogen Korea Inc. Forward and reverse sequences were manually checked using Sequencher v4.9 (Gene Codes Corporation) and aligned using CLUSTALW implemented in BIOEDIT 7 (Hall, 1999). Evidence that authentic mtDNA copies were sequenced instead of nuclear insertions (Numts) included observations that: multiple electrophoretic peaks were not present in the sequences; both tissue and faecal samples produced the same sequences; for the black-and-white colobus, the same haplotypes were produced using two different primer sets, and; cloning and sequencing of PCR products supported the evidence that none of the sequences used in the study were nuclear copies (see supplementary material). Haplotype sequences were deposited in GenBank database (XXXXXXXX).

2.3.3. Genetic diversity and social group structure

Genetic diversity was analyzed across all social groups through the number of alleles (N), observed (H_o) and expected heterozygosity (H_e), and unbiased allelic richness (AR), using FSTAT (Goudet, 2001). The inbreeding coefficient (F_{is}) was estimated using GenAlEx 6.41 (Peakall and Smouse, 2006). Analysis of molecular variance (AMOVA), using a codominant genotypic distance matrix to calculate $\Phi_{PT,}$ was also implemented in GenAlEx 6.41 (Peakall and Smouse, 2006) to understand how diversity was partitioned within and among social groups. Significant tests were performed through 9999 permutations. This analysis was carried out for complete social groups and for each sex separately.

Nucleotide (π) and haplotype diversity (H_d) were calculated for the entire species sample and for each social group. Number of polymorphic sites and mean number of pairwise differences among sequences were also estimated. Diversity measures were computed in DnaSP version 5 (Rozas et al., 2010). AMOVA, using pairwise differences, was carried using Φ-statistics as implemented in ARLEQUIN version 3.1 (Excoffier et al., 2005). For the *C. polykomos*, a two-level AMOVA (among and within social groups) was performed and for the *P. b. temminckii* we carried a three-level AMOVA out where sex was also considered. Additionally, we used BAPS (Bayesian Analysis of Population Structure) v5 (Corander and Tang, 2007; Corander et al., 2008) in order to corroborate the inference of social group structure. Here we did not incorporate geographical information and used stochastic optimization allowing K to vary between one and the total number of social groups: each analysis was repeated 10 times. The evolutionary relationships between haplotypes for both species were determined using a median-joining haplotype network calculated with NETWORK 4.5 (Bandelt et al., 1999) with $\varepsilon = 0$ and all variable sites weighted equally. Frequencies and geographic distributions of different haplotypes were used to depict geographical and potential ancestor-descendant relationships among haplotype sequences.

2.3.4. Assignment tests

Mean corrected assignment indices (mAI_c) were calculated and compared between males and females using FSTAT (Goudet, 2001) and GenAlEx 6.41 (Peakall and Smouse, 2006). The assignment index is the probability that an individual's multilocus genotype originated in the sampled population (Favre et al., 1997). Because mAI_c is corrected to zero, genotypes less likely to belong to the population

(e.g. immigrants) are expected to show negative mAI_c whereas positive values are expected for natal individuals. The dispersing sex should also show higher values of variance of the corrected assignment index (vAI_c) as a consequence of the presence of both immigrants and residents (Favre et al., 1997; Goudet et al., 2002). Because such analysis assumes that only post-dispersal individuals are present and in our case it was not possible to distinguish adults and juveniles, we used one habituated *P. b. temminckii* social group (Focal), where samples were known to belong to adult and juvenile individuals, to serve as a control. Therefore, the same analysis was run with adults and juveniles as well as just adults, and results compared. GenAlEx 6.41 was used to preform assignment tests with the P. b. temminckii Focal group dataset.

2.3.5.Relatedness

Mean pairwise relatedness was estimated using Kingroup v2_101202 (Konovalov et al., 2004) and Coancestry v1.0 (Wang, 2011). The relatedness estimator of Queller and Goodnight (1989) was used for all possible dyads in the population and for the comparison between females and males from the same social group. Because similar results were obtained with the two programs, here we refer only to the results from Coancestry, where 1000 per-locus bootstraps were performed to achieve 95% confidence interval for each dyad. The module "Test Group Difference" was used to statistically compare mean pairwise relatedness between males and females from the same social group. Statistical significance was achieved through 1000 bootstraps for a 95% confidence interval. The test for significance was only possible for groups that have three or more individuals of each sex. Because estimators of relatedness are likely to be biased by group size (the philopatric sex might only be detected to have higher within group pairwise relatedness if the group is small: Valsecchi et al., 2002; Lukas et al., 2005) and the presence of pre-dispersal individuals increases mean pairwise relatedness for both sexes, the number of highly related dyads for each of the sexes formed by individuals from the same or different social groups were also used as an additional indicator of sex-biased dispersal. We calculated the percentage of parent-offspring, full-siblings and half-siblings dyads of the same sex present within and among social groups. In order to estimate the number of such pairs of individuals, a likelihood ratio test was implemented in Kingroup v2_101202 (Konovalov et al., 2004), where the null hypothesis of "Unrelated" was tested against the primary hypotheses of "Parent-offspring", Full-siblings and "Half-siblings". One would expect that a higher percentage of such dyads would occur within social groups for the most philopatric sex and amongst social groups for the dispersing sex.

2.4 Results

2.4.1 Genetic diversity and structure

Genotypes were derived from unidentified pre- and post-dispersal individuals and diversity indices are summarized in Table 2.1. *P. b. temminckii* showed slightly higher mean number of alleles, H_o, H_e and Fis than *C. polykomos,* although such differences were not significant. However, the allelic richness, which is a diversity measure that controls for differences in the sample size was, significantly higher for *C. polykomos* (t= 4.08; d.f.= 13; p=0.001). For *C. polykomos*, significant structure among social groups was found (Φ_{PT} = 0.165, *P*< 0.001), although variance among individuals within the same social group explained most of the variation. This pattern was maintained when the AMOVA was conducted for females (Φ_{PT} = 0.184, *P*< 0.001) and males (Φ_{PT} = 0.193, *P*= 0.001) separately. Red colobus social groups also showed significant genetic variance among groups, although less than *C. polykomos* (Φ_{PT} = 0.057, *P*< 0.001). While females *P. b. temminckii* exhibit similar levels of genetic structure (Φ_{PT} = 0.053, *P*< 0.001), there was some evidence that *P. b. temminckii* males are the more structured sex (Φ_{PT} = 0.199, *P*< 0.005).

Table 2.1. Diversity indices for the microsatellite loci

Locus	# Alleles	Allele size range (bp)	Ho[1]	He[2]	Fis[3]	Allelic R[4]
C. polycomos						
D1s548	5	200-216	0.669	0.622	-0.197	2.495
D1s1665	4	164-176	0.589	0.537	-0.182	2.265
D4s2408	5	259-283	0.650	0.560	-0.261	2.103
D13s321	6	158-182	0.730	0.651	-0.224	2.586
D6s474	4	122-134	0.638	0.564	-0.212	2.465
D10s611	5	177-193	0.527	0.487	-0.176	2.213
D2s1326	5	215-239	0.580	0.590	-0.062	2.381
D11s2002	4	174-186	0.641	0.494	-0.381	2.215
D12s372	2	226-230	0.127	0.114	-0.209	1.185
D6s503	4	293-329	0.135	0.162	0.058	1.339
D6s1056	3	265-293	0.414	0.380	-0.180	1.984
D10s676	5	247-267	0.356	0.630	0.340	2.376
D10s1432	3	210-222	0.568	0.543	-0.145	2.103
Fesps	4	138-158	0.057	0.056	-0.098	1.122
Mean	4.2	-	0.475	0.415	-0.138	2.059
P. b. temminckii						
D1s548	7	192-216	0.927	0.783	-0.327	1.795
D1s1665	5	187-203	0.382	0.555	0.225	1.612
D4s2408	6	275-311	0.398	0.464	0.046	1.581
D13s321	2	165-169	0.289	0.321	0.008	1.307

D6s474	4	131-183	0.689	0.576	-0.281	1.571
D10s611	6	181-217	0.642	0.736	0.034	1.760
D2s1326	7	215-239	0.836	0.736	-0.247	1.750
D11s2002	4	158-182	0.544	0.503	-0.171	1.484
D6s503	7	294-338	0.586	0.777	0.107	1.748
D6s1056	3	269-277	0.327	0.453	0.138	1.476
D10s676	5	230-266	0.678	0.688	-0.130	1.745
D10s1432	7	211-247	0.671	0.837	0.055	1.803
Fesps	5	146-162	0.540	0.561	-0.056	1.613
Mean	5.2	-	0.538	0.508	-0.045	1.634

[1] Observed heterozygosity, [2] Expected heterozygosity, [3]Inbreeding coefficient, [4] Allelic richness.

For all *C. polykomos* individuals, 478bp of the HVI domain of the control region were successfully amplified. Only three haplotypes were detected (BW1, BW2, BW3) with two segregating sites (transitions) and a mean number of nucleotide differences between two sequences of 0.17 mutational steps. Haplotype (H_d = 0.17 ± 0.065) and nucleotide diversity (π = 0.00038 ± 0.0001) were consequently remarkably low (Table 2.2). Haplotype BW2 was present in 51 individuals and in all but Deep Amidara social group. Haplotypes BW1 (one individual) and BW3 (four individuals) were found in one social group each (Cambeque and Deep Amidara, respectively). Four hundred and forty-eight bp of control region was successfully amplified from all 79 *P. b. temminckii*. In contrast to the *C. polykomos*, 9 haplotypes were identified, with 45 polymorphisms (42 transitions and 3 transversions). The mean number of nucleotide differences between sequences was 16.5 mutational steps, exhibiting high haplotype (H_d = 0.82 ± 0.017) and nucleotide diversity (π = 0.037 ± 0.002; Table 2.2). The most common haplotypes (RC1 and RC4) were present in five and seven social groups, respectively. There were four haplotypes found in only one social group (RC6-RC9).

Table 2.2 – Summary of mitochondrial DNA diversity

Social Group	**N[1]**	**# Hapl[2]**	***hd*[3]**	**π [4]**
C. polycomos	58	3	0.16	0.00036
Focal	9	1	0	0
Neighbour	10	1	0	0
Cambeque	11	2	0.18	0.0004
Cancira	7	1	0	0
Muna	6	1	0	0
Deep Amidara	4	1	0	0
Cangode	3	1	0	0
Amidara	6	1	0	0
Bushmeat	2	1	0	0

P. b. temminckii	86	11	0.83	0.038
Focal	29	5	0.77	0.03
Madina	19	3	0.70	0.02
Cambeque	11	5	0.78	0.04
Muna	2	2	1.00	0.05
Cangode	9	6	0.89	0.05
Cungha	9	3	0.64	0.04
Bushmeat	7	4	0.81	0.05

[1] Number of sequences, [2] Number of haplotypes, [3] Haplotype diversity, [4] Nucleotide diversity

The *C. polykomos* AMOVA for mtDNA yielded high fixation indices between social groups (Φ_{st} = 0.80, P< 0.001), with 80% of the variation being partitioned between groups. As this population revealed low genetic diversity, this result is almost exclusively due to individuals from Amidara that exhibited an exclusive haplotype. Structure was not detected when this social group was excluded from the analysis (data not shown). BAPS analysis supported the existence of three clusters matching the three haplotypes found (p = 0.5, Fig. 2.2a). As depicted by the minimum-spanning network (Fig. 2.3a), BW2 was the most widespread haplotype in the population and the other two haplotypes were rare and likely to have recently originated from BW2. For *P. b. temminckii* there was weak genetic structure between social groups (AMOVA, Φ_{st} = 0.13, P< 0.005), with 87% of the total variation within social units. Only 16% of the variation was found within social units and none explained among males and females from the same group. The BAPS results supported the weak genetic structure found by AMOVA since the three clusters identified (p= 0.699, Fig. 2.2b) had no correspondence with social groups. Furthermore, more than two haplotype clusters were found in all social groups. All others were shared between two or more social units, reinforcing the lack of mitochondrial DNA structure between *P. b. temminckii* social groups. The minimum-spanning network showing relationships between the 9 haplotypes revealed three very divergent haplogroups with a high level of haplotype sharing between social groups (Fig. 2.3b) in agreement with AMOVA and BAPS results.

2.4.2. Assignment tests

When comparing mAI_c values for *C. polykomos* social groups there was no strong evidence for sex-biased dispersal (Table 2.3). The mAI_c was positive for females (mean=0.019) and negative for males (mean=-0.027). The vAI_c was very similar and low for both sexes. The mAI_c and vAI_c values were not significantly different between sexes. For *P. b. temminckii,* when the three social groups with both sexes (Focal, Madina, Cangode) were analyzed, mAI_c was positive for females (mean=0.229)

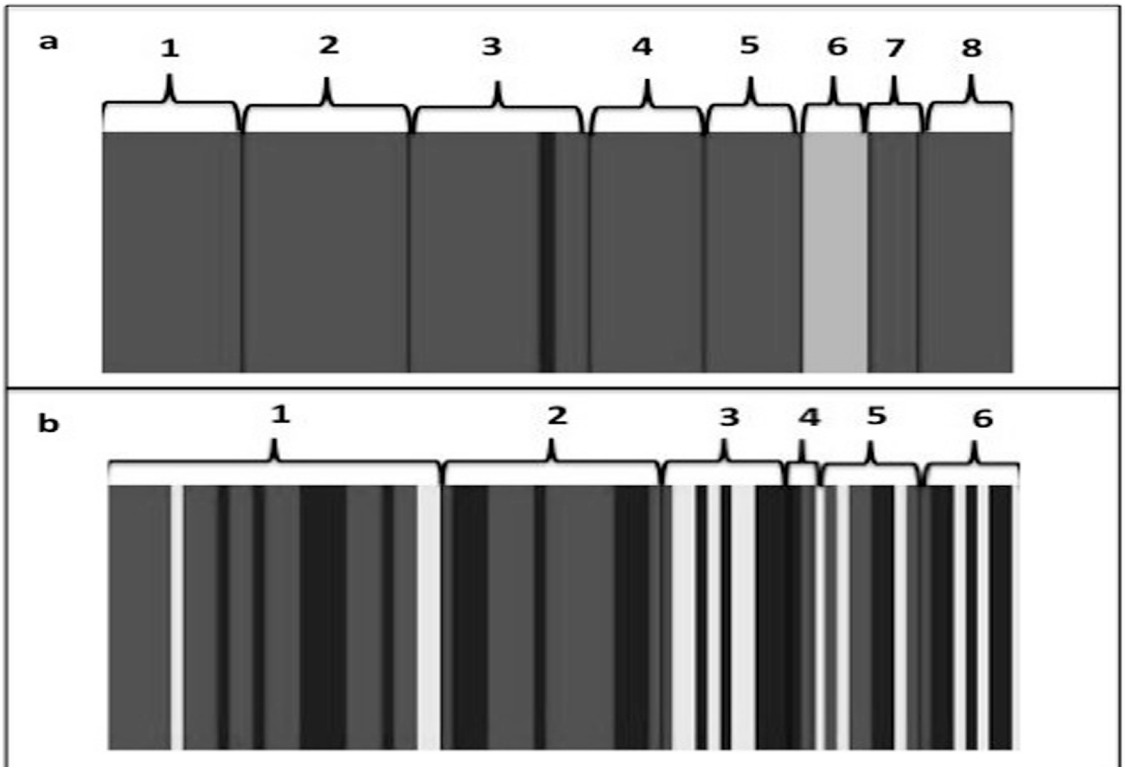

Figure 2.2 – BAPS results for each black-and-white colobus social group (a): 1 Focal, 2 Neighbour , 3 Cambeque, 4 Cancira, 5 Muna, 6 Deep Amidara, 7 Cangode, 8 Amidara,; and red colobus social groups (b) (number of clusters in parenthesis): 1 Focal (3), 2 Madina (2), 3 Cambeque (2), 4 Muna (2), 5 Cangode (3), 6 Cungha (3).

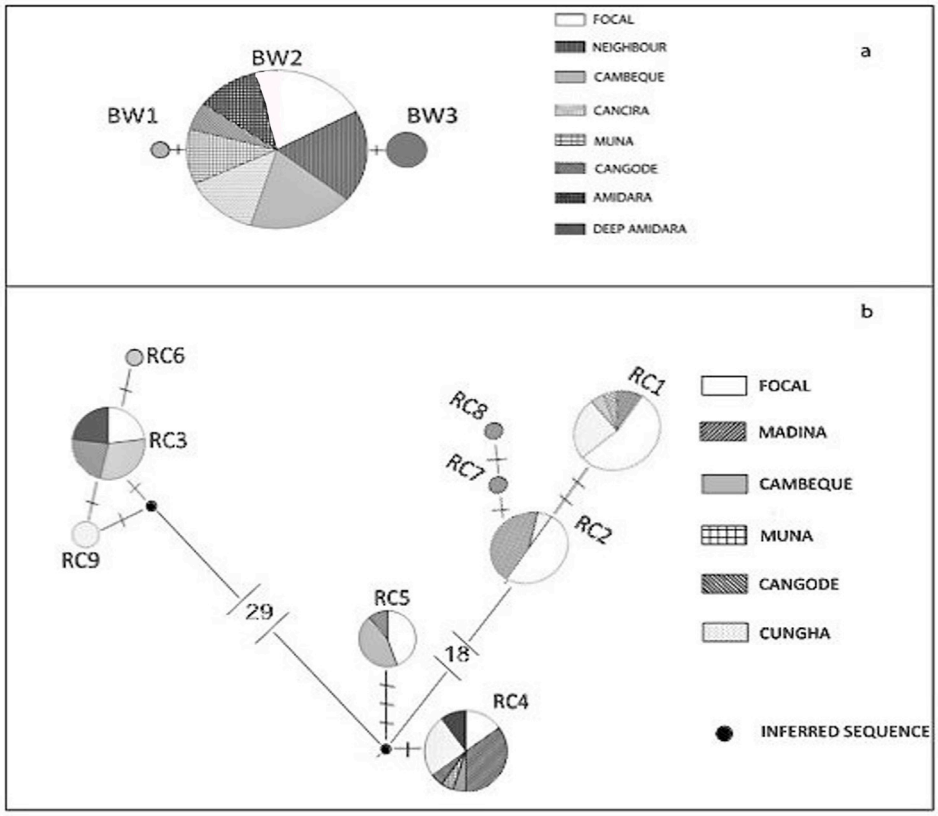

Figure 2.3 - Haplotype network for black-and-white (a) and red colobus (b) based on mtDNA d-loop sequences. Circle diameter is proportional to the frequency of the haplotypes. Different color patterns represent different social groups. Lines between haplotypes represent the number of mutational steps.

and negative for males (mean=-0.918), but the differences were not statistically significant (Table 2.3). However, vAI_c was higher for females indicating than despite a positive mAI_c, there is greater variation in the assignment index for *P. b. temminckii* females. When the same analysis was performed for the best-studied social group (Focal), and included both adults and juveniles, a positive mAIc was obtained for the females (mean=0.016) and a negative value was obtained for the males (mean=-0.050), mimicking the pattern found for the previous analysis using the three social groups. However, repeating the analysis only with Focal adult individuals, mAI_c values was negative for females (mean=-0.496) and positive for males (mean=0.868), as is expected for a female-dispersal system .

Table 2.3 – Tests of sex-biased dispersal carried in FSTAT

C. polykomos	**# Individuals**	**mAIc[1]**	**P-value**	**vAIc[2]**	**P-value**
Females	30	0.01916	0.95	6.11120	0.82
Males	21	-0.02738		6.94474	
P. b. temminckii					
Females	44	0.22963	0.36	15.00585	0.52
Males	11	-0.91851		10.70524	

[1] Mean corrected assignment, [2] Variance of the mean corrected assignment

2.4.3. Relatedness within and among social groups

C. polykomos did not show a clear pattern of sex-biased dispersal when differences in within group sex relatedness were considered (Table 2.4). Females showed higher, but not significant, levels of mean pairwise relatedness than males for the social groups where among sex comparisons were possible, displaying negative values for the Neighbour and Muna social groups. Additionally, females were found to have a higher percentage of their mother/daughters (77.8%) and full sisters (52.4%) in a different group to their own. Also, 42% of *C. polykomos* half-sisters were in different groups. For males, a higher percentage of all close relatives was found in different social groups (father-son: 55.6%, full-brothers: 66.7%, half-brothers: 66.7%; Table 2.5), meaning that a higher proportion of closely related dyads was found between individuals from different social groups for both females and males . Due to the limited number of *P. b. temminckii* males in this study, comparison of the mean pairwise relatedness between males and females was only possible for two social groups (Table 2.4). Females had higher levels of mean pairwise relatedness than males for both social groups. Again, Focal group results when only adult individuals were analysed are more in agreement to what would be expected from a female dispersal system. Statistical tests were only possible for the Focal group

and were not significant. Although 85.2% of mother-daughter dyads were present within groups, this percentage dropped to 45.7% and 46.9% when full- and half-sisters are considered, respectively (Table 2.5). Nonetheless for males, father-son dyads were only found within groups and only 28.6% of full-brothers and 16.7% of half-brothers were of individuals that belonged to different social groups (Table 2.5).

Table 2.4 – Pairwise relatedness for males and females within each social group

Social Groups	R Females[1]	R Males[2]
C. polycomos		
Focal	0.22810 (4)	0.06769 (5)
Neighbour	-0.04087 (4)	0.25135 (4)
Cambeque	0.20834 (6)	0.07127 (3)
Muna	-0.23223 (3)	0.09378 (4)
Deep Amidara	0.10617 (4)	NA (1)
Cancira	0.15390 (4)	0.57710 (2)
Cangode	0.06880 (2)	NA (1)
Amidara	0.39853 (3)	0.18690 (2)
P. b. temminckii		
Focal	0.06641 (22)	0.05035 (8)
Focal adults	-0.08382 (11)	0.05195 (4)
Madina	0.01628 (13)	-0.13510 (2)
Cambeque	0.00036 (10)	NA (0)
Muna	NA (0)	0.39700 (2)
Cangode	0.00319 (9)	NA (1)
Cungha	0.24948 (5)	NA (0)

Note: Numbers in brackets () correspond to the number of individuals used in each class
[1] Female pairwise relatedness, [2] Male pairwise relatedness

Table 2.5 – Percentage of closely related dyads of individuals of the same sex

	Parent-Offspring		Full-siblings		Half-siblings	
C. polykomos	**Intra-group**	**Inter-group**	**Intra-group**	**Inter-group**	**Intra-group**	**Inter-group**
Females	22.2 (4)	77.8 (14)	47.6 (10)	52.4 (11)	57.9 (11)	42.1 (8)
Males	44.4 (4)	55.6 (5)	33.3 (2)	66.7 (4)	33.3 (2)	66.7 (4)
P. b. temminckii						
Females	85.2 (46)	14.8 (8)	45.7 (48)	54.3 (57)	46.9 (46)	53.1 (52)
Males	100 (3)	0 (0)	71.4 (5)	28.6 (2)	83.3 (5)	16.7 (1)

Note: Numbers in brackets () correspond to the number of individuals used in each class

2.5 Discussion

2.5.1 Comparison of levels of genetic diversity and structure

The low mitochondrial diversity in the *C. polykomos* population and the high diversity found for *P. b. temminckii* is concordant with a male dispersal system in black-and-white colobus and female-mediated dispersal in red colobus. For *C. polykomos*, reduced mitochondrial gene flow as a result of female philopatry, together with the stochastic events of mutation, genetic drift and lineage sorting should result in restricted levels of genetic diversity within local populations of male dispersing species (Avise, 1995, 2000). AMOVA results show that mitochondrial haplotypes are unevenly distributed across social units resulting in 80% of the variation being explained among groups. Melnick and Hoelzer (1992) found that for the male dispersal system of *Macaca mulatta* (Rhesus monkey), mitochondrial sequence differences between populations (2.45%) were an order of magnitude larger than those within populations (0.23%), where 91% of the diversity was explained between populations. The *C. polykomos* microsatellite data showed weaker structure among social groups with most of the variation found within social units. This pattern could be concordant with what is expected for species where dispersal is mainly male mediated: female philopatry does not allow the mitochondrial genes to be distributed throughout the population, but dispersal by males homogenizes the nuclear genome (Di Fiore, 2003). However, the fact that the strong mitochondrial structure of is solely due to one social group (Deep Amidara) and that the genetic diversity is extremely low, makes it difficult to infer dispersal among social groups of the Cantanhez *C. polykomos* from mtDNA alone. It could be argued that forest fragmentation and not male dispersal is responsible for the fact one haplotype is exclusive from Deep Amidara, but microsatellite data indicate that, at least currently, females from Deep Amidara have closely related individuals in other social groups (data not shown) so emigration from this social group remains possible. In contrast, the fact that *P. b. temminckii* showed high levels of mitochondrial diversity and no genetic structure for either of the markers suggests that the females introduced novel genetic mitochondrial information in the population and are homogenizing both genomes through dispersal among social groups (Di Fiore, 2003; Lawson Handley and Perrin, 2007). In a study conducted on the closely related Asian colobine, the snub-nosed monkey (*Rhinopithecus roxellana*), one of the populations also showed comparable levels of mtDNA diversity to the red colobus population, as expected for a population where females emigrate (H_d= 0.88 and π = 0.04; Li et al., 2007). The same trend has also been found in other female dispersal primates, as the Proboscis monkeys

(*Nasalis larvatus*; see Munshi-South and Bernard, 2011), Hamadryas baboons (*Papio hamadryas*; see Hapke et al., 2001) and bonobos, where Eriksson et al. (2004) revealed high levels of haplotype (0.78 to 0.92) and nucleotide diversity (0.023 to 0.038) where approximately 70% of the variation was found within sampled communities.

2.5.2. Sex-biased dispersal: population- and individual-based tests

Assignment tests were not able to identify any sex bias in dispersal for the *C. polykomos* population, for which system males are thought to be the main dispersers, but where episodes of female dispersal have also been reported (Dasilva, 1989; Korstjens et al., 2002). We found no significant difference between male and female mAI_c. In accordance, vAI_c was also very similar for males and females. Goudet et al. (2002) showed that tests based on mAI_c and F_{st}, are only able to detect the sex bias when this is strong. Therefore, if a sex bias in dispersal exists in this species, we can only conclude that is not strong enough to be detected by population-based methods. Further, we obtained the same indication of an absence of sex bias in the dispersal for the relatedness analysis. Females only showed higher levels of within group pairwise relatedness than males in half of the groups and we found a similar percentage of highly related females in both within and among group dyads. These results are indicative that females might disperse to some extent. Ultimately, when inspecting the distribution of dyads of close relatives ("parent-offspring", "full-siblings" and half-siblings"), a high percentage was found of individuals belonging to different social groups for both sexes, in agreement with the mAI_c and F_{st} results. These results are in line with Harris et al. (2009)'s findings for the only other black-and-white colobus population (*C. guereza*, Uganda) where the genetic signature of the dispersal system has been studied to date. Their results based on pairwise relatedness within and among social groups also revealed the presence of some highly related females dyads in different social groups. However, *C. guereza* related female dyads were more likely to be found within groups and males were on average less related within groups than females. Harris et al. (2009) explained the dispersal system as being complex, where males might disperse longer distances and the less extensive female dispersal being more restricted to neighboring groups. They also explained the existence of highly related female dyads in different groups as a possible consequence of group dilution. Even if this event could explain some of the relatedness patterns found for the *C. polykomos* females in the Cantanhez population, this phenomenon alone cannot explain the extensive among group relatedness found for these females, unless it is extremely common. Moreover, highly related female dyads were found between more pairs of groups (N pairs=16) than male dyads (N pairs =12)

(data not shown), adding evidence for dispersal by both sexes in this population.

For the *P. b. temminckii* population, both population- and individual-based analysis indicate that females should be the main sex promoting dispersal. Although with the mean corrected assignment tests, no significant differences were found between males and females, females exhibited negative values of mAI_c for the Focal group when only adult individuals were considered. When this group contained both adult and juveniles, we obtained a similar pattern to the total database where the mAI_c was positive for the females but where the vAI_c was also higher for this sex. For *P. b. temminckii*, conclusions should be taken with caution as the limited number of males in the sample, can uncover the dispersal pattern (Goudet et al., 2002). Moreover, the low number of *P. b. temminckii* males did not allow an extensive comparison of within group female and male pairwise relatedness and may be responsible for an underestimation of among group "male dispersal". Nonetheless, results obtained for the well-sampled Focal group when only adult individuals are considered, suggest that females are less related than males, thus being the candidate sex to conduct dispersal, at least in this social group. In a well-studied group of spider monkeys (*Ateles belzebuth*; see Di Fiore et al., 2009), males were found to be more related within groups, with assignment tests also suggesting female-dispersal. This pattern was not found for a second well-studied group in the same population, indicating that the sex that disperses might change for different groups. Adding to the evidence that dispersal might be mainly mediated by females in the Cantanhez *P. b. temminckii* population, is the fact that no among group father-son dyad was found and, only few dyads of full- and half-brothers were constituted by individuals from different social groups. Clearly, more males from different social groups are needed to fully understand this dispersal system. However, our data indicate that males might not be completely philopatric as is thought for red colobus. Nevertheless, we were able to show that in *P. b. temminckii,* females should be the main sex promoting dispersal, as demonstrated by the AMOVA, assignment tests and pairwise relatedness. Adding to the evidence from the nuclear markers, the lack of mitochondrial structure among social groups and high genetic diversity for this molecular marker also supports the extensive female dispersal for red colobus.

2.5.3. Current and historical or long-range dispersal

If the analysis of the mitochondrial DNA indicates historical or long range dispersal by males in *C. polykomos* and by females in *P. b. temminckii*, this signal becomes more complex when analyzing current within population dispersal. The combination of both low mitochondrial haplotype and nucleotide diversity in *C.*

polykomos population suggests a scenario where the colonization of the peninsula was accomplished by one or a few mitochondrial lineages (Grant and Bowen, 1998). We suggest that the fact that the males were the primary sex to immigrate into the population may not have allowed for new mitochondrial haplotypes to be established in the population. As a consequence, historical and/or long-range dispersal mainly mediated by *C. polykomos* males is reflected not only in the present pattern of mitochondrial diversity of the population but has also left its signature in the colonization history of the Cantanhez Peninsula. Moreover, levels of haplotype and nucleotide diversity exhibited by *P. b. temminckii* population suggest the existence of, either a large stable population with deep evolutionary history, or secondary contact between divergent lineages (Grant and Bowen, 1998). The shape of the network supports the latter since three very divergent lineages were found within the population. The coexistence of large mitochondrial differences in the same geographical area can be explained by the secondary admixture between differentiated lineages (Avise, 1987). Their immigration into Cantanhez Peninsula may reflect the species dispersal pattern during or after the colonization process.

Table 2.6 – Summary of main results explaining the patterns of dispersal found for the two colobus populations from Cantanhez

	C. polycomos		***P. b. temminckii***	
Historical/long-range dispersal (mtDNA)	Male dispersal	Extremely low genetic diversity within the population	Female dispersal	High genetic diversity within the population with three divergent haplogroups
Within population dispersal (mtDNA)	Not conclusive (very low diversity)	Low genetic diversity within groups	Female dispersal	High genetic diversity within groups
		Social groups highly structured		Lack of structure between social groups
Current within population dispersal (microsatellite loci)	Both sexes	No sex diferences in genetic structure among social groups	Female dispersal but with some evidance of less extensive male dispersal	Females less strctured among social groups than males
		No sex differences in within group relatedness		Negative mAIc for adult females (Focal group)
		No sex differences in mAIc		Negative intra-group adult female relatedness (Focal group)
		High percentage of same sex close relatives are of individuals from different social groups for both males and females		Most dyads of highly related females were of individuals from different social groups but most higly related males were found within groups

Evidence from microsatellite data, which can be used to measure sex-biased dispersal in one generation (Lawson Handley and Perrin, 2007), is that within the *C. polykomos* population, dispersal is mediated by both sexes, whereas for *P. b. temminckii*, females seem to be the main dispersing sex, although we have evidence of some male dispersal. The forest in Cantanhez National Park is highly fragmented and episodes of colobus being hunted for bushmeat consumption have been recorded (Minhós et al., unpublished data; Hockings and Sousa, 2011). Both species occupy patches of forest where anthropogenic pressure is high and the two main threats to their survival (habitat loss/fragmentation and poaching) co-exist. Consequently, the possibility that the colobus are changing their dispersal patterns in response to recent changes in their habitat cannot be excluded (Goossens et al., 2006). In addition, stochastic demographic events (e.g. high mortality due to hunting) might have altered within- and among-group relatedness (Di Fiore et al., 2009). This might be the case for the colobus monkeys in Cantanhez as mitochondrial DNA data indicates historical and/or long-range dispersal by one sex but nuclear data suggests that currently both sexes may disperse within the population. It is recognized that the bias in dispersal might vary with geographical scale (Lawson Handley and Perry, 2007) with males being able to disperse larger distances than females (Waser, 1985), and this black-and-white colobus might also illustrate this situation (long-range dispersal by males but within population dispersal by both sexes). The fact that most behavioral studies on black-and-white colobus species report only very few episodes of females dispersing, suggests that in those populations females disperse in a lesser extent than the males, and consequently, the extensive female dispersal evidenced by the nuclear markers in Cantanhez may correspond to local behavioral adaptation in response to changes in the environment. Proximate causes could include inbreeding avoidance and/or kin competition for resources, as dispersing over short distances is sufficient to avoid both problems (*reviewed in* Lawson Handley and Perrin, 2007; Korstjens and Schippers, 2003). Ultimately, genetic data integrated in the study of dispersal patterns can provide great insights not only on the socio-genetic dynamics of a species but also on the effect that anthropogenic disturbance might have on its endangered populations.

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Chapter 3

How important is kinship shaping the intra-group social dynamics of two sympatric African colobus species?

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Key-words: *Colobus polykomos*; *Piliocolobus badius temminckii*; kinship; time-budgets; social behaviour; Guinea Bissau

3.1 Abstract

Temminck's red colobus (TRC: *Piliocolobus badius temminckii*) and western black-and-white colobus (BWC: *Colobus polykomos*) belong to Colobinae subfamily, often live in sympatry but exhibit very different social systems. TRC live in large multi-male multi-female social groups where dispersal is mainly female-biased. In contrast, BWC live in much smaller groups with one to three adult males and dispersal can be mediated by both sexes. We investigated how the patterns of kinship may influence the intra-group social dynamics of these two colobus monkeys living in sympatry. Focal and *Ad libitum* data were collected for a social group of each species in Cantanhez National Park, Guinea Bissau, between October 2008 and June 2009. Intra-group pairwise relatedness was estimated using faecal DNA from nine BWC individuals and 15 TRC individuals genotyped for 15 microsatellite loci. If kinship is the determinant factor shaping these groups' social dynamics we should expect individuals to direct their affiliative interactions (e.g. grooming) to their related counterparts. However, although we could not exclude kinship as being an important factor determining the BWC focal group's social interactions, that was not the case for the TRC. Our results showed that despite the fact that most of TRC females are not related and most of males are, females engaged in grooming events with other females very frequently and males only groomed each other very rarely. By combining analysis on the time budgets, social interactions and relatedness we showed that the intra-group behavioural patterns of the TRC group is different from other studied red colobus, suggesting that anthropogenic and/or ecological factors may be important shaping these groups' social bonding.

3.2 Introduction

Although phylogenetically related and very often living in sympatry, temminck's red colobus (TRC, *Piliocolobus badius temminckii)* and western black-and-white colobus (BWC, *Colobus polykomos)* exhibit very different social systems. Western black-and-white colobus live in relatively small groups, never exceeding 20 individuals, comprising 1-3 adult males and 4-6 adult females (DaSilva 1989; Galat & Galat-Luong 1985). Western black-and-white colobus have well defined home ranges but these can overlap extensively with other groups as well as with other species. (Galat & Galat-Luong 1985; Korstjens 2001; Korstjens et al. 2005a, b). Within social units, BWC females have been described to either maintain closer relationships with one another than they do with males (Oates 1977; Struhsaker

& Leland 1979) or to exhibit looser social bonds with no preference to interact affiliatively with other females (Korstjens et al. 2002). When there is more than one adult male in the group, affiliative interactions among males are almost non-existent and they display a clear dominance hierarchy (Dasilva 1989). As in all other *Colobus* species, although dispersal is thought to be mainly male-biased (Korstjens et al. 2002), episodes of female migration have also been reported (e.g. *Colobus polykomos*: Minhós et al. unpublished data; *Colobus satanas*: Fleury & Gautier-Hion 1999; *Colobus guereza*: Harris et al. 2009; *Colobus vellerosus*: Teichroeb et al. 2009). It is believed that BWC females only disperse when the costs of staying in their natal group are high (e.g. inbreeding avoidance: Korstjens et al. 2005a). If females are philopatric to some extent, social groups are composed by at least some related females, and therefore more extensive social interactions within this sex are expected. Korstjens et al. (2002) reported that agonistic interactions were relatively common among BWC females, especially in the context of foraging. This group, in Tai National Park, showed weak bonding among females with no preference for association or grooming. These results contrast with other study groups of the same species and other *Colobus* species (Oates 1977; Struhsaker & Leland 1979) and provide evidence that different strategies can be adopted by this species as a response to different ecological and/or sociological constraints.

Temminck's red colobus live in large multi-male, multi-female groups with numbers ranging from 12 to 65, comprising several adult males and females (Galat & Galat-Luong 1985; Korstjens 2001; Struhsaker 1975; Struhsaker & Oates 1975). As in all other red colobus, *P. b. temminckii* dispersal is mainly female-biased, and their society has been described as patrilineal (Marsh 1979). Social interactions among females are rare and "allogrooming" is more frequent among males. Within groups, males often cooperate with each other in aggressive encounters with members of other groups but females usually do not take part (Struhsaker & Leland 1979) although in Abuko Nature Reserve, The Gambia, Starin (1991, 1994) found evidence for active female participation in aggressive intergroup encounters. There was no inter-male grooming or proximity. Males only cooperated when either an alien male or a neighbouring troop was in proximity. The differences exhibited by the population from Abuko Nature Reserve compared to other red colobus groups highlight the importance of understanding the factors shaping these primates' social dynamics.

The assumption that individuals should preferentially address their affiliative and cooperative behaviour to more closely related counterparts constitutes a starting point for many of the models that try to explain the evolution of primate

social systems, since cooperating with kin increases the inclusive fitness of the individual (e.g. Chapais 2001; Gouzoules & Gouzoules 1987; Hamilton 1964a, b; Silk 1987, 2002). Nevertheless, accurately testing the hypothesis that more affiliative and cooperative interactions reflect closer relatedness between individuals has only been possible with the development molecular techniques for quantifying relatedness (Lynch & Ritland 1999; Pamilo & Crozier 1982; Queller & Goodnight 1989).

For many long-lived primate species, long-term studies on mating systems and dispersal patterns are difficult. By allowing the assessment of paternity, kinship and population structures, molecular data can provide insights into these features of social systems (Di Fiore 2003). Long-term studies on chimpanzee *(Pan troglodytes)* communities have demonstrated the existence of strong affiliative and cooperative bonds among philopatric males (e.g. Watts & Mitani 2001; Wrangham & Peterson 1996;), suggesting that 'related' males exhibit closer affiliations than 'unrelated' females. However, two studies that evaluated intra-community relatedness of chimpanzees at Gombe using a molecular approach (Gagneux et al. 1999; Vigilant et al. 2001) found no significant difference between male-male and female-female relatedness. These results suggested that affiliative and cooperative behaviours observed among males did not arise as a direct result of kin selection, suggesting the existence of other evolutionary mechanisms (e.g. mutualism, reciprocal altruism). There are other species where co-operation in the absence of kinship has been demonstrated (e.g. baboons: Bercovitch 1988; Noe 1990; dolphins: Connor et al. 2001; long-tailed manakin: McDonald & Potts 1994), reinforcing the notion that intra-group relatedness is not always enough to explain groups' social dynamics.

Although African colobines have been subject to various socio-ecological studies throughout their geographic distribution (e.g. Uganda: Struhsaker 1975; Tanzania: Clutton-Brock 1972; Ghana: Teichroeb et al. 2003; Ivory Coast: Galat & Galat-Luong 1985; Sierra Leone: Dasilva 1989; Senegal: Struhsaker 1975; The Gambia: Starin 1991; Zanzibar: Silkiluwasha 1981), we present the first socio-genetic study conducted on TRC and western BWC in Guinea Bissau, where both species occur in sympatry. Genetic analysis has shown that dispersal is mainly mediated by females in TRC population from Cantanhez and by both sexes for the BWC population (Chapter 2). Here we examine the relationship between intra-group relatedness and social affiliation in order to understand if the frequency of social interactions within and among sexes for these two threatened colobine species (*Colobus polykomos*: Vulnerable, Oates 2008a; *Piliocolobus badius temminckii*: Endangered, Oates 2008b) in Cantanhez National Park (CNP), Guinea Bissau is kin-based. This hypothesised

relationship derives from the assumption that kinship plays a major role on the evolution of primate social systems, where individuals cooperate and establish stronger bonds with more related counterparts due to the benefits of the inclusive fitness (Di Fiore 2003).

3.3 Material and Methods

3.3.1Study site and social groups

Cantanhez National Park comprises a mosaic of savannah, forest and mangrove habitat and covers an area of 1.067 ha in the southwest of Guinea-Bissau (Fig 3.1) and is a highly populated area with several villages and extensive agriculture throughout the park. As a consequence, the forests are severely fragmented comprising several patches of various sizes (ranging from 47,5 to 2500 ha; Simão 1997). The annual temperature ranges from 28ºC to 31ºC and the annual rainfall is 2000 to 2500mm (Simão 1997).

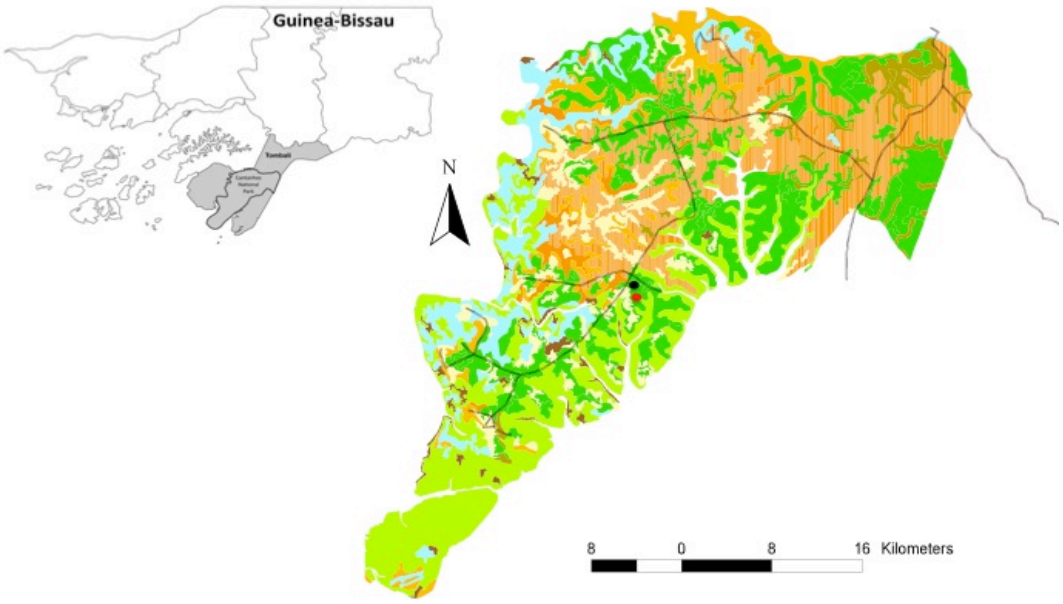

Figure 3.1 Map of Cantanhez National Park in Guinea Bissau. Black and red circles indicate BWC and TRC groups locations, respectively.

Observations were made on one group of TRC and one group of BWC. Both groups' home range overlapped with a village that is also a tourist site and were not provisioned. As a result, the groups were already partially habituated, allowing observations. It is possible that such proximity with the tourist site provides them some protection from local hunters. In CNP both species are hunted for bushmeat

consumption and trade although this pressure seems to be more intense for the TRC than for the BWC (Hockings & Sousa 2011; T. Minhós personal observation) For TRC, it was possible to count a minimum of 27 animals in the group of which three were adult males, 10 adult females, six juveniles and eight infants. However, it was not possible to see all group members at the same time and accurately determine the size of the group. For BWC we were able to individually recognize all group members. The group comprised one adult male, one sub-adult male, four adult females, two juvenile males, one juvenile female and one infant. In March 2009, the adult male, sub-adult male, a juvenile male and an adult female with the infant left the group and had did not return.

3.3.2 Behavioural data

3.3.2.1. Time budgets

Focal sampling was carried out between March and June 2009. Each focal observation was of four hours duration thus three sessions were carried *per* day. Each activity performed by the focal individual (feed, rest, move, travel, social) and its duration was recorded on a continuous basis. When the individual under the focal observation was out of sight, the sampling was paused and continued after finding the individual again. If the focal individual was out of sight for more than 30 minutes the focal sampling was stopped. In the BWC focal group all group members were identified. For the TRC focal group some individuals were not individually recognized, so the data presented was based on a sex- and age-class classification for both focal individual and its partners. Time budgets were calculated using focal data based on 528 hours of observation. For BWC 276 hours of focal observations were carried out on one adult male, one sub-adult male, four adult females, two juvenile males and one juvenile female. For TRC, 252 hours of observation were performed on adult females and adult males.

3.3.2.2. Social interactions

Ad libitum data (Altmann 1974) was collected between October 2008 and March 2009 on a daily basis from 7:00 to 19:00. Date, time, habitat, site, individual, activity and partner were recorded. For the individual and partner, identification of the sex and/or age class of unknown individuals was attempted. Data were collected continually during the day, every time a social interaction was observed

and corresponds to 19 full days of observation for each species. Since observation of all group members simultaneously was not possible for TRC, the largest subset of temporarily adjacent individuals was observed to record the maximum interactions possible. We consider the following activities here: aggression (aggressive and submissive interactions involving two individuals), grooming, social fight (aggression involving three or more individuals), copula, play and vocalisation. For grooming exchanged with a peer we considered only one event for each individual even if the grooming direction changed several times. For all behavioural categories, if the interaction was interrupted for less than 3 minutes, it was considered as the same event (Korstjens et al. 2002).

3.3.3 Behaviural data analysis

Statistical analyses were implemented in R v. 2.12.0 [R Development Core Team 2009]. Non-parametric tests were employed since data could not be transformed to conform to a normal distribution. Two-tailed tests were employed throughout. Mann-Whitney U-tests were applied to compare between the two species. Kruskal–Wallis one-way ANOVA tests were used to analyse differences among the three sex-classes and when significant differences were found, a pairwise Wilcoxon rank sum test was implemented, with p-values adjusted using the Holm-Bonferroni method.

Only adult and sub-adult individuals were included when analysing social interactions using the *ad libitum* data. The proportion of each activity was compared between the two species and grooming and agonistic interactions were analysed within each species, while considering the sex of the individuals. Since some TRC group members were not recognizable individually, we considered each observation day and each focal as the sample unit for the *ad libitum* and focal data, respectively. When interpreting the results, one needs to be aware that some pseudo-replication may result from this approach for the unknown TRC individuals. Such issue may be more relevant for the adult females than for the adult males. I was able to individually recognize at least 3 TRC adult males that should represent approximately half of the number of adult males present in the group. As group size in TRC was much larger than in BWC, the number of social interactions was much higher in TRC. In order to correct for this bias, social interactions results are expressed as the proportion of each social activity relative to the total of intra-group observed social interactions for each observation day. Time budget results are presented as the mean percentage of time that a focal individual spent in each activity.

3.3.4. Relatedness analysis

Faecal samples were collected for all known individuals of BWC focal group and several individuals from TRC which sex and age class was known. DNA was extracted using the QIAampDNA Stool Kit (Qiagen, Valencia, CA) following the manufacturer's instructions and stored at -20 ºC. All samples were genotyped for 15 human-derived microsatellite *loci.* The genotyping procedures and the information on the microsatellite *loci* used are described in detail on Chapter 2. In the BWC focal group we were able to genotype one adult male, one sub-adult male, three adult females, two juvenile males, one juvenile female and one adult male that joined the group in 2010. For the TRC focal group we were able to genotype 11 adult females and four adult males. The relatedness coefficient of Queller and Goodnight (1989) was estimated for all intra-group dyads using Kingroup v2_101202 (Konovalov et al., 2004). Whereas for the BWC relatedness analyses correspond to the full genetic characterization of the social group, for the TRC those are just minimum estimates as there were several other adult individuals in the group from which we were not able to obtain genotypes.

3.4 Results

3*.4.1 Time budgets*

Both species spent more than half of their daily time resting (BWC: 56.78% ± 12.64; TRC: 53.23% ± 20.04) and more than one quarter on feeding activities (BWC: 36.05% ± 13.78; TRC: 26.60% ± 16.57). BWC spent slightly more time on feeding activities but this was not significantly different to TRC. TRC spent a significantly higher percentage of their time socializing when compared with BWC (12.02% ± 13.47 and 2.60% ± 1.91, respectively) (Mann- Whitney U-test, $U= 435$; $p<0,01$). Travelling occurred at a low frequency and was not different between the groups (Fig 3.2).

3.4.2 Social interactions

3.4.2.1. Between-species comparisons

For TRC a total of 828 interactions were recorded (mean: 43.63 interactions/ day, S.D. ± 16.68). For BWC we recorded 321 interactions (mean: 16.89 interactions/ day, S.D. ± 12.08). Both species engaged in grooming frequently, although this

behaviour was higher for TRC (BWC: 0.57 ± 0.26, $N_{interactions}$=210; TRC: 0.71 ± 0.12, $N_{interactions}$=600) (Fig 3.3). TRC also engaged significantly more in agonistic interactions than BWC (0.27 ± 0.17, $N_{interactions}$=209 and 0.18 ± 0.17, $N_{interactions}$=47, respectively) (Mann-Whitney U-test, U = 102; p<0.05). Both groups showed low levels of social fights and copulation although these were slightly more frequent within TRC.

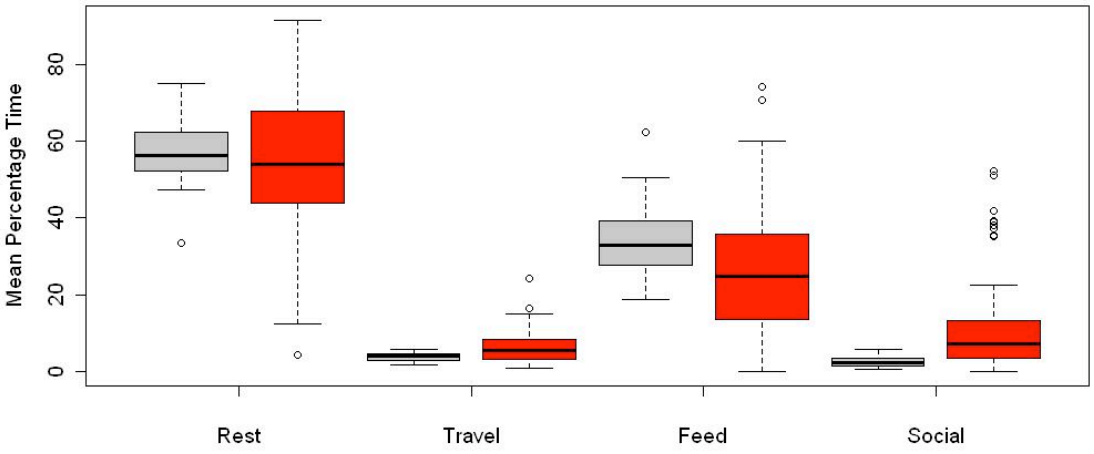

Figure 3.2 – Mean percentage of time dedicated to each activity for each focal group (BWC: grey, TRC: red). Results based on focal data (N=276h for BWC and N=252h for TRC)

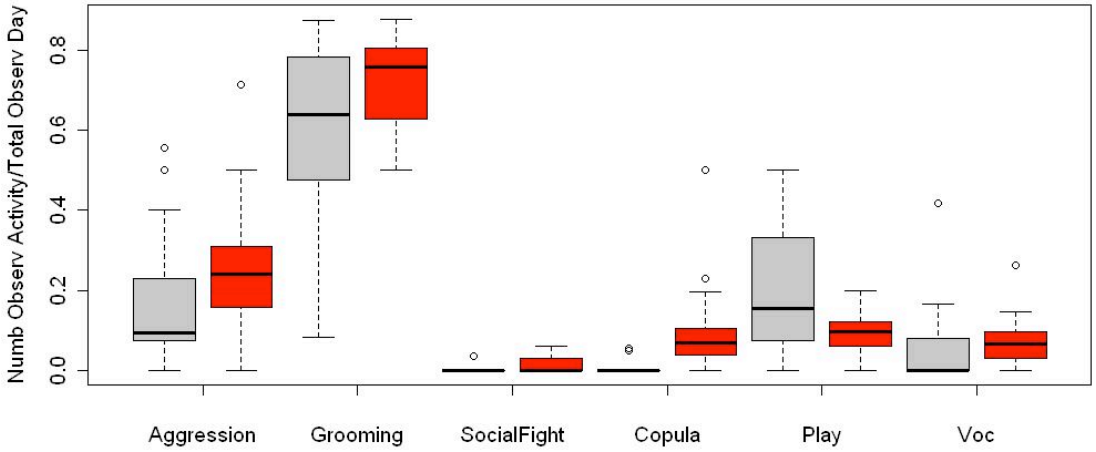

Figure 3.3 - Mean proportion of each social activity in the BWC (grey) and TRC (red) focal groups relative to the total number of social interactions recorded *per* observation day (N days=19).

3.4.2.2. Within-species comparisons

When sex-classes were considered was clear that agonistic and grooming events between adults had different patterns in the two species (Fig 3.4 and 3.5). In BWC, there was no difference among same sex dyads in aggressive behaviour

(Kruskal-Wallis chi-squared = 2.33 $p>0.05$) in contrast to grooming exchange (Kruskal-Wallis chi-squared = 7.20 $p<0.05$). Agonistic interactions between the adult and sub-adult male (0.053 ± 0.23, $N_{interactions}=1$) were less frequent than between females (0.21 ± 0.42, $N_{interactions}=6$) and female-male pairs (0.21 ± 0.42, $N_{interactions}=5$), although this was not significant. However, the adult and sub-adult male groomed each other significantly less (0.089 ± 0.24, $N_{interactions}=6$) than they changed grooming with females (0.31 ± 0.33, $N_{interactions}=38$) or than females groomed each other (0.29 ± 0.32, $N_{interactions}=37$) (Wilcoxon rank sum test, $p<0.05$). There was no difference in grooming exchange among females or the different sexes (Wilcoxon rank sum test, $p>0.05$).

In TRC, there was no significant difference in aggressive interactions between sex-class dyads (Kruskal-Wallis chi-squared = 5.11 $p>0.05$). However, a highly significant difference was found for grooming (Kruskal-Wallis chi-squared = 36.47 $p<0.01$). Females groomed each other (0.59 ± 0.25, $N_{interactions}=81$) significantly more often than they groomed males (0.36 ± 0.22, $N_{interactions}=41$) (Wilcoxon rank sum test, $p<0.01$). Adult males engaged in grooming events with other adult males very rarely (0.004 ± 0.02, $N_{interactions}=1$) and significantly less than when females are involved (Wilcoxon rank sum test, $p<0.01$).

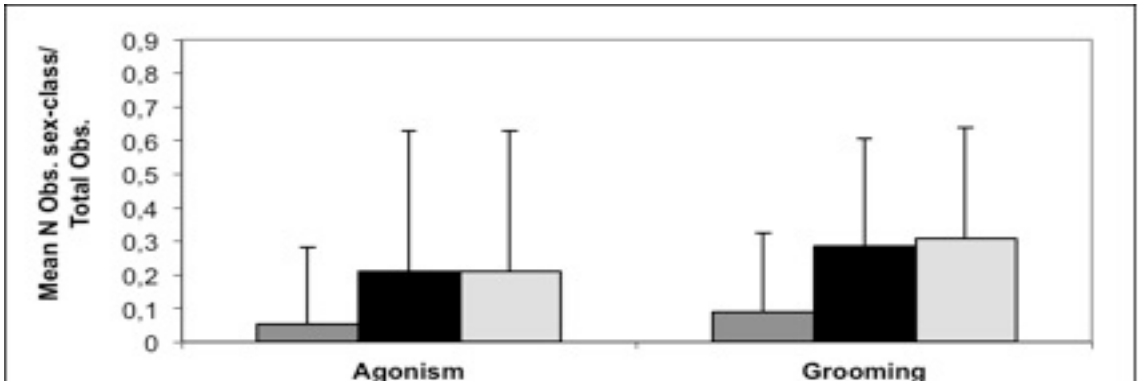

Figure 3.4 - Mean proportion of the number of agonistic and grooming events for each sex-class dyad (male-male: grey, female-female: black, male-female: light grey) in the BWC focal group (N days=19).

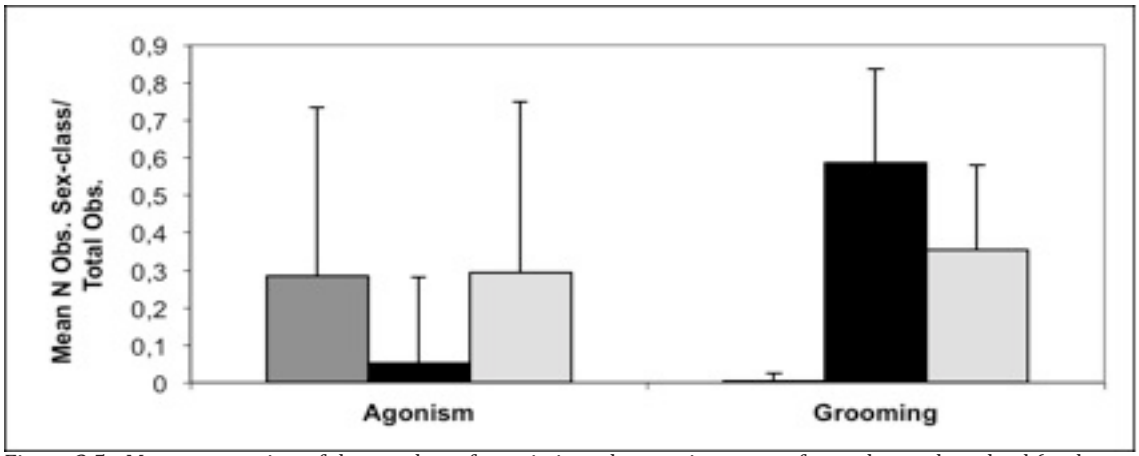

Figure 3.5 - Mean proportion of the number of agonistic and grooming events for each sex-class dyad (male-male: grey, female-female: black, male-female: light grey) in the TRC focal group (N days=19).

3.4.3. Intra-group relatedness

In the BWC focal group, from all 36 possible dyads only 8 (22.2%) are significantly related (Fig 3.6). From those, there is only one dyad of related adult females (r = 0.40, out of three possible dyads), meaning that there is one of the three adult females that have no adult female related in the group. Additionally, the adult male is significantly related with two of the adult females (r = 0.83 and r = 0.44, the same ones that are related to each other) and the juvenile female (r = 0.75). The sub-adult male is only related with the third adult female (r = 0.68) and the immigrant male that joined the group in 2010 has no related individuals in the group.

In the TRC focal group, from all 55 possible dyads of adult females only seven pairs are significantly related (12,7%; Fig 3.7). On the other hand, for the adult males, four out of six possible dyads (66,7%) are significantly related (Fig 3.7).

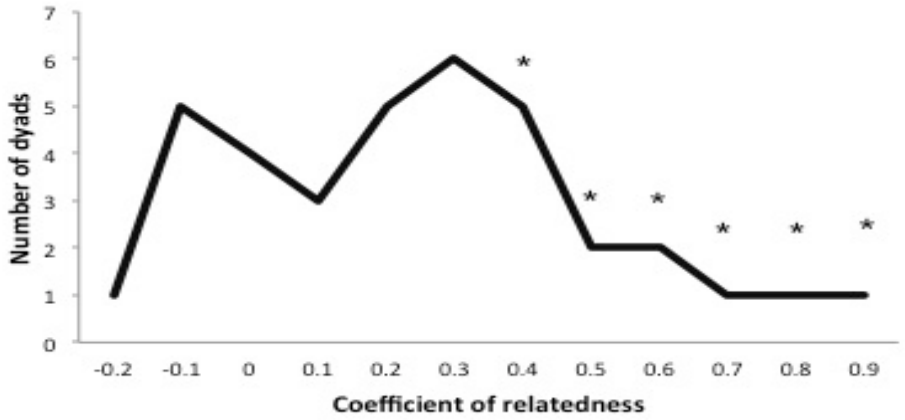

Figure 3.6 - Distribution of the number of dyads according with the pairwise relatedness value in the BWC focal group. These pairwise estimates include all individuals of the group. Stars (*)correspond to significant values. Relatedness is significant for all dyads between 0.5 and 0.9. For R=0.4 only one of the 5 dyads is significant.

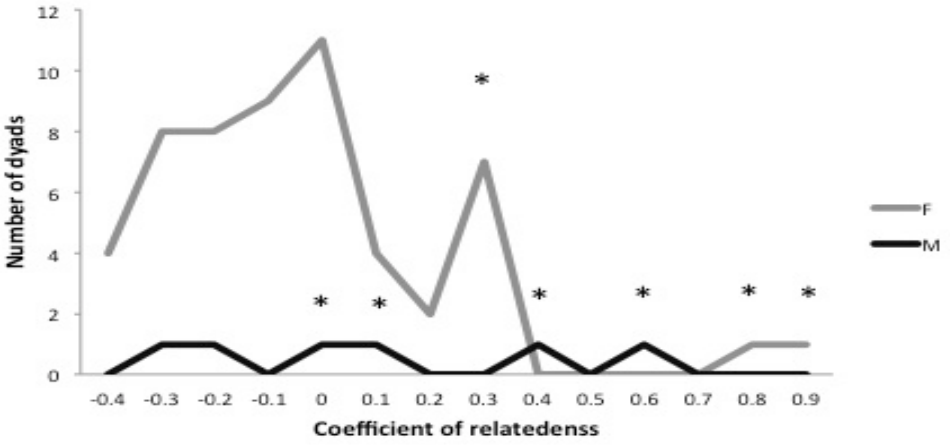

Figure 3.7 - Distribution of the number of dyads according with the pairwise relatedness value in theTRC focal group. Pairwise relatedness is estimated for females (grey) and males (black) seperatly. Stars (*)correspond to significant values. Relatedness is significant for all dyads between 0.4 and 0.9 and between 0 and 0.1. For R=0.3 only five of the seven dyads is significant.

3.4. Discussion

3.4.1. Time budgets

The two species exhibit similar patterns in the time they dedicated to each daily activity. Both spent most of their time resting followed next by feeding activities. Although both species are mainly folivorous they have shown some differences the in their dietary components. Black-and-white colobus' diet relies mainly on seed and mature leaves whereas red colobus consume higher percentages of young leaves and fruit pulp (Korstjens & Dunbar 2007) with a higher ratio of protein-to-fiber, known to be a good indicator of food quality for colobines (Waterman & Kool 1994). As a consequence of a lower quality diet, which requires more time to be digested, black-and-white colobus usually spend a higher percentage of their time resting than red colobus (reviewed in Korstjens & Dunbar 2007). This was not the case in the present study. The black-and-white colobus group spent a similar percentage of time resting (57%) to other *Colobus* species (43% - 70%) but the red colobus group resting time (53%) is amongst the highest values for all species studied (30-55%; reviewed in Korstjens & Dunbar 2007) but is most similar to the two western red colobus populations studied (Tiwai Forest: 55% (Davies at al. 1999) and Abuko 52% (Starin 1991). In the Tiwai population, for example, it was found no difference in the annual proportions of the different food types in the two species diet and therefore, one of the possible explanations for the increase of time dedicated to resting could be the adoption of a more BWC type of diet. There was no significant difference found in time spent in feeding activities between the two species although BWC spent around 10% more of their time in these activities. Compared with other conspecific populations, Cantanhez BWC showed the highest percentage of time dedicated to feeding which could be an indication of lower quality or less available food in this groups' home range. However, TRC exhibited a pattern comparable to other conspecific populations and not the same trend found for the BWC. Therefore, the reasons underlying the BWC feeding patterns cannot be fully understood with the data available. Both species spent a similar percentage of their time traveling and both values are amongst the lowest found for other studied populations (reviewed in Korstjens & Dunbar 2007). This finding is in agreement with the direct observation that both groups had very limited movement, never leaving the core area. This could be explained either due to: 1) severe forest fragmentation that prevents these individuals from easily moving to another area, 2) by intergroup competition with neighbouring groups or 3) by the safety provided by the village environment

against potential hunters. There were two neighbouring BWC and one TRC groups but aggressive encounters were never observed. However, the two closest areas where the focal groups could move into were known to be frequently visited by local hunters, so it is possible that all these factors contribute to the limited movement patterns of both studied groups.

The fact that TRC dedicated more of its time to social activities that BWC is a pattern that does not seem to be clear among other populations from the two species (reviewed in Korstjens & Dunbar 2007). Here, the time that BWC spent in social interactions is amongst the lower values (2.6%) compared with other sites (1-14%), with the opposite being true for TRC. Therefore, is seems that BWC individuals decreased their social time whereas TRC individuals spent more time strengthening social bonds. From the comparison with other study sites, it seems that in BWC the decrease in the time spent socializing and traveling was being invested in feeding activities whereas in TRC the time saved moving was being invested in social interactions. The fact that time was spent feeding in BWC and resting (known to be important for food digestion in colobines) in TRC may indicate lower food quality/quantity in these focal groups' home ranges. In TRC, the distribution of time spent in each activity resembled the Abuko Nature Reserve population more than any other studied so far. Both populations belong to the same subspecies, inhabit a severely fragmented area and spend significant social time with otherwise uncommon intra-group female and male bonding.

3.4.2. Role of kinship on social dynamics

Within the BWC focal group, we found that the adult and the sub-adult male are not related. Therefore the little grooming observed between them does not contradict the expectation that these individuals direct their grooming preferentially to related counterparts. Additionally, two of the adult females are related to each other and to the adult male, and the third adult female is only related to the sub-adult male. Therefore, since the group is composed by pairs of related females and females related to males, the fact that adult females do not show any preference to groom other adult females rather than males supports the idea that there is no evidence that kinship is not playing a major role shaping this group social dynamics.

On the other hand, in the TRC groups although only 12.7% of all pairs of adult females are significantly related they showed a preference to groom each other rather than to groom males. Moreover, despite the fact that 66.7% of the pairs of adult males are significantly related, adult males only groom other adult males very

rarely. These patterns of relatedness within each sex come in agreement with the pattern of mainly female dispersal found for the TRC population of Cantanhez and for this focal group in particular (Chapter 2). Although there are some related adult females, their proportion in the group seems to be very small in order to be able to justify the extremely high frequency of grooming exchange between females. Additionally, if kinship is the main factor shaping these group social dynamics we would expect the grooming between males to be more frequent, as it is in other studied red colobus groups. In fact, in this social group, the higher level of grooming between females than between males or different sexes contrasts with most studies that have reported higher levels of grooming between males and that females mostly target their grooming towards males (Struhsaker 1975; Struhsaker & Leland 1979; Were 2000). The paucity of grooming exchange between males observed in this red colobus group was also described for red colobus males in both Abuko (Starin 1991) and Jozani, Zanzibar (Siex 2003) but it was only in Abuko that females also showed preference to groom other females rather than males. Starin (1991) showed that Abuko red colobus had a stronger female-female bond and weaker male-male bond than is often described for groups where dispersal is female-biased. Some explanations have been put forward, namely, that of females transferring into the group along with other females from the same natal group (parallel dispersal, van Hooff 2000). In addition, the low number social units in this highly fragmented habitat favour the presence of immigrant females that share the same natal group occurring within the same group as adults. This observation could explain the existence of closely related females within a group and thus the stronger bonding between them. The behavioural patterns obtained for TRC focal group are more similar to those observed for Abuko Nature Reserve than for any other red colobus' population studied so far. It is possible that parallel dispersal occurs and such females prefer to groom each other instead of grooming non-related females. However, due to the low percentage of related females, parallel dispersal and kinship cannot fully explain the social dynamics found among these females. Consequently, there might be other factors besides kinship (e.g. high resource competition) that are strong enough for it to be advantageous for these females to establish strong social bonds with non-related females. The differences found in the time budgets between our study groups and colobus groups from other studies also support the evidence that there might be some strong intra-group resource competition. Before the foundation of the CNP, red colobus were a target species for human hunting. The fact that these particular groups have their home range overlapping the village indirectly protects them from both humans and others predators (e.g. chimpanzees). Poaching has never been

reported in these groups as opposed to several other groups in the surroundings (Hockings and Sousa, 2011). This may have favoured the increase of red colobus density in this area, therefore increasing the intra-group competition for resources.

By combining data on the time budgets, social interactions and patterns of relatedness we suggest that at least for the TRC focal group, when intense enough, ecological and/or anthropogenic-related pressures and not kinship can act as a major factor shaping social dynamics in this species. Nevertheless, the study of more social groups of both BWC and TRC in Cantanhez National Park would be important to confirm and strengthen our findings. Additionally, it would also be important to have data on individually recognized red colobus in order to understand if pseudo-replication is having a major impact biasing our results.

3.5. Acknowledgements

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Chapter 4

The interaction of social system, genetic structure and habitat fragmentation in two threatened primate species

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4.1 Abstract

The ability of forest dwelling primates to adapt to changes in their habitat is being increasingly challenged by the rapid pace of forest degradation due to human activities. The Western black-and-white colobus (BWC, *Colobus polykomos*) and Temminck's red colobus (TRC, *Procolobus badius temminckii*) are two forest-dependent threatened species, whose populations are reported to be declining throughout their range due mainly to habitat destruction and hunting. These two species share most of their ecological requirements but exhibit contrasting social systems in terms of group size and dispersal. We compared the genetic structure of these two sympatric species in a fragmented habitat in one of the last pockets of forest in Guinea Bissau. Using microsatellite genotype data from six black-and-white colobus and eight red colobus social groups in Cantanhez National Park we examined genetic structure at a variety of spatial scales. Only one genetic unit was found for each species. However, differences were found between the two species when the fine-scale spatial structure was investigated. In BWC geographic distance was the main factor determining the distribution of genetic diversity throughout the population. The combination of a pattern of "isolation-by-distance" with the fact BWC groups were found in most areas of the park visited seem to indicate that this species is still able to use and survive on the patches of available forest. On the contrary, TRC showed no correlation between pairwise relatedness and geographic distance. Moreover, TRC females relatedness is higher between immediately adjacent groups suggesting that females dispersal in this species is restricted to neighbouring areas, which could explain the disruption of the "isolation-by-distance" pattern. The combination of the fine-scale spatial genetic structure found for the TRC with the fact that this species was absent from the more degraded forest patches in the park, seems to suggest that higher susceptibility of TRC to cope with habitat loss and fragmentation when compared with BWC.

4.2 Introduction

Habitat loss and fragmentation are amongst the main contemporary threats to biodiversity (Goossens at al., 2006; Craul et al., 2009). Although natural processes can cause population fragmentation and structure, expansion of human land use is occurring at such at rapid pace that most natural populations are likely to be negatively affected (Gerlach & Musolf, 2000; Fahring, 2003; Epps et al., 2005). Besides reducing the total area of suitable habitat, the negative effects of landscape

modification include habitat fragmentation that splits populations into several units, isolated from each other and usually of smaller size than the original (Frankham, 2006; Frankham et al., 2002). As result, a decrease of genetic diversity is expected to occur, reducing the evolutionary potential of the population and increasing the risk for extinction (e.g. Frankham et al., 2002; Keller & Waller, 2002; Johansson et al., 2007). Population genetic data can provide insights on the challenges faced by fragmented wild populations (Chikhi & Bruford, 2005; Goossens et al., 2005; Quéméré et al., 2010; Zhu et al., 2010). Studying the spatial distribution of genetic variation potentially allows the identification of habitat discontinuities responsible for disruptions in demographic structure (Manel et al., 2003; Chikhi & Bruford, 2005). The use of highly variable molecular markers in such analyses makes it possible to infer dispersal patterns (Lawson Handley & Perrin, 2007), which provides crucial information for conservation plans for threated populations.

The extent to which habitat fragmentation affects a species is highly dependent on its ability to disperse, the degree of habitat fragmentation and the matrix of habitat between patches (Frankham et al., 2002; Frankham, 2006). Species that are capable of maintaining high dispersal rates despite habitat fragmentation are likely to be the least affected (Kareiva, 1987; Debinski & Holt, 2000; Villard, 2002). On the other hand, less mobile species or species highly dependent on the fragmented habitat are more likely to experience populations isolation (e.g. Goossens et al., 2006; Liu et al., 2009). Western black-and-white colobus (BWC; *Colobus polykomos*) and Temmink's red colobus (TRC; *Procolobus badius temminckii*) are expected to illustrate the latter situation, since they are threatened (Oates et al., 2008a,b) forest-dependent species that are often found in sympatry. They share most ecological requirements having predominantly arboreal lifestyles within tropical woodlands (Oates, 1994), feed mainly on young foliage, seeds, some mature leaves and flowers and fruits (Oates et al., 1994). However, they feature contrasting social systems: BWC live in groups that often comprise one male and multiple females and never exceed 16 individuals (Oates, 1994) and TRC live in much larger multi-male, multi-female groups of 25-40 individuals (Oates, 1994). Additionally, BWC dispersal is reported to be predominantly male mediated, although some episodes of female dispersal have been noted (Korstjens *et al.,* 2002, Minhós et al., Chapter 2), whereas in TRC females are the main dispersers (Starin, 1994; Minhós et al., Chapter 2). Since these species have contrasting social systems, but at the same time share most ecological requirements, makes them interesting models to test whether different social systems affect a species' ability to adapt to habitat loss and fragmentation. A survey of the eastern black-and-white and red colobus (*Colobus guereza* and *Piliocolobus*

pennantii, respectively) in a highly fragmented habitat in Uganda, revealed that they respond differently to landscape changes (Onderdonk & Chapman, 2000): while *C. guereza* were found in all forest patches surveyed (0.8 – 130 ha), *P. pennantii* groups were absent from most, implying that red colobus may be more sensitive to habitat fragmentation than black-and-white. The ability of black-and-white colobus to adapt their diet, home range and group size to smaller forest patches was highlighted as a possible explanation for their higher resilience to such degraded forest and similar flexibility was also described for the same species in Ethiopia (Dunbar, 1987) and Kibale (Uganda; Struhsaker, 1997). In Guinea Bissau BWC and TRC live in sympatry and their groups often overlap in home range (Gippoliti & Dell'Omo, 1996, 2003; Minhós et al., Chapter 3). Both species are reported to have a patchy but broad distribution in the country (Gippoliti & Dell'Omo, 2003) but the most recent census indicates that they are disappearing from most areas (Casanova & Sousa, 2007). Cantanhez National Park (CNP), in South-West of Guinea Bissau is one of the last areas in the country where forest, although highly fragmented, still persists and is home of the biggest populations of both species (Fig. 4.1; IBAP, 2007).

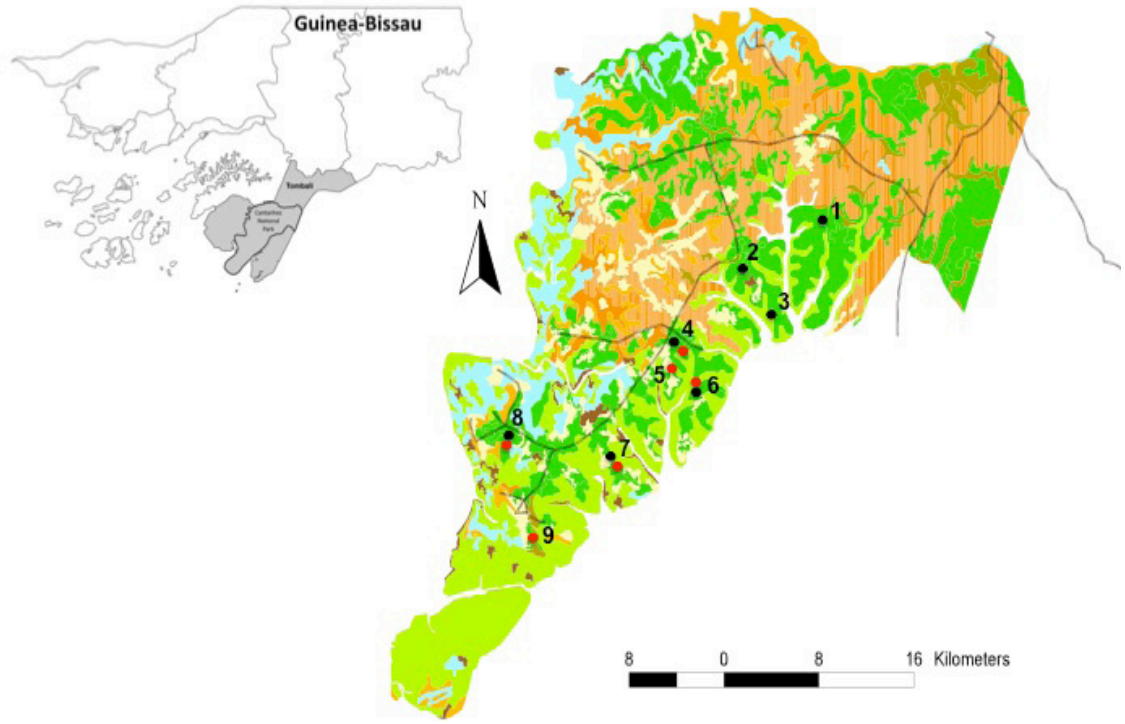

Figure 4.1 Map of the land cover for Cantanhez National Park (provided by INEP): dark green – forest; light green – mangrove; yellow – savanna; blue – rice fields; beige – crops; brown – tannes. Black circles: black-and-white colobus sampled groups; red circles: red colobus sampled groups. 1 – Cancira; 2 – Amidara; 3 – Deep Amidara; 4 – Focal and Neighbor; 5 - Madina; 6 – Cambeque; 7 - Cangode; 8 – Muna; 9 – Cungha.

Here we investigated the genetic structure of these two sympatric species and its interaction with forest fragmentation in CNP, using non-invasive molecular techniques. First, we analyzed population genetic variation in order to detect large-scale genetic structure. Secondly, we analyzed individual pairwise relatedness at a fine spatial scale to detect discontinuities in dispersal. If dispersal is conducted in a stepwise manner, a pattern of "isolation-by-distance is expected. However, when dispersal is very extensive or restricted, such genetic pattern can be erased (Hutchison & Templeton, 1999). Due to their high dependence on the forest, we expect both species to be affected by forest fragmentation in Cantanhez. Nevertheless, TRC population was expected to be affected more heavily than BWC, as already reported in East Africa. If the effect of habitat fragmentation is recent we may not detect major population differentiation. However, if the species ability to disperse within CNP is being restricted, we should expect a disruption of the "isolation-by-distance" pattern (Hutchison & Templeton, 1999), where the pairwise relatedness at adjacent, but not distant groups, is higher than expected (Peakall et al., 2003; Vignieri, 2007). This comparative study was carried out to infer the interaction between social system and species adaptation under anthropogenic habitat disturbance and to suggest conservation priorities in these threatened populations.

Table 4.1. Expected relation between geographic and genetic distance under different levels of dispersal

Level of dispersal	Isolation-by-distance	Explanation
"Stepping stone"	Observed	If the geographic distance is the only to constraint to dispersal, individuals will tend to disperse to geographical close areas in a stepwise manner continuously throughout the population
Extensive	Disrupted	If the rate of dispersal is very high and individuals are able to disperse long distances, there will be no correlation between geographic and genetic distance
Interrupted	Disrupted	If there is any barrier (e.g. geographic or anthropogenic) limiting or interrupting dispersal, the correlation between geographic and genetic distance will be disrupted

4.3 Material and Methods

4.3.1 Study site and social groups

Cantanhez National Park is located on a peninsula (total area: 1.067 Km^2), in south-western Guinea-Bissau (NE limit: 11°22'58''N, 14°46'12''E; SW limit:

11°2'18''S. 15°15'58''W; Fig. 4.1), where the forest is now fragmented into patches (ranging from 47.5 to 2,500 ha; Simão, 1997) most of which are connected to adjacent patches in some way. Before being declared a National Park in 2008, Cantanhez forests were considered critical sites for biodiversity conservation in Guinea-Bissau (e.g. IUCN, 1993), with some of the fragments being protected by local people. Despite being a National Park, where logging activities and hunting are illegal, the national management authority (IBAP) does not operate in the park due to financial constraints. As a result, deforestation and primate hunting remain current threats to the colobus populations (Hockings & Sousa, 2011; TM personal observation).

Extensive surveys were conducted in the park between February 2009 and April 2010. Patches where both species could not be found on the first visit were revisited several times until sampling could be carried out or an assumption of absence in the area could be made. A total of 380 faecal samples were collected. One social group per forest patch was sampled in each fragment where colobus were detected (Fig. 4.1). Only feces that were 2m or more apart were collected in order to minimize the chances of sampling the same individual multiple times. Samples were stored using the two-step approach (Roeder et al., 2004). A total of eight BW and six RC social groups were analyzed (Fig. 4.1).

4.3.2 DNA extraction and microsatellite genotyping

Detailed description on DNA extraction procedure, PCR amplification of 15 microsatellite *loci*, molecular sex-identification and electrophoretic analysis are provided in Chapter 2. Methods used to deal with genotyping errors due to low quantity/quality DNA extracted from faecal samples and the presence of repeated genotypes in the sample were as in Chapter 2. After excluding low quality samples (see Chapter 2) 52 BWC individuals from eight social groups (11-14 *loci,* 97.2% complete genotypes, mean quality index of 0.84; Table 4.1) and 72 TRC individuals from six social groups (10-13 *loci*, 96,5% complete genotypes, mean quality index of 0.77; Table 4.1) were analysed. Detection of null alleles was conducted in Micro-Checker (van Oosterhout et al., 2004) and exact tests for Hardy-Weinberg and linkage disequilibrium were performed using FSTAT v2.9.3.2 (Goudet, 2001). When sampling social groups, the presence of highly related individuals may induce population substructure resulting in a deficit of heterozygotes (e.g. Chikhi & Bruford, 2005). After removing such related individuals from the sample, none of the *loci* showed evidence for null alleles, linkage disequilibrium or deviations from Hardy-Weinberg equilibrium.

4.3.3 Data analysis

Population genetic structure was investigated using two Bayesian model-based approaches and the consistency of results was compared. Since these approaches use multi-locus genotypes to cluster individuals into populations that minimize Hardy-Weinberg (HW) and linkage disequilibrium (LD), any departure from random mating leads to subdivision of the data into sub-populations (Pritchard et al., 2000; Beaumont & Rannala, 2004). We first used STRUCTURE 2.2 (Pritchard et al., 2000), without a spatial prior, to detect the optimal number of genetic clusters (K). We allowed K to range from one to ten, using five independent runs, with 1,000,000 Markov chain Monte Carlo (MCMC) iterations, after a 100,000 burn-in period. Runs were performed under the admixture module and allele frequencies were assumed to be correlated. The most appropriate K was chosen following the summary statistic ΔK described by Evanno et al. (2005), which is based on the rate of change in the log probability of the data between successive K values. Secondly we used a spatially explicit approach using the software BAPS 5 (Corander et al., 2004). We ran BAPS using individual spatial clustering with five replicates varying the maximum K value at 5, 10, 15 and 20. The value of K that best explains the genetic partition of the data is given by its highest probability. When sampling social groups, the presence of highly related dyads of individuals in the sample induces family-based structure that does not correspond to a true population (Pritchard & Wen, 2004; Bergl & Vigilant, 2007; Anderson & Dunham, 2008). In order to control for this bias, both packages were first run with all set of individuals and after, with a restricted dataset where one individual belonging to each dyad related above 0.5 was removed.

We estimated mean pairwise relatedness using COANCESTRY v1.0 (Wang, 2011). The Queller and Goodnight (1989) relatedness estimator was used with 1000 *per-locus* bootstraps to achieve the 95% confidence interval for each dyad. Analysis was run for all dyads within group, and females and males separately.

We investigated fine-scale spatial genetic structure by analysing isolation-by-distance and spatial autocorrelation using the entire genetic dataset and for the female and male subset of dyads separately. GenAlEx 6.41 (Peakall and Smouse, 2006) was used to perform a Mantel test to estimate the correlation between the Queller and Goodnight relatedness estimator and geographical distance. The significance of results was assessed by 9999 permutations. Spatial autocorrelation of individual genotypes were compared with those from neighbouring areas at set distance intervals. Any deviation of these relationships from zero indicates that individuals are more (positive values) or less (negative values) related than expected

at random. This analysis was conducted in SPAGeDI 1.3 (Hardy & Vekemans, 2002) and spatial categories based on the even distribution of the number of dyads within ten categories for BWC and eight for TRC. Permutation tests (10 000) were conducted to establish 95% confidence intervals. The first distance class corresponds to comparison of dyads of individuals from the same social group. All other classes comprise individuals belonging to the next most adjacent group.

4.4 Results

4.4.1 Genetic structure at the population level

Analysis of population structure using STRUCTURE and BAPS revealed concordant results but different genetic partitions were obtained when using the entire and reduced dataset, especially for TRC. STRUCTURE analysis with the entire BWC dataset showed highest ΔK at four genetic clusters, although no difference in the distribution of population assignment values was found among individuals, suggesting an absence of overall population structure (Fig. 4.2a). This pattern was supported by BAPS spatial analysis (Fig. 4.2c) where only one genetic cluster was detected, but with low probability (p=0.36884). After removing BWC individuals belonging to dyads with relatedness above 0.5 (analysis carried out with 37 individuals), the same K=4 was obtained using STRUCTURE (Fig. 4.2b). BAPS also supported the absence of population genetic differentiation (K=1) but with much stronger support (p=0.99998) than obtained with the complete dataset. For TRC K=6 was obtained for the entire dataset (Fig. 4.3a) with clear differentiation for at least three genetic clusters but with no correspondence to social groups. Results from BAPS reinforced the existence of genetic structure, inferring nine differentiated clusters (p=0.7161) also non-coincident with social groups (Fig. 4.3c). Both analyses suggested the existence of genetically differentiated individuals within social groups. However, when dyads related above 0.5 were excluded from the analysis, all signatures of population genetic differentiation were lost. Although in STRUCTURE the ΔK was highest for two clusters, the distribution of population assignment values for all 40 individuals used in the analysis were similar (Fig. 4.2b) implying that all belonged to the same genetic population. BAPS supported this lack of differentiation, as the scenario of a single genetic entity (K=1) best explained the reduced genotypic dataset (p=0.98064) (Fig. 4.3d). These data, taken together, strongly indicate that for both species, all individuals in Cantanhez National Park are part of the same genetic unit. The apparent genetic differentiation, especially between TRC individuals was a consequence the extensive sampling of family members.

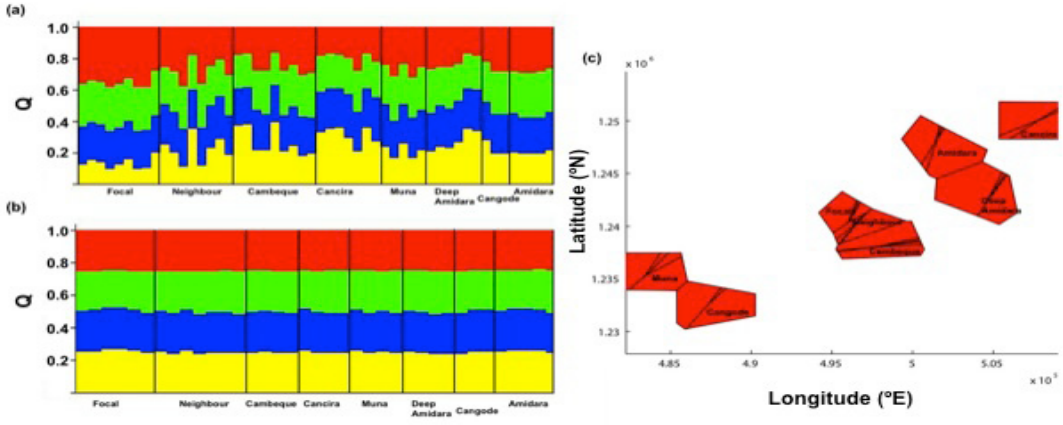

Figure 4.2 Bayesian population genetic clustering of black-and-white colobus using SRUCTURE (non-spatial; a,b) and BAPS (spatial; c) softwares. Different colours represent different genetic clusters and the names represent different social groups. With STRUCTURE, each column represents a different individual. Both analyses were run with the total dataset (a, c) and excluding individuals that belong to dyads related above 0.5 (b,c).

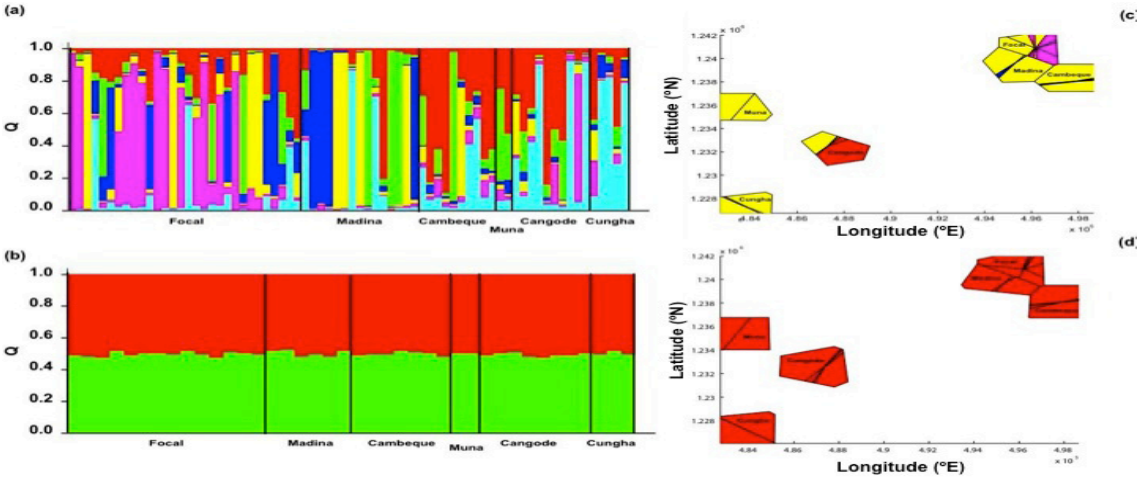

Figure 4.3 Bayesian population genetic clustering of red colobus using SRUCTURE (non-spatial; a,b) and BAPS (spatial; c,d) softwares. Different colours represent different genetic clusters and the names represent different social groups. With STRUCTURE, each column represents a different individual. Both analyses were run with the total dataset (a, c) and excluding individuals that belong to dyads related. above 0.5 (b,d).

4.4.2 Fine-scale genetic structure

The within-group relatedness analysis revealed that overall, BWC showed higher levels of group relatedness than TRC (Table 4.2). BWC exhibited mean group pairwise relatedness of approximately 0.15 in six of eight sampled groups (Fig. 4.4), whereas for TRC, four of the six social groups showed mean dyad relatedness close to zero (Fig. 4.5). Pairwise relatedness was not systematically higher for either sex in both species (see details in Chapter 2). For BWC, within-group relatedness was highest in the Amidara group and it was also in this group that females were more related to each other. However, for the most northerly group (Cancira) males showed higher relatedness. For TRC, the Muna and Cungha social groups comprised

individuals that were more related. However, both these groups are represented by small sample sizes and only one sex (two males in Muna and five females in Cungha).

Table 4.2 Pairwise relatedness for all individuals, males and females within each social group.

Social Groups	R Social Group	R Females	R Males
Western black-and-white colobus			
Focal	0,1665 (9)	0.22810 (4)	0.06769 (5)
Neighbour	0,1629 (8)	-0.04087 (4)	0.25135 (4)
Cambeque	0,1576 (9)	0.20834 (6)	0.07127 (3)
Muna	-0,0058 (7)	-0.23223 (3)	0.09378 (4)
Deep Amidara	0,0239 (5)	0.10617 (4)	NA (1)
Cancira	0,2217 (6)	0.15390 (4)	0.57710 (2)
Cangode	0,1704 (3)	0.06880 (2)	NA (1)
Amidara	0,3021 (5)	0.39853 (3)	0.18690 (2)
Temmink's red colobus			
Focal	0,05695 (30)	0.06641 (22)	0.05035 (8)
Madina	-0,00764 (15)	0.01628 (13)	-0.13510 (2)
Cambeque	0,00197 (10)	0.00036 (10)	NA (0)
Muna	0,3988 (2)	NA (0)	0.39700 (2)
Cangode	-0,02525 (10)	0.00319 (9)	NA (1)
Cungha	0,25486 (5)	0.24948 (5)	NA (0)

Note: Numbers in () correspond to the number of individuals used in each class

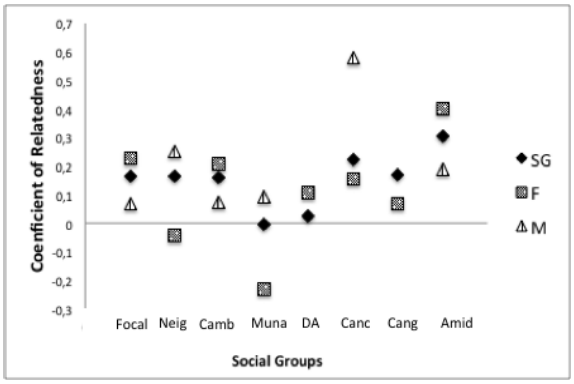

Figure 4.4 Mean pairwise relatedness within black-and-white colobus social groups considering: females and males together (SG), only females (F) and only males (M). Names on the x axis represent the eight social groups: Focal, Neighbour (Neig), Cambeque (Camb), Muna, Deep Amidara (DA), Cancira (Canc), Cangode (Cang) and Amidara (Amid).

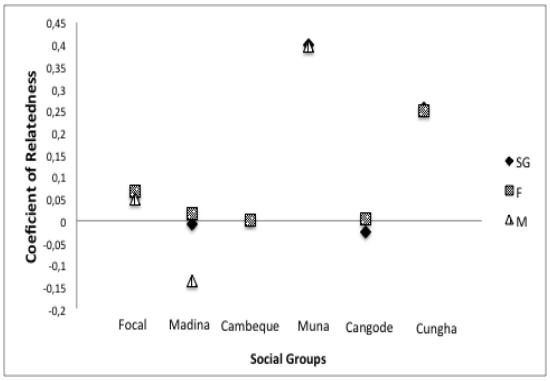

Figure 4.5 Mean pairwise relatedness within red colobus social groups considering: females and males together (SG), only females (F) and only males (M). Names on the x axis represent the six social groups.

Mantel tests provided contrasting results for the two species. BWC showed a significant negative correlation between pairwise relatedness and geographic distance, for social groups, males and females (Fig. 4.6), implying that for BWC, geographic distance plays an important role in shaping the distribution of genetic diversity in the population. In contrast, for TRC no correlation was found between dyadic relatedness and geographical distance (Fig. 4.6). This was especially true where entire social groups and only females were considered (Fig 4.6a-b). For TRC males, there was a tendency for a negative correlation, which may not have reached significance due to the limited number of males in the sample (Fig. 4.6c).

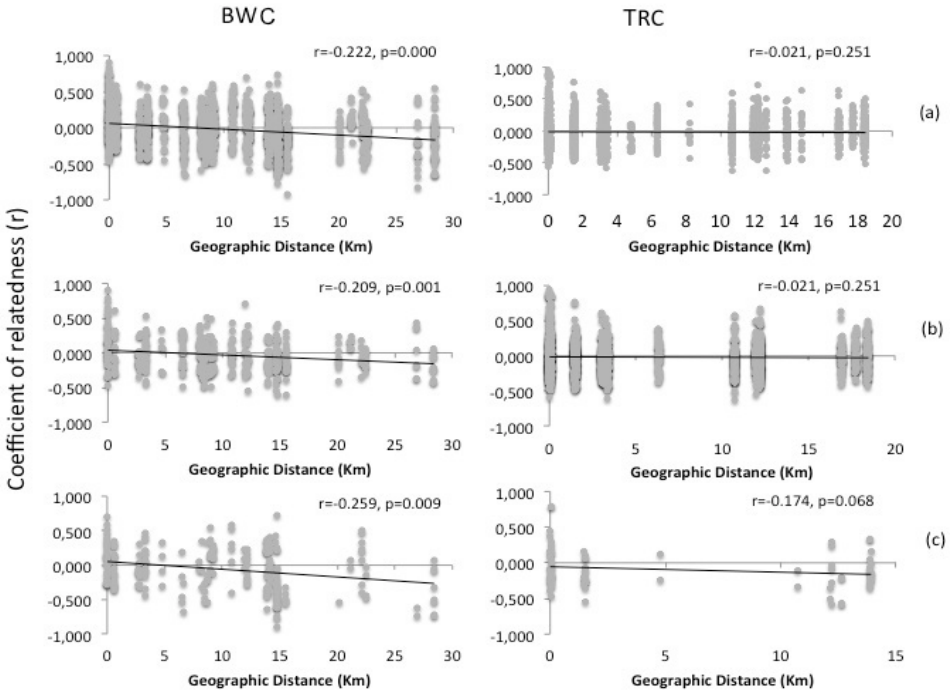

Figure 4.6 Correlation (r) between pairwise relatedness (Queller and Goodnight, 1989) and geographic distance (Km) for black-and-white colobus (BW) and red colobus (RC). Each dot represents a dyad of individuals. Mantel tests were performed for males and females together (a), only females (b) and only males (c).

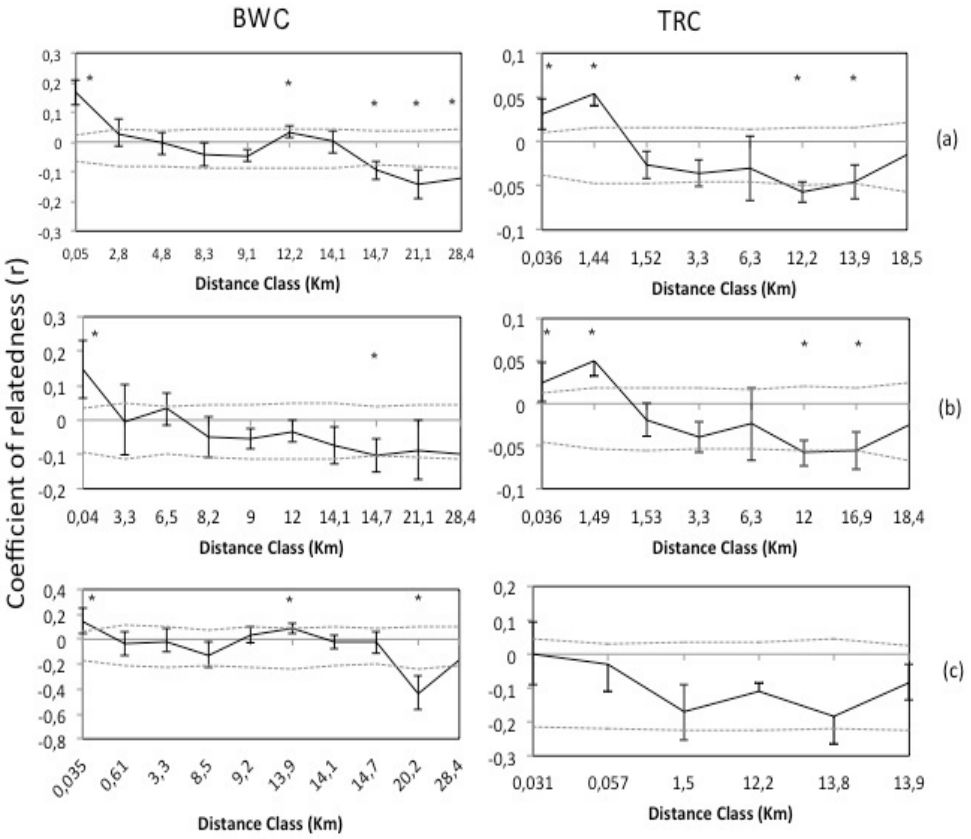

Figure4.7 Spatial autocorrelation using pairwise relatedness (Queller and Goodnight 1989, black line) and pairwise geographic distance (Km) for black-and-white colobus (BW) and red colobus (RC). Stars (*) represent significant deviation from the 95% interval (dashed grey lines) to the null hypothesis of relatedness=0. Analyses were performed for males and females together (a), only females (b) and only males (c).

For the first distance class of the spatial autocorrelation analysis (corresponding to within group dyads), all individuals of both species were significantly positively related to each other (except TRC males), as is expected from group-living organisms (Fig. 4.7). In BWC, with all individuals included (N=132-133 dyads), relatedness was higher than expected at the considerable distance interval of]9.1 – 12.2] Km (r=0.034, p<0.05). At distances further than 14.1Km all BWC were less related than expected at random (p<0.01). Female BWC (N=43-44 dyads) were found to be less related at the interval of]14.1-14.7] Km (r=-0.103, p<0.05), whereas males (N=23-24 dyads) showed a significant positive relatedness at]9.2-13.9] Km (r=0,085, p<0.05) and negative at]14.7-20.2] Km (r=-0.438, p<0.0001). Because females constitute most of the TRC sample, spatial autocorrelation patterns of females were similar to the ones obtained for entire social groups. In the second distance class (max. 1.5km), RC ($N_{social\ group}$=319-320; $N_{females}$=213-214) were still significantly more related than expected at random ($r_{social\ group}$=0.055, p<0.001; $r_{females}$=0.050, p<0.0001) and were negatively related at further distances between]6.3-16.9] Km (p<0.05). However, no deviation from expectation was observed at the maximum distance of 18.4 km ($r_{social\ group}$=-0.016, p=0.504; $r_{females}$=-0,024, p=0.609). No deviation from the expected relatedness patterns for RC males was found (N=13) for any of the distance classes.

4.5 Discussion

4.5.1 Absence genetic structure at the population level

Overall we found an absence of broad population structure in the two colobus species in Cantanhez National Park. Both clustering approaches used (spatial and non-spatial) were concordant. The differences rested however when accessing the population subdivision using the total dataset or a reduced subset. This difference was particularly important for the TRC population where evidence for population structure was found with the entire dataset, a signal that disappeared with the reduced subset of samples. The bias imposed by the presence of family members in the dataset proved to have great influence in inducing genetic structure. Initially, STRUCTURE suggested the existence of six genetic clusters whereas BAPS found nine. Regardless of the differences in number of clusters found with the two approaches, both clearly indicated genetic subdivision in the population. However, both approaches converged on a single genetic unit after the removal of highly related individuals, clearly indicating that the structure initially found was a family induced structure rather than a true population subdivision. A few studies have accounted

for the bias of extensively sampling family members when interpreting population subdivision (e.g. Berry et al., 2004; Bergl & Vigilant, 2007; Quéméré et al., 2010), and, to our knowledge, there is only one that specifically addresses this problem by analyzing real and simulated data in rainbow trout (*Oncorhynchus mykiss*) using STRUCTURE (Anderson & Dunham, 2008). These authors found that the presence of family members in the sample led to the inference of K>1 even when all individuals were from the same population, and that this bias was stronger when full siblings where present in the dataset. They noticed that the non-authentic population structure was more likely to occur within populations with high reproductive skew and where a large fraction of the population was sampled. The colobus from Cantanhez may illustrate both problems. First, as in most primates, it is very likely that high variance in the reproductive success exists, especially for males. Secondly, the fact that both are endangered forest dwelling species and that Cantanhez National Park is a relatively small and isolated area of fragmented forest implies the existence of small populations and that a large fraction of both were sampled. The authors only tested spurious structure induced by family members with STRUCTURE. They suggested that other clustering softwares such as BAPS and PARTITION may behave similarly, although this has not been tested yet. Here, we have confirmed this bias with STRUCTURE using empirical data and have shown that BAPS is as sensitive to this type of sampling as STRUCTURE. Our results highlighted the fact that such clustering approaches are an important tool to be used as exploratory analyses (Chikhi & Bruford, 2005; Anderson & Dunham, 2008), but their outcome should be interpreted with caution.

4.5.2 Spatial genetic structure in a fragmented habitat

One of the main contrasting results we obtained with this comparative study was that the distribution of the genetic diversity in BWC followed an "isolation by distance" model, whereas this was not the case for TRC. The negative correlation between pairwise relatedness and geographic distance found in BWC males and females shows that BWC individuals tend to disperse in a stepwise manner and that gene flow is possible throughout the park with no major barriers. Despite the absence of continuous forests in the park, some connectivity is still maintained between most of the patches. Our results suggested that BWC may still be able to cope with this level of fragmentation and use the available forest corridors. The ability of BWC to persist in fragmented forest has already been noted in previous studies (Dunbar, 1987; Struhsaker, 1997; Onderdonk & Chapman, 2000). Additionally, the fact that

a linear correlation between relatedness and geographic structure exists for both sexes agrees with previous evidence for dispersal mediated by both sexes (Chapter 2) in this population. The spatial autocorrelation analysis showed some degree of fine-scale genetic structure. Overall, BWC individuals were more related than expected at random between 9 and 12km distance, a pattern also found for males but not in females. This may indicate that males are able to disperse longer distances if habitat connectivity exists. If females disperse in order to avoid kin competition for resources, dispersing over short distances may be enough to solve such problem. Additionally, by staying in the surroundings of their natal social group they avoid travelling and foraging in unfamiliar areas (Lawson Handley & Perrin, 2007). At further distances (14 to 30km) the analysis with all individuals included showed that individuals are negatively related, as expected when dispersal is conducted in a stepwise manner.

In TRC, the lack of a correlation between pair-wise relatedness and geographic distance suggests that there might be other factors shaping population genetic structure. In populations with a "stepping-stone" mode of dispersal some pattern of isolation by distance is expected. Both extensive or interruption of gene flow can disrupt the expected correlation between genetic and geographic distance (Hutchison & Templeton, 1999). For males, inference must be cautious due to their limited number and distribution in our sample. Nevertheless, TRC females, contrary to BWC females do not seem to be able to disperse continuously throughout the park. Red colobus were more related than expected at the first distance interval where pairwise comparisons include individuals from adjacent groups (approx. 1.5Km), and then relatedness decreases considerably when pairwise relatedness includes individuals from other social groups rather than just the neighbour group. This may be the reason for the disruption of the isolation-by-distance pattern, indicating that in this female dispersing population (Chapter 2), TRC females are restricted by some unknown factor (e.g. geographical, anthropogenic) to disperse to groups that are immediately adjacent to their natal territory. The absence of an isolation-by-distance pattern together with the genetic structure found with the spatial autocorrelation analysis indicates that some discontinuities in gene flow may exist for this species. Similar dispersal dynamics among red colobus females living in highly fragmented habitats has also been described by Starin (1994) in Abuko Nature Reserve, The Gambia. The fact that this strong structure was not found for BWC females (where geographic distance plays a major role), suggests the lower ability of TRC females to disperse to social groups other than their immediate neighbors.

4.5.3 Response of the two social systems to forest fragmentation

Our results were able to demonstrate the importance of using these two sympatric colobine species as a model to understand the response of different social systems to the same human altered habitat. Both species are highly dependent on the existence of forest and share great similarities in their ecological requirements. They contrast however in their social system which should contribute to their different sensitivity to habitat fragmentation. Previous studies were not conclusive in explaining why red colobus are more sensitive to habitat modification. The fact that they live in bigger social groups and demand a more varied diet are the main possibilities in explaining their lower ability to cope with habitat degradation and living in small forest patches (Onderdonk & Chapman, 2000). Using genetic data we were able to show that the two species may respond differently to the same habitat pressures. The fact that no broad population differentiation was found for either species may indicate that the effect of habitat fragmentation on red colobus genetic structure is still either recent and/or not so severe, only being detected with fine-scale analyses. Additional evidence supporting the fact that TRC are more affected by habitat degradation is the fact that they were not found in all areas of the park (see Fig. 4.1). Whereas BWC existed in almost all patches, TRC were not found north of Iemberem (center of the park). In the northern region of the park the forest is more fragmented and illegal hunting and logging is less controlled. While local communities have historically preserved forest fragments and their biodiversity south of Amidara, in the northernmost forests, land use and resources exploitation are not controlled (Temudo, 1998, 2009). Some social groups were detected around Amidara in 2008 and 2009 but not in 2010 (TM, personal observation). The lack of samples in the northern of the park may suggest either lower density or even the absence of this species in these more altered areas. However, BWC are still able to persist there. Even if TRC groups are still present in the deeper areas of these forests, the fact that they were not found together with BWC in more degraded habitats supports the notion of their higher susceptibility to this threat. Although fragmented, Cantanhez comprises one of the few forest pockets of Guinea Bissau. Hence it is most probably the major focus for colobus populations in the country (IBAP, 2007). Residual populations may still exist outside CNP but recent reports indicate that they are severely threatened and rapidly decreasing (Casanova & Sousa, 2007; IBAP, 2007; Ferreira da Silva, 2012). Even though we cannot exclude the possibility that the fragmentation of the forest is already affecting BWC in Cantanhez, our results suggested that the response to this threat is stronger in TRC. The higher flexibility of

black-and-white colobus to change their mode of dispersal (Chapter 2), group size and diet in smaller and fragmented areas of suitable habitat may determine their better survival in anthropogenically disturbed habitats.

4.5.4 Conservation implications

In the present study we have shown the absence of major barriers to colobus dispersal in Cantanhez despite increasing habitat fragmentation. If this may suggest good news, analyses of fine-scale spatial genetic structure suggests the TRC population is more affected by the degradation of their habitat. Although BWC seems to adapt more easily to such a level of habitat degradation, we caution that in the case of TRC this situation could become critical very soon. As noticed from previous visits, TRC groups are decreasing or even disappearing from areas where they used to be found. Cantanhez is the last area in Guinea Bissau where colobus still exist in viable numbers and with some suitable habitat (IBAP, 2007). Consequently the timing could be critical for conservation measures to avoid the extinction of red colobus from Guinea Bissau. Although BWC may be more resilient, they will most likely face the same threat if no habitat protection is conducted. While is imperative that the existent corridors are maintained in order to assure the gene flow through the park, it is important that forest is restored, especially in the northern part of the park, in order for TRC groups to reestablish. In addition to forest loss and fragmentation, both colobus are also targets for hunting and bushmeat consumption. Again, although this threat seems to affect TRC more than BWC (Appendix five), it will only accelerate the population decrease of both species. Therefore, future conservation plans should not only consider habitat connectivity and rehabilitation but also law enforcement towards illegal hunting.

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CHAPTER 5

General Discussion

This dissertation has examined the dispersal patterns, social dynamics and the genetic structure in social groups of two sympatric and endangered colobus monkeys in Cantanhez National Park, Guinea Bissau. Western black-and-white colobus (BWC; *Colobus polykomos*) and Temminck's red colobus (TRC; *Procolobus badius temminckii*) are both forest dwelling species that share most of their ecological requirements but exhibit contrasting social systems. I tested whether such differences could lead to different patterns of genetic structure and diversity and by inference, to different abilities to adapt to the same fragmented habitat. Combining genetic, behavioural and spatial data I provided the most complete study on population and conservation genetics of African colobus populations. A picture of spatio-temporal patterns of dispersal, intra-social group relatedness, social interactions and time budgets was produced. Finally, broad and fine-scale spatial genetic structure of both populations was revealed. Besides contributing to the understanding of the evolution and adaptation of primate societies, the present study provides important insights for the conservation of colobus in Guinea Bissau.

5.1 Overview of main results

5.1.1 Chapter 2: Genetic evidence for spatio-temporal changes in the dispersal patterns of two sympatric African Colobine monkeys

In this chapter the spatio-temporal patterns of dispersal were examined by analysing genetic data using different molecular markers. The hypothesis that genetic data would support observations of mainly male dispersal in BWC and female dispersal in TRC was tested. Analyses of the mitochondrial DNA suggested historical and/or long-range dispersal for males in BWC and females in TRC. This was evident from the extremely low mitochondrial diversity (only three haplotypes) found within the BWC population. On the other hand, eleven haplotypes were found for the TRC population, belonging to three very divergent haplogroups and with most of the genetic variation being found within social units. However, both population- (AMOVA and assignment tests) and individual-based (within group sex relatedness) tests for current sex-biased dispersal gave evidence for dispersal by both sexes in BWC and mainly females for TRC, although with evidance of some male dispersal. While historical and/or long-range patterns of dispersal for both species were in agreement with dispersal mainly by one sex (e.g. Starin, 1991; Korstjens, 2001), evidence was found for a change in the current dispersal mode of the colobus

monkeys from Cantanhez National Park. Some episodes of female dispersal were already described in other studied black-and-white colobus populations (Korstjens *et al*., 2002; Harris *et al*., 2009), but none reported the extensive female dispersal found in BWC from Cantanhez. This forest is currently under high anthropogenic pressure, where the two main threats to colobus survival (habitat loss/fragmentation and poaching; Oates *et al.,* 2008a,b), co-exist. Consequently, the possibility that BWC are changing their dispersal patterns in response to recent changes in their habitat cannot be excluded, as was already observed in other primates (Goossens *et al.,* 2006). As inbreeding and kin competition for resources are likely to be intensified in degraded habitats, female dispersal within the BWC population could represent a local behavioural adaptation in order to avoid such problems (Lawson Handley & Perrin, 2007).

One of the limitations faced with the data analysis of this chapter was the fact that both pre- and post-dispersal individuals were present in the dataset. The fact that for most social groups I was not able to see the individuals defecating while collecting the faecal samples, did not allow me to attribute each sample to an age-class. Consequently, the tests for sex-biased dispersal (mAIc and relatedness) were conducted including individuals of all ages instead of just adults. I tried to understand the extent of this limitation by comparing the relatedness and mAIc results when using only adult individuals from the well-known TRC focal group or using all individuals in the group. For example, Hammond et al. (2006), tested several measures to detect sex-biased dispersal in hamadryas baboon (*Papio hamadryas hamadryas*), showing that when using pre-dispersal individuals no difference between males and females is obtained. This confounding effect most likely uncovered the real pattern of sex-biased dispersal. Despite the constraints, the combination of different molecular markers, different analyses and different datasets allowed me to understand the dispersal mode for both species. However, one should be aware that this type of sampling scheme could induce to misleading results when analysing sex-biased dispersal if such bias was not taken into consideration.

5.1.2 Chapter 3: How important is kinship shaping the intra-group social dynamics of two sympatric African colobus species ?

In this Chapter I aimed to understand how the patterns of intra-group kinship could affect the social dynamics. Under the assumption that kinship plays a major role shaping the social group behavioural patterns, I investigated the relationship

between intra-group relatedness and social affiliation in order to understand if the frequency of social interactions within and among sexes was kin-based. This hypothesis was tested using behavioural data from one focal group of each species and estimating pairwise relatedness among individuals from each of the focal groups studied. For the BWC focal group I found that females did not prefer to direct the grooming towards other females nor males and that the group was composed by pairs of related females and females related to males. Consequently, the fact that adult females do not show any preference to groom other adult females rather than males did not contradict the hypothesis that kinship is playing a major role shaping this group social dynamics.

For the TRC focal group, although most adult females were not related they groomed each other very frequently whereas the mostly related males only groomed each other very rarely. These results contrasted with most of the previous studies investigating intra-group social dynamics in red colobus which reported absence of female bonding and social interactions and stronger bonds among ault males (Struhsaker, 1975; Struhsaker & Leland, 1979; Werre, 2000). So far, only one other study has shown the same pattern of female preference for grooming other females rather than males, which focused on TRC from the Abuko Nature Reserve in The Gambia that were also being affected by habitat fragmentation (Starin, 1991). The uncommon behavioural pattern found amongst TRC females suggested that the social dynamics in this focal group may be shaped by other factors besides kinship. If there is stronger resource competition as a result of habitat degradation, it may be advantageous for these females to establish solid social bonds with unrelated females. Habitat fragmentation may be also forcing some of the females to immigrate together to this focal group (parallel dispersal) wich could explain the existent related adult females. Nevertheless, I believe that the high frequency of grooming among adult females could not be exclusively due to grooming exchange between the few pairs of of related females. However, our present data does not allow us to fully clarify this question. The fact that for the TRC focal group I was not able to identify all individuals did not allowed the direct correlation between behavioural interactions and the coefficient of relatedness between pairs of individuals. This question, unfortunately, could only be fully solved by investing several months in individually identifying all members of the focal group.

Differences in time budgets were also found when the BWC and TRC focal groups were compared with groups from other populations (Korstjens & Dunbar, 2007). The BWC group spent less time traveling and socializing and invested in time spent feeding. The TRC group spent less time travelling than other red colobus groups and

invested their spare time in resting (known to be important for the food digestion process in colobines) and social interactions. Overall, the fact that BWC needed more time to feed and TRC to rest could be an indicator of low food quality/quantity in their home range. Additionally, the high level of social interactions observed among TRC females could be explained as a mechanism to overcome the increased within group competition resulting from limited resources. Interestingly, both TRC time budgets and social dynamics resembled the patterns found for the population in the fragmented forest of Abuko Nature Reserve (Starin, 1991) reinforcing the evidence that habitat degradation may be having a major role on these groups' behavioural patterns.

5.1.3 Chapter 4: The interaction of social system, genetic structure and habitat fragmentation in two threatened primate species.

Here, multilocus genotypes were used to investigate the response of the two colobus species to forest fragmentation. This was accomplished by analysing broad-scale (spatial and non-spatial Bayesian clustering methods) and fine-scale spatial genetic structure (Mantel test and spatial autocorrelation). I tested the hypothesis that forest fragmentation was important shaping these colobus population genetic structure, by decreasing their dispersal ability through more fragmented areas. Overall, no major genetic discontinuities for either of the species were found, as all individuals were part of the same genetic unit. However, fine-scale spatial genetic structure analysis revealed some level of genetic structure for TRC through: i) a lack of correlation between pairwise relatedness and geographical distance and ii) significant positive relatedness between individuals from neighbouring groups and lower relatedness at larger distances. Negative correlation between pairwise relatedness and geographic distance is expected in cases when gene flow is carried in a stepwise manner (Hutchison & Templeton, 1999). The fact that TRC females tend to disperse to immediately adjacent groups (approx. 1,5 Km) together with the absence of an "isolation-by-distance" pattern suggested some constraint in their dispersal throughout Cantanhez National Park (Hutchison & Templeton, 1999; Peakall et al., 2003; Vignieri, 2007). Contrary to predictions, the same trend was not found for BWC since: i) the distribution of their genetic diversity follows an "isolation-by-distance" model, ii) individuals are more related then expected within their social group and then significantly negatively related at larger distances iii) males but not females were positively related at intermediate distances. The negative correlation between pairwise relatedness and geographic distance showed

that BWC are able to disperse in a stepwise manner throughout their distribution supported by the fact that individuals were negatively related at longer distances. Despite habitat fragmentation and contrary to TRC, BWC seemed to be able to use the existent forest corridors between patches. The higher ability for black-and-white colobus to persist in degraded habitats was already been reported in populations from East Africa (Dunbar, 1987; Struhsaker, 1997; Onderdonk & Chapman, 2000). In these studies, black-and-white colobus groups were found in most of the forest patches visited whereas red colobus groups were absent from a large fraction. It is not completely clear why red colobus are more sensitive to habitat modification. The fact that they live in bigger social groups and demand a more varied diet, are the main candidates explaining the lower ability to cope with habitat degradation and persist in small forest patches (Onderdonk & Chapman, 2000). The absence or decreased density of TRC in the northernmost forestry areas in CNP, where BWC were still found to be present, also supports the evidence that TRC are being more affected by forest degradation. Local communities have historically preserved forest fragments south of Amidara (see Fig 4.1) and its biodiversity, exploiting the resources in a sustainable way. This was not the case in the northernmost forests, where land use and resources exploitation was never controlled (Temudo 1998, 2009). The forest fragments where TRC groups were found completely overlapped with the forests that were historically preserved (Fig 4.1), supporting the evidence that TRC demand a more pristine forest to be able to persist. Altogether, the results from this analysis indicated that TRC population is responding more strongly to the forest degradation than BWC. Although we cannot exclude the possibility that BWC are also being affected or will be, if deforestation does not cease in the park, their higher flexibility in terms of dispersal mode (see Chapter 2), group size and diet, seems to allow them to better persist in disturbed habitats. Nevertheless, outside Cantanhez, remaining populations of both species may have already been severely affected. Previous studies have reported intense forest destruction outside Cantanhez and the disappearance of both colobus species from areas where they used to be present (Casanova & Sousa 2007; IBAP, 2007; Ferreira da Silva 2012). This demonstrates that both species survival in Cantanhez will be compromised with the increasing of forest degradation.

The fact that I only addressed the Cantanhez populations and did not sampled other areas in Guinea Bissau where colobus may still present, did not allow me to investigate the impact of forest fragmentation at a wider scale or to access migration rates between populations. These are questions that need to be addressed in the near future in order to define conservation priorities, not only for Cantanhez National

Park, but also for the remaining, and most likely scarce, colobus populations in Guinea Bissau. The evidence obtained in this study that TRC is already being affected by forest fragmentation in Cantanhez and BWC will most likely be affected in the near future, highlights the urgency to address other colobus populations that occupy even more degraded areas. Additionally, estimation of the effective population size and demographic history should also be carried out in order to fully understand the genetic patterns currently observed.

5.2 Evidence for adaptation to forest degradation?

Overall, these two closely related species were found to adopt different strategies to respond to the same ecological/anthropogenic pressures, as summarized below:

Table 5.1 Summary of the contrasting patterns found for the two species

	BWC		TRC	
Historical/long-range migration (mtDNA)	Male-biased	Low genetic diversity	Female-biased	High genetic diversity
Current migration (mtDNA)	Not conclusive	Social groups highly structured	Female-biased	Lack of structure between social groups
Current migration (microsatellite *loci*)	Males and females	No sex differences in within group relatedness	Female-biased	Negative mAIc for females (R<0.3 database)
		No sex differences in mAIc		Negative within group female relatedness (R<0.3) for most social groups
Social bonding determined by kinship	Not contradicted	Adult females groom and are related to other females and males equally	Contradicted	High levels of grooming among adult females, despite most are unrelated; Grooming is rare among adult males although most are related
Time budgets	Different from other populations	Increase in time spent feeding	Different from other populations	Increase in time spent resting and socializing
Effect of forest fragmentation on genetic structure	Not evident	Isolation by distance	Evident	Absence of isolation by distance
		Higher relatedness within group and lower at greater distances		Higher relatedness among individuals from adjacent groups

In addition, evidence was found in the two species for behavioural changes that could represent local responses to forest degradation.

In black-and-white colobus:

a) Current dispersal of both sexes contrasts with historical and/or long-range dispersal by males. This may be a result of females being

forced to disperse to avoid inbreeding or resource competition with kin and, that dispersal over short distances is enough to avoid such problems;

b) In comparison with other studied groups, BWC spend less time travelling and more time feeding, which could be indicative of reduced habitat availability and low quality food.

In red colobus:

a) Substantial high levels of adult female affiliative interactions (grooming) in TRC, where females are the main dispersers and most adult females are not related. High intra-group competition for limited resources could favour females to establish strong social bonds with non-related females. Forest fragmentation, could be also be forcing or favouring some related females from the same social group to immigrate into TRC which is supported by the evidence that TRC females tend to disperse to adjacent groups.

b) TRC focal group spent more time resting and socializing than other studied populations. Resting is known to be important in the colobines digestion process and the observed increase in the resting time could be indicative of a lower quality diet in this TRC group. Additionally, the increased time spent in social activities, could also be a consequence of increased intra-group competition resultant from inhabiting a degraded habitat.

All the above changes or unexpected behavioural patterns can be explained by ecological constraints resultant from habitat degradation. However, measures on habitat quality, food availability, colobus home-ranges and density would be necessary to clarify the proximate causes underlying such patterns. However, the evaluation of these parameters was out of the scope of the present study.

The high dependence of both species on forestry habitat supports the fact that forest degradation is the most likely cause underlying such unusual behaviour patterns. It was demonstrated that TRC dispersal movements were already being constrained inside the park and its distribution also restricted to more conserved forest fragments. Altogether, this study showed that these two different social systems are being differently affected by forest fragmentation and provided evidence for the use of different strategies to respond to the same constraints.

5.3 Conservation considerations

Both western black-and-white and temminck's' red colobus are threatened taxa (Oates *et al*., 2008a,b) with populations declining throughout their distribution. In Guinea Bissau the situation does not seem to be different. Although colobus groups were described to be present throughout the country (Gippoliti & Dell'Omo, 2003), the most recent reports indicated that both species have disappeared in several areas where they used to exist (Casanova & Sousa, 2007; IBAP, 2007; Ferreira da Silva, 2012). Local communities have described entire groups being killed and trapped in human-induced fires. The fact that colobus are easy to hunt because they do not run away, unlike baboons or vervet monkeys, has also been mentioned (Ferreira da Silva, 2012). Such reports reinforce the evidence that the persistence of colobus populations in Guinea Bissau is severely threatened. Despite the high levels of fragmentation, the forests of Cantanhez National Park are one of the remaining forest pockets in the country, possibly encompassing the only viable population in the country (IBAP, 2007). The park was gazetted in 2008 but due to economic constraints, management and park guards have not been functional until now (IBAP, personal communication). As a result, uncontrolled deforestation for logging activities and hunting of primates are still occurring (TM, personal observation, Hockings & Sousa, 2011). I have personally witnessed the disappearance of social groups of the two species from one of the forests around Iemberém, and was not able to find red colobus in Amidara in 2010 in the areas where I had seen them in 2008 and 2009. The fact that red colobus are much more scarce or even absent in the northern part of the park shows how limited is the habitat available for this population. All these facts, together with the results produced in this study highlight the fact that we are reaching a critical time where conservation of colobus populations in Guinea Bissau remains just possible. If unmanaged forest cutting and poaching are not stopped, colobus will most likely become extinct in the country. It is important that IBAP gets financial support in order to be able to hire a park director and park guards to patrol and control resource exploitation. Additionally, it is crucial that the forest is restored in the northernmost part of the park in order to increase the habitat availability especially for red colobus. It is also essential that the remaining forest corridors are maintained and new ones are created connecting presently isolate patches (e.g. Cungha). Future research on the Cantanhez colobus population should include a population census, evaluation of the habitat and food resources availability as well as the distribution and home-ranges of the colobus groups. The mapping on the forest areas and corridors distribution is also essential,

as it is the periodic monitoring on the two species, since this would help the detection of major demographic or geographical distribution changes. At the same time the risk of logging and hunting in such monitored areas would decrease.

There is one national NGO based in the park (AD, Acção para o Desenvolvimento) that works in the development of the local communities and in conservation programs, including eco-tourism projects. Cantanhez National Park is highly populated and local communities are very dependent on the natural resources (Temudo, 2009). Besides eco-tourism and small food shops (mostly owned by immigrants), activities involving natural resource exploitation such as agriculture, fishing or logging, constitute the only financial income for the local people. Therefore, any conservation plan, in order to be viable in the long-term, should not only consider habitat and species conservation, but also create alternatives to the subsistence of the communities living inside the park. Eco-tourism activities are obviously one of the alternatives. AD has been developing an eco-tourism program in the park, which involved the construction of a tourist lodge and training several tourist guides. The problem is that such program benefits only few people in the community (14 tourism guides and few people to clean and cook for the lodge), so the majority do not appreciate the benefits of preserving the forest and its biodiversity. Research can also be a great opportunity to create new jobs, conduct environmental education through training and involve the community in the conservation of their own territory. There is a research team mainly involved on primate conservation (from which this study is part of), which has been employing local people to assist in the research. Intensive training and workshops have been carried and some of these people have been employed more or less on a regular basis. Long-term research as well as long-term conservation programs involving managing and monitoring of natural resources carried out by local people, could help create more job opportunities, therefore benefiting a bigger part of the community for conserving their forests.

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APPENDICES

Appendix one: Additional information on fieldwork methods

Focal groups

In the first incursion to the field I focused my attention on one social group of each species. This option was due to the fact that these two social groups have their home range overlapping the village where I was staying. The proximity with humans made both social groups less afraid of humans facilitating the habituation process.

The observation period was divided between the two social groups since I had to follow one at the time. I would follow one group during one week (from Saturday to Thursday) and then change to the other group in the following week. I had two guards of the park working with me in the field . Each of them was always following the same group, wich helped in the habituation proces. The focal groups were followed from 7:00 to 19:00.

Habituation, identification and behavioural data

During the first month both groups were followed with the purpose of habituation and identification only. No behavioural data was collected during that period. One of the guards would be following the red colobus group and the second guard following the black-and-white colobus group. I would change from one to another every week. During this period I would spend the day following the social group, trying to count them and filling up the identification forms. The observation of the individuals was made using binoculars by both me and the guards. I would focus my attention on an adult individual that was still at that moment and observed it for as long as it was possible. During observation I would register their physical characteristics: sex, body size, body marks, shape and size of the tail, colours patterns of the face, presence of infant and its age. I would only consider an individual as identified and named when I was able to recognize it at least two times after the first identification.

When groups stopped running away in our presence I started to collect behavioural data. I decided to collect only *ad libitum* (ad lib.) data because I wanted to have a general idea of the frequency and type of social interactions of both species. For the ad lib. method I would record the observer, date, time, habitat, site, individual, activity, partner and relevant notes. For the individual and partner I would identify the sex and/or age class of unknown individuals. When none of these characteristics was possible tobe observed, the animal would be named as Individual. I considered

the following sex- and age-classes:

Adult (AD) – Large body size and secondary sexual characters.
Juvenile (JUV) – Smaller, thinner and more active than adults.
Infant (INF) – Smaller than juveniles and most of the times very close to the mother.
Female (F) – Presence of breasts and absence of testis and/or penis.
Male (M) – Absence of breasts and with testis or/and penis.
Adult Male (M AD) – When it is possible to identify both male and adult characteristics.
Adult Female (F AD) – When it is possible to identify both female and adult characteristics.
Juvenile Male (M JUV) – When it is possible to identify both male and juvenile characteristics.
Juvenile Female (F JUV) – When it is possible to identify both female and juvenile characteristics.
Individual (IND) – When it is not possible to identify either sex or age class.

I recorded the *ad libitum* data on a continuous basis during the day and every time that a social interaction took place. I considered the following activities (based on Nowak K. 2007):

Aggression – Includes all antagonistic interactions with or without physical contact such as threat, biting, pushing, shoving or brushing another individual or individuals.
Displacement – Supplant another individual.
Grooming – Pass hands, fingers or mouth on other individual in a picking motion.
Copulation – Male with adult female engaging in a sexual act.
Play – Involves at least one more individual and is performed in a spontaneous manner.
Presentation – Displaying rump to another individual's head. Usually involves lifting of tail and lowering upper portion of body/torso.
Allomothering – One female handles and takes care of other's female infant.
Vocalize– Emitting call.
Social Fight – When an aggressive event involves more than two members of the social unit.
Intergroup Fight – When members of the focal group engage in aggressive events with members of another social unit.
Interspecific Association – When different primate species are overlapping the space of activity and/or travelling together with no need of interactions between

individuals of each species.

Sampling and storage of faecal samples

The initial plan was to collect faecal samples only from the identified animals. However, because identification was difficult I decided also to collect samples from unknown individuals and the sex- and age-class were identified. In these cases is possible that the same individual was sampled more than once. Samples were collected only when the focal individual was observed defecating and right after defecation. Collection of faeces was made using a sterile wooden stick, a pair of sterile gloves and a face mask for each sample. A code was given to each sample and, date along with time of defecation, individual identity, sex- and age-class, name of the observer and collector were recorded. As a consequence of the constraints involving the extraction of DNA from faecal samples, the first season of field work had also the goal of testing different storage methods. Regarding this, each sample was subdivided and stored using four sampling methods described as follows:

Ethanol Method: Two to three pellets of the faecal material were collected and stored in a 15ml falcon tube containing approximately 8ml of absolute ethanol and kept at room temperature. Once the tube was capped it was sealed with Parafilm to prevent leakage.

Silica Method: Two to three pellets of the faecal sample were collected and stored in a 15ml falcon tube containing roughly 8g of silica gel beds (Sigma) and kept at room temperature. Once the tube was capped it was sealed with Parafilm to prevent air contact with the exterior.

Two Steps Method (Roeder et al., 2004): Two to three pellets of the faecal material were collected and stored in a 15ml falcon tube containing approximately 8ml of absolute ethanol and kept at room temperature for 24 hours. The faecal material was then transferred to a 15ml falcon tube containing about 8g of silica gel beads and kept like this for the rest of the field period. This method has the advantage of quickly dry the sample. By preserve it in silica afterwards it prevented DNA degradation by ethanol and problems of international transportation.

Wash Method (Palomares et al., 2002): Two to three pellets of the faecal sample was collected and stored in a 60ml containing 40g of silica gel beads. Once the tube

was capped it was sealed with Parafilm to prevent air contact with the exterior. These samples were stored in silica in order to test an extraction method that uses a solution that results from washing the sample surface with a PBS (Phosphate Buffered Saline) solution.

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Appendix two: Demographic history based on mitochondrial DNA

Methods

Past demographic events were explored using mtDNA with Tajima's D (Tajima, 1989) and Fu's *Fs* (Fu, 1997), calculated in ARLEQUIN version 3.1. Mismatch distribution profiles (distribution of pairwise sequence differences; Rogers and Harpending 1992) were also generated in ARLEQUIN, where the hypothesis under a sudden demographic expansion was tested. Demographic processes were examined for the entire dataset of each species and for each divergent haplogroup in the red colobus.

Results

The negative results for both Tajima's *D* and Fu's *Fs* neutrality test (Table 1), in BW, indicate either presence of selection, or more likely in this study, a signal of population expansion. A unimodal observed mismatch distribution (Fig 1a), very similar to the simulated curve (*p-values* non-significant; Table 1) also supported a recent demographic expansion for this BW population. In RC, Tajima's *D* and Fu's *Fs* tests did not reveal any deviation from neutrality (Table 1). A tri-modal mismatch distribution pattern was obtained (Fig 1b), differing significantly from the simulated values (Table 1), indicating a stable demographic history for the whole population.

Discussion

Despite the low haplotype and nucleotide diversity found in the BW population (Chapter 2), signs of a sudden population expansion were detected. Sudden demographic expansions can lead to star-shaped phylogenies and unimodal distributions of pairwise differences (Slatkin and Hudson, 1991; Rogers and Harpending, 1992) as well as a decrease in the number of segregating sites (Bertorelle and Slatkin, 1995; ArisBrosou and Excoffier, 1996; Tajima 1996), as found for the CNP BWC. The negative results for both the neutrality tests (Tajima's *D* and Fu's *Fs*) and the unimodal mismatch distribution for observed pairwise sequence differences, suggests a possible sudden demographic expansion that might have occurred after a bottleneck or founder effect. This idea is supported by the existence of only three haplotypes found in the population differing by only one mutation step (see Chapter 2). The combination of both low haplotype and nucleotide diversity is concordant with

Table 1 Number of sequences (N), haplotypes, haplotype diversity (hd), nucleotide diversity (π), test of selective neutrality (Tajima's D, Fu's FS), population parameters (θ0 and θ1) and sum of square deviation (SSD) and raggedness index (HRI) for overall black-and-white (BW) and red colobus (RC) dataset and for each social group

Group	N	Nº Haplotypes	*hd*	*π*	*D*	*Fs*	*ϑ0*	*ϑ1*	SSD	HRI
BW	**58**	**3**	**0.16**	**0.00036**	**-1.07**	**-1.67**	**0.00**	**0.20**	**0.0008**	**0.49**
Focal	9	1	0	0	....	....	....	....	....	....
Neighbour	10	1	0	0	....	....	....	....	....	....
Cambeque	11	2	0.18	0.0004	....	....	....	....	....	....
Cancira	7	1	0	0	....	....	....	....	....	....
Muna	6	1	0	0	....	....	....	....	....	....
Deep Amidara	4	1	0	0	....	....	....	....	....	....
Cangode	3	1	0	0	....	....	....	....	....	....
Amidara	6	1	0	0	....	....	....	....	....	....
Bushmeat	2	1	0	0	....	....	....	....	....	....
RC	**86**	**11**	**0.83**	**0.038**	**2.31**	**18.33**	**0.00**	**28.91**	***0.09***	***0.17***
Focal	29	5	0.77	0.03	0.95	14.22	0.00	4.87	0.13	*0.29*
Madina	19	3	0.70	0.02	2.66	12.94	0.00	2.86	0.19	0.42
Cambeque	11	5	0.78	0.04	1.46	7.12	0.00	99999	*0.16*	*0.26*
Muna	2	2	1.00	0.05	0.00	3.04	....	....	...	....
Cangode	9	6	0.89	0.05	1.49	3.50	0.00	157.2	*0.08*	0.14
Cungha	9	3	0.64	0.04	1.19	10.27	1.23	1.99	*0.30*	0.46
Bushmeat	7	4	0.81	0.05	1.38	5.60	0.00	99999	*0.12*	0.16
Haplogroup A	29	2	...	...	1.92	5.50	0.00	99999	*0.39*	0.70
Haplogroup B	38	4	...	...	0.57	1.07	0.00	1.85	0.15	*0.58*
Haplogroup C	17	3	...	...	-0.70	-0.62	0.00	99999	0.01	0.18

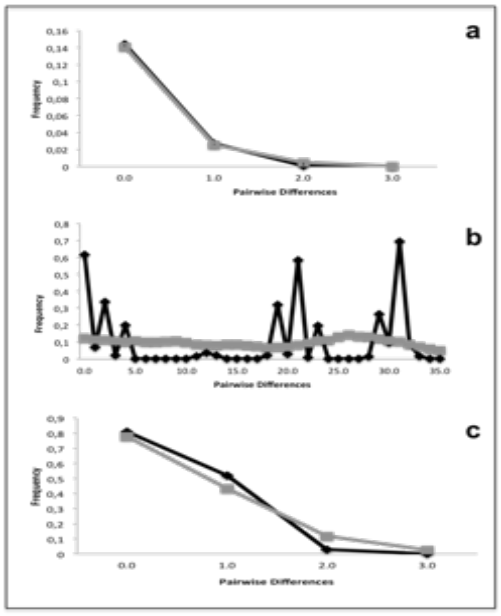

Figure 1 Mismatch distribution for black-and-white colobus (a), red colobus (b) and red colobus haplogroup C (c). Black lines represent observed and grey lines represent simulated distributions for the sudden expansion model

a scenario where the colonisation of the peninsula was accomplished by one or a few mitochondrial lineages (Grant and Bowen, 1998). The fact that the males were the primary sex to disperse did not allow new mitochondrial haplotypes to be established in the population. If the population has remained stable over time, less frequent haplotypes might have disappeared from the population due to genetic drift and lineage sorting (Avise et al., 1987). Only two low frequency haplotypes were detected with only one mutation accumulated between them and this enhances the idea that the population expansion of the Cantanhez BW population might be very recent (see Chapter 2). This results suggest that historical dispersal mainly mediated by males is reflected not only in the present pattern of mitochondrial diversity and

within-population structure of the black-and-white colobus but has also left its signature in the colonisation history of the Cantanhez peninsula.

A multimodal pattern of mismatch distribution was obtained for the RC, suggesting a stable population and the neutrality tests corroborated this scenario. The levels of haplotype and nucleotide diversity exhibited by this population suggest the existence of, either a large stable population with deep evolutionary history or secondary contact between divergent lineages (Grant and Bowen, 1998). The shape of the network supports the latter since four very divergent lineages were found within the population (see Chapter 2).

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Appendix three: The trade and ethnobiological use of Chimpanzee body parts in Guinea-Bissau: implications for conservation. Traffic Bulletin 2012, 24 (1): 31-34.

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INTRODUCTION

Guinea-Bissau represents the western-most limit of the endangered West African Chimpanzee *Pan troglodytes verus* (Sousa *et al.,* 2005). During the 1980s, Chimpanzees were erroneously considered extinct in the country due to a total absence of information owing largely to political and civil unrests (Lee *et al.,* 1988). In 1994, a preliminary survey was conducted and the presence of Chimpanzees was reconfirmed (Gippoliti and Dell' Omo, 1995; 1996). More recently, research has been carried out in co-operation with national and local authorities, establishing a system for the systematic monitoring and management of this great ape (Casanova and Sousa, 2007). Within the country, Chimpanzees are distributed across the south of the Corubal River. Their presence is confirmed in two protected areas Cantanhez National Park (CNP) and Cufada Lagoons Natural Park (CLNP) in the southwestern region, and in the eastern region of Boé (Casanova and Sousa 2007; Brugiere *et al.*, 2009).

Due to high levels of exploitation, loss of habitat and habitat quality as a result of human activities, this subspecies is estimated to have experienced a significant population reduction in the past 20 to 30 years (IUCN, 2011). However, no recent data are available to allow for an estimation of rates of decline (IUCN, 2011). The most recent figures available, from 1996 (Gippoliti *et al.*, 2003), estimate that the number of Chimpanzees in Guinea-Bissau ranges from between 600 and 1000 individuals. It is estimated that Chimpanzee density in the southern area of CNP is of 2.34 nest builders/km^2 in a total area of 17.225 km^2 corresponding to 40 individuals (Sousa *et al.,* 2011), while in the neighbouring east area of Gadamael, just outside the CNP area, this value decreases to 0.89 nest builders/km^2 in a total area of 36.513 km^2, which corresponds to 33 individuals (Sousa, 2009). However, the exact number of individuals and communities for the whole CNP and the rest of the country remain unclear; with the aid of a molecular census, however, it will be possible to infer its effective population size (Sá *et al.,* 2009).

Anthropogenic disturbances such as habitat loss and fragmentation (e.g. logging activities and shifting land occupation for the purposes of agriculture and food production, e.g. cashew nuts, the hunting of infant animals for the pet trade, and casual deaths from crop raiding allied to extrinsic factors such as disease are the main threats, not only to Chimpanzees but to all non-human primates in Guinea-Bissau (Gippoliti *et al.,* 2003; Casanova and Sousa, 2007; Brugiere *et al.,* 2009). The species is classified by IUCN as Endangered, and listed in CITES Appendix I, being also protected at national level. Even though most primate species in Guinea-Bissau are traded for meat consumption, there is no evidence that this is the case for Chimpanzees (Minhos *et al.,* in prep.).

The following paper reports on the use and trade of Chimpanzee body parts in Guinea-Bissau for traditional practices (e.g. for nutritional, medicinal or ritual purposes, or "animistic myths"). Informal interviews were conducted and observations made with a view to providing insight into how these human traditions and myths might pose an additional threat.

METHODS

Visits were made to Bandim market, the largest market in Bissau, the capital, during two weeks in September 2008 and a similar period in June 2010. Some 10–15 men were found to be offering wild animal body parts for sale (e.g. skin, bones, teeth, horns and scales). Where possible, morphological identification of the specimens viewed was made and photographs taken.

An ethnoprimatological approach (i.e. the study of human and non-human primate interactions) aims to understand the incorporation of non-human primates into folklore, myths, the hunting of non-human primates for food, keeping non-human primates as pets, indigenous knowledge of non-human primate behaviour, among others (Wolfe and Fuentes, 2007; Fuentes and Hockings, 2010). In this study, the authors were interested in understanding and placing into context the social inclusion of Chimpanzee body parts for human traditional practices using informal interviews and ethnographic observations, although not enough data were collected to provide an in-depth analysis for such an approach.

Most of the vendors encountered were male. Five urban vendors in Bandim and 17 rural informants in villages in the CNP and the Boé region were informally interviewed following an unstructured script, in order to document the geographical origin and use of Chimpanzee body parts, prices and the scale of the trade, i.e.

whether at a national, regional, or transnational level. Direct observations of the trade were conducted in the market and field notes were taken. Informants were ensured that the purpose of the work was not to condemn or report their practices to the local authorities. Every observation heard and/or seen was recorded and notes/interviews organized into social demographic categories (e.g. urban traders, local villagers, gender, ethnic group). Only information relevant to the research topic was assigned to these categories (Rubin and Rubin, 1995).

RESULTS AND DISCUSSION

Traded species

During market visits (seven visits of approximately four hours each) morphologically identified dried Chimpanzee skins were found being sold for traditional medicinal purposes. Additionally, dried skins from Temminck's Red Colobus monkeys *Piliocolobus badius temminckii*, Guinea Baboons *Papio hamadryas papio* and Olive Baboons *Papio hamadryas anubis* were also found. The authors also detected trade of dried skins of several non-primate species such as Leopard *Panthera pardus*, Nile Crocodile *Crocodylus niloticus*, African Civet *Civettictis civetta*, Elephant *Loxodonta* sp., Hare *Lepus* sp., African Buffalo *Syncerus caffer*, Spotted Hyaena *Crocuta crocuta* and several species of antelopes, snakes and lizards, as well as skins alleged to be of Wild Dog *Lycaon pictus* and Lion *Panthera leo* (Fig. 1).

Other animal body parts observed included bones, spines of Crested Porcupine *Hystrix cristata*, teeth, antelope horns, scales of Pangolin *Manis* sp., mollusc shells, fish bones and feathers. Morphologically specific identification was not possible in most cases due to the similarity of those body parts to other species, as well as to their condition. A few sellers mentioned that some of the bones being offered for sale were from primates.

All the species mentioned above are reported as occurring in Guinea Bissau except for Olive Baboons, whose western limit of their distribution is reported to be in Mali and Republic of Guinea (IUCN, 2011). The observed olive baboon skin was morphologically quite different to the other baboon skins found at the market. While Guinea Baboon skins presented red/brownish coloration, the Olive Baboon (*Papio anubis*) skin was more grey-greenish, typical of what has been described for the subspecies (Groves 2001).

Figure 1: Trade of animal body parts in Bandim Market (legend includes IUCN 2008 conservation status and CITES protection level)

1 – Leopard (*Panthera pardus*) (1a – complete) - Near Threatened, CITES Appendix I)
2 – Chimpanzee (*Pan troglodytes ssp. verus*) – Endangered, CITES Appendix I)
3 – Crocodile (*Crocodylus niloticus*) - Lower Risk/least concern, CITES Appendix I and II)
4 – Guinea baboon (*papio*) - Lower Risk/ Least Concern, CITES Appendix II)
5 – Possibly Lion (*Panthera leo*) - Vulnerable, CITES Appendix II)
6 – Possibly African civet (*Civettictis civetta*) - Least Concern, Appendix III)
7 – Antelope's horns

Costs, origin and scale

Interviews with urban traders revealed that the cost of a piece of Chimpanzee skin (Fig.1) was relatively high, ranging from 1500 CFA Francs (XOF) to XOF90 000 (USD2.9 to USD173.96, based on an exchange rate in 2008 of XOF460.77 to USD1). The average monthly wage in 2008 was XOF40 000 (approximately USD88.00) (UNDP, 2010).

All urban vendors reported that the Chimpanzee and other animal body parts originated from the "southern part" (with the exception of the elephant hide seen which, the authors were told, came from Senegal), and they frequently mentioned the regions of Cantanhez and Gabú. Vendors considered south of the country every location south of Bissau. It should be noted that elephants have not been seen in Senegal for 15 years [OK to include. anonymous reviewer info, citation to be checked if we include]. Furthermore, most vendors said that consumers were of both sexes, different ethnic groups and social status.

It was apparent to the authors that witchdoctors are not the only people to buy animal-derived products for traditional medicine or protection fetishes. For example, according to statements from three vendors:

"All sort of people buy. Men and women, poor or rich… Fulas, Pepel, Balanta, even Europeans. Every kind. Not only djamba kuss [witchdoctors] to please the irans [magical and religious entities]."

According to Robillard in litt., July 2011, it is common practice in Africa for people who are unwell to buy their own products based on a list provided by the traditional doctor.

Two of the vendors also mentioned that individuals from neighbouring countries such as Senegal, Guinea or Gambia are involved in the trade within in the country: *"Other foreigners also buy and sell their own plants, shells or skins".*

Symbolic and medicinal use

Most male informants in rural CNP and Boé villages associated the use of Chimpanzee-derived products with the needs of women, as revealed by one elder Fula respondent in Béli, Boé:

"Dári [chimpanzee] is mezinho [traditional medicine] of women."

Three Balanta women in CNP confirmed that Chimpanzee skin is used to: *"prepare a cleansing mixture against hideousness when they are pregnant or their children are still babies in the event they see a lonely chimpanzee cross their way"*. Likewise, another woman said that *"the leaves of the nest where a menstruated female chimpanzee sleeps can be applied to heal mental problems"*.

One informant admitted that he uses a stitched amulet made of chimpanzee body parts to help provide awareness to protect him and his friends while in the bush (Rui Sá, personal observation 2008).

Guinea-Bissau in the context of previous studies

One possible explanation for the lack of information on magic practices and traditional–medicines using animal body parts in Guinea-Bissau is the difficulty in collecting information on such an undisclosed subject and also the lack of interest and in-depth studies undertaken. As a result, the authors' observations are opportunistic. However, the use of animals' body parts for medicinal purpose could seriously threaten the biodiversity of Guinea-Bissau and, in particular, constitutes an additional and significant threat to Chimpanzee populations already menaced by habitat lost and fragmentation, the pet trade and crop raiding conflicts. Therefore, this phenomenon deserves to be thoroughly investigated (Cá 2008). Although previously not reported for Guinea Bissau, the use of non-human primate body

parts in traditional medicine is not unusual elsewhere in the world (Alves *et al.,* 2010; Leypey and Fomine, 2010). In a recent review, Alves *et al.,* 2010, reported the use of 101 species of primates in folk/magic-religious practices, most frequently in Africa, Latin America and Asia. Although Cercopithecidae species are the most affected, Chimpanzees are also referred to as a remedy for diseases and for use in folk medicine (Alves *et al.,* 2010).

In Nigeria, Mali, Sierra Leone, Congo and Guinea, Chimpanzee body parts are used to cure male impotency, epilepsy, bone fractures and infertility in women (Dedeke and Aboyami, 2006). In Cameroon, the Bakweri people believe that by using the liquid derived from boiled Chimpanzee bones, the bones of children or babies will become stronger (Leypey and Fomine, 2006). Additionally, in the forested areas, people use Chimpanzee body parts in birth and circumcision rituals (Mallart Guimera, 1981). The Yoruba people of south-western Nigeria believe in the magical properties of Chimpanzee body parts in appeasing witches and fortune tellers (Dedeke and Aboyami, 2006). However, it is not easy for people to obtain these remedies or to gain access to these animals. In Central Africa, the consumption of Chimpanzee meat is taboo for young men, pregnant women and children.

The presence in Bandim market of the skin of an Olive Baboon suggests a foreign origin for some of the animal body parts being offered for sale. While the distribution area for this species (*Papio anubis*) includes neighbouring Guinea and Mali, it does not occur in Guinea-Bissau (Soewu 2008). The Guinean Baboon, *Papio papio* is the only baboon subspecies reported and observed in the country (IUCN, 2011). There are striking differences in morphology between both baboons species (namely coat coloration, Grooves 2001), which enables distinction based on its skins. Furthermore, in Colobane and Boucotte markets at Senegal (in Dakar and Ziguinchor, respectively), several species of reptiles and mammals, including primate species (data not shown) were found in trade for use in traditional medicinal practices and/or magical ceremonies [Fernando Sousa, personal observation 2008]. According to information provided by the sellers, those animal body parts were brought from Niger, Nigeria, Ivory Coast and Mali. Chimpanzee skins were also found in these Senegalese markets [Fernando Sousa, personal observation 2008]. The respondents pinpointed Cassamance (on the border between Senegal and Guinea-Bissau) as the putative origin of Boucotte market Chimpanzee skins and Guinea-Bissau and Republic of Guinea as the possible origin of the Chimpanzee skins being sold at Colobane market. The possibility that the Chimpanzee skins found in Bandim market could also be from the Republic of Guinea cannot be excluded since sellers mentioned the "south" as the origin but not specifically the south of Guinea-Bissau.

Implications for conservation

The suggested transnational interest for Guinea-Bissau Chimpanzee skins may constitute an even bigger threat for the conservation of this population. Since Chimpanzee populations are declining in West African countries (IUCN, 2010), foreign hunters could be attracted to Guinea-Bissau and the hunting of Chimpanzees could therefore increase in the near future. Biodiversity management authorities in Guinea-Bissau (*IBAP* and *Direcção Geral de Florestas e Fauna)* have introduced new laws to regulate the trade in wild meat (e.g. recently, the hunting of primates throughout the country was prohibited (Angola Press, 2011). However, the lack of resources and lack of awareness of management authorities and politicians are hindering law enforcements in the country. At the international level, conservation agencies should re-examine their strategies to mitigate this trade, and, at the national level, specific programmes should be designed and applied to empower all actors involved (e.g. park rangers, Customs officers, the military, police, etc.), complemented at the same time by provision of environmental education for the local communities.

Further work by the authors will include the molecular determination of the origin of the skins observed in the markets and of the species involved. This will assist in evaluating the scale of the trade. Finally, an ethnographic study specifically centred on the use of non-human primate body parts by traditional medicine using more in-depth techniques such as participant observation or long-term observation will allow the authors to draw up possible differences in the use of distinct animal parts and determine how such practices are disseminated.

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Appendix four: Using genetics as a tool in primate conservation. Nature Education Knowledge 3(6):10.

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Teaser

"Here I am, in the middle of this forest and I can't see the primates… How can I learn more about the species that I am studying?" This question can be answered with the help of recent and exciting developments in non-invasive molecular genetics.

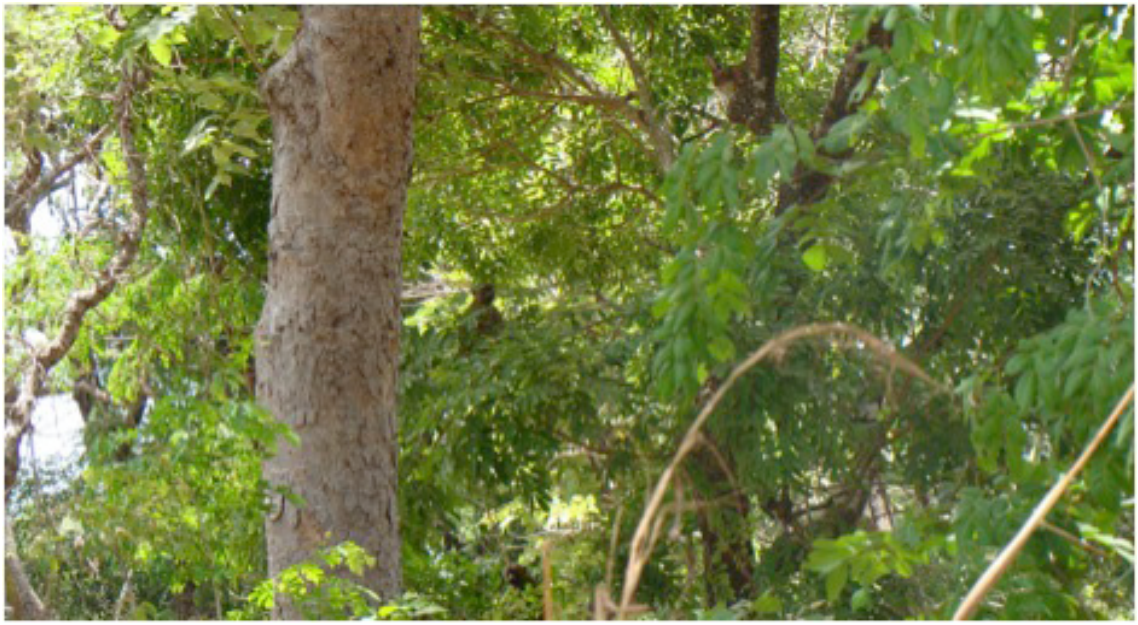

Fig. 1: Elusive animals. Guinea baboons (Papio hamadryas papio) live in dense forested habitats and are very hard to observe. By using molecular tools, primatologists can gather information about this elusive species.

Key words: conservation genetics; non-invasive genetics; primates; molecular tools; habitat fragmentation; hunting; diseases; population census; ESUs; MUs; population viability analysis

Introduction

The current conservation status and decline of nonhuman primates is alarming (Norconk et al. 2011) due to factors such as disease, habitat destruction, hunting, illegal trade and climate change (WWF 2010). Unfortunately, their restricted geographical range, resource requirements long lifespan and slow breeding,

dispersal needs and degree of specialization make primates more susceptible to extinction than many other species (Cowlishaw & Dunbar 2000; Harcourt et al. 2002; Gibbons and Harcourt 2009). Moreover, the geographical range of threatened primate species often overlaps with areas of high human density (Harcourt & Parks 2003). Recent studies state that 48% of primate species are in danger of becoming extinct, making well-informed conservation measures crucial for ensuring their long-term survival (Mittermeier *et al*. 2009). Aside from their contribution as models for human evolutionary research, their value to ecotourism and their charisma, primate species play important ecological roles, especially as seed dispersers (Chapman & Russo 2007). Some studies suggest that maintaining this role could be important for ecosystem resilience because monkeys and apes visit trees in social groups where they tend to stay longer during feeding periods than other mammals or birds (Lambert 2011).

Non-invasive genetics analysis

The relatively recent development of non-invasive genetics has allowed primatologists to better understand the population and group dynamics of wild primates, simply by the fact that it is now possible to obtain genetic information by extracting DNA from by-products such as feces, shed hair and urine. The first study of this kind was in chimpanzees (*Pan troglodytes*) in which Morin *et al*. (1993) analyzed patterns of gene flow in the Gombe chimpanzee community (Tanzania). Since then, major technical improvements in non-invasive genetics have greatly expanded our capacity to address a wide range of questions about the structure of primate populations, their evolutionary histories, and adaptation while allowing the study of wild populations without direct contact with the animals (Charpentier et al., 2007; Tung et al., 2008). More importantly, the combination of genetics with long-term socio-ecological data has enabled comprehensive analysis at an individual and social group level for a wide range of primate species.

DNA obtained non-invasively can be analyzed in individuals and populations for a wide range of molecular genetic markers such as microsatellites, minisatellites, mitochondrial DNA, amplified fragment length polymorphism (AFLP) and the major histocompatibility complex (MHC). By using a variety of software to analyze the genetic data produced, primatologists can now obtain information on effective population size, parentage, relatedness, sex, dispersal, population structure, population assignment and gene flow. This is crucial if we are to fully understand population dynamics at a local scale and evaluate the threats and suggest appropriate conservation measures (Goossens & Bruford 2009).

Threats to primate conservation

Habitat Fragmentation

Habitat fragmentation can influence key features of primate populations: 1) their diet, 2) the social group size or density and 3) the dispersal and gene flow between social groups or subpopulations (Marsh 2003; Frankham *et al.* 2002). Consequently, the capacity for populations to persist in fragmented landscapes is related to a species' particular characteristics (Marsh 2003) (Fig. 2). It is theoretically possible for primate populations to increase in size within fragments (for example, if natural predators disappear, Strier 2007) but more frequently, they decrease or become extinct. This can be due either to direct mortality (caused by an increased hunting pressure, since isolated areas become accessible for humans (Marsh 2003, Strier 2007)) or due to genetic changes (Frankham *et al*. 2002) (Fig. 3). In the long term, fragmentation can lead to a reduction in genetic diversity and increased genetic differentiation. This results from the decrease of gene flow between breeding groups and the action of random genetic drift and/or inbreeding (Frankham *et al*. 2002). Each population fragment may show different levels of genetic diversity and significantly different allele frequencies from the other fragments. The risk of inbreeding depression is increased if the population is smaller and isolated, with lower genetic diversity. Migration of individuals between fragments and subsequent reproduction will introduce new alleles into the population (increasing genetic diversity) and it will counterbalance the effects of genetic drift and inbreeding, preventing complete fixation of alleles (Frankham *et al*. 2002). By using non-invasive genetic methods it is possible to identify the genetic structure of a fragmented population and levels of gene flow between units and determine whether ecological corridors should be created/maintained or individuals should be translocated (e.g. Bruford *et al.* 2010). In the fragmented range of the Cross River Gorilla (*Gorilla gorilla diehli*) three sub-populations have been uncovered using microsatellite markers (Bergl and Vigilant 2007). Although this genetic structure corresponds broadly to the pattern of habitat fragmentation, migrants between fragments could be identified. Since different levels of genetic diversity were found between the sub-populations, it was suggested that the conservation of the most genetically diverse sub-population should be prioritized. Also, habitat corridors between fragments along with measures to control hunting in areas between fragments were recommended (Bergl *et al.* 2008). The Bornean orang-utan (*Pongo pymaeus*), living in forest fragments of the Lower Kinabatangan flood plain in Sabah, Malaysia, shows a different pattern: high levels of heterozygosity within fragments with a relative scarcity of rare alleles, suggesting

that this population was large in the past and has suffered a recent major reduction (Goossens *et al*. 2005). Goossens *et al*. (2006) using extensive non-invasive sampling across the area and 14 microsatellite loci showed that the Bornean orang-utans population has decreased in size by 95% over the last decades or centuries, due to anthropogenetic fragmentation of the habitat. Therefore, the high genetic diversity found is transitory and may disappear if forest corridors alongside the riverbank are not established (Bruford *et al*. 2010).

Fig. 2: Behavioural adaptation to habitat fragmentation. Black-and-white colobus are almost exclusively arboreal. Fragmentation may force the adaptation to new habitats or result in local extinctions.

Fig. 3: Habitat loss and deforestation. Deforestation is happening at an accelerated rate and can be caused by logging, collection of non-timber forest products and fires. It not only promotes isolation of populations but also increases hunting pressure and contributes to climate change.

Hunting

The impact of hunting pressure on primate populations is often difficult to evaluate. Although information on the amount of harvested primates can be obtained by counting carcasses in urban bushmeat markets, morphological identification can be hindered if a carcass has been processed or if the meat has been smoked (Fig. 4).

Primatologists can use molecular PCR-based tools to taxonomically identify unknown specimens. After extracting DNA and amplifying a specific DNA fragment, these fragments can then be compared with other DNA fragments obtained from specimens of known species. The comparison can also be accomplished by verifying the presence/size of the fragment after PCR (for a review of the techniques see Fajardo *et al*. 2010).

Fig. 4: Bushmeat markets. Hunting of primates is occurring at very high rates. Primate meat is consumed in rural areas for subsistence and in urban centers as a delicacy. It is the result of an illegal organized trade.

Fig. 5: Molecular Identification of bushmeat. Morphological identification can be difficult if carcasses have been processed. Molecular identification is the easiest and most reliable tool available.

In many cases however, the researcher might not have access to specimens of known species. To overcome this difficulty, it is necessary to amplify a standard gene fragment that can be compared with fragments from voucher species deposited in public databases. A fragment of 648bp from the mitochondrial cytochrome c oxidase (COI) gene was proposed by Hebert *et al*. (2003) as a standard fragment for DNA barcoding the data for which are deposited in the Barcode of Life Database (HYPERLINK "http://www.barcodeoflife.org/"http://www.barcodeoflife.org/) as well as in public databases such as GenBank. Lorenz *et al*. (2005) tested the use of this mitochondrial DNA region to identify the species of primate samples.

All samples, representing 56 primate species, amplified with at least one of the 3 different primers used and, with few exceptions, the fragments obtained clustered together with sequences retrieved from GenBank (Fig. 5). More recently, Rönn *et al.* (2009) proposed the use of a micro-array system to assign samples of primates to the genus level, using both nuclear and mitochondrial genes. This technique uses 111 diagnostic nucleotide positions to perform a hierarchical assignment of samples. This method can be used to process a large number of samples at a relatively low cost, and 45 out of the 64 samples were correctly assigned to their Primates genus.

Diseases

Disease is another important aspect for primate conservation. Tha Ebola and Anthrax outbreaks that have occurred in Central Africa in recent decades caused a dramatic decline in gorilla and chimpanzee populations (Leendertz et al. 2006; Bermejo *et al*. 2007; Campbell *et al*. 2008). Additionally, recent studies on parasite infection dynamics have demonstrated an association with hunting, human population growth, and fragmentation in wild primates (Gillespie & Chapman 2006; Goldberg *et al*. 2007; Gillespie *et al*. 2008; Riley and Fuentes 2011). With the incorporation of molecular approaches to epidemiology, Johnston *et al*. (2010) have demonstrated cross species transmission of *Giardia duodenalis* between humans, livestock and wild primates in Western Uganda. Likewise, Goldberg *et al*. (2009) discovered three novel retroviruses in red colobus monkeys, shedding light on the dynamics of primate retroviral transmission. More recently, Yildirim *et al*. (2010) unveiled the gut microbial community of three nonhuman primate species by sequencing the small subunit rRNA unit from fecal samples allowing future analysis on comparative and evolutionary studies of human gut microbes and other primates. Furthermore, in an innovative method (that combines a single-genome amplification of *Plasmodium* sp. recovered non-invasively from fecal material of great apes), Liu *et al*. (2010) inferred that the origin of the human malignant malaria *Plasmodium falciparum* is gorilla-derived. This result argues against the previous study from Prugnolle *et al*. (2009) that showed that *P. falciparum* emerged from *P. reichenowi* by a single transfer from chimpanzees. Similarly, HIV/AIDS is the result of a cross-species transmission event of simian immunodeficiency virus (SIV) to humans from non-human African primates and much attention has been paid to the understanding of the evolutionary history of these emerging infection diseases (Gao *et al*. 1999; Damond *et al*. 2004; Liu *et al*. 2008). By using a molecular dating technique, Wertheim and Worobey (2009) estimated a surprisingly recent common ancestor of infectious SIV in chimpanzees (between 1266 to 1685 years) and sooty mangabeys (between 1729 to 1875 years) the reservoirs of HIV-1 and

HIV-2, respectively. Conversely, human transmitted pathogens to great apes such as: bacteria (eg. *Streptococcus pneumoniae*) or viruses (eg. human metapneumovirus) are causing fatal respiratory outbreaks (Chi *et al*. 2007; Kaur *et al*. 2008; Köndgen *et al*. 2008; Köndgen *et al*. 2011; Palacios *et al*. 2011) and to mitigate the risk of disease transmission the use of face masks by researchers, tourists and staff is advocated as a good practice (Macfie & Williamson 2010). These studies emphasize the fact that there is much to be learned concerning disease transmission and its implications for wild primates and those molecular tools will give a clearer insight.

Applying conservation genetics

Primate Census

Abundance and density of wild primate populations are key parameters for assessing their conservation status and management (Arandjelovic *et al*. 2010). Biomonitoring and molecular censusing allows the determination of population size estimates as well as individual movements in the landscape (Storfer *et al*. 2007; Vigilant & Guschanski 2009). Guschanski *et al*. (2009), using a panel of 16 microsatellite loci, estimated that the population size of the endangered mountain gorillas (*Gorilla beringei beringei*) was 10% less when compared to the classical nest-count methods. All molecular census estimates in primates have shown a population size smaller than previously accessed by traditional methods. In contrast, Zhan *et al*. (2006), comparing the numbers of traditional survey methods with molecular censusing, demonstrated that the DNA-based estimate for a well-studied giant panda (*Ailuropoda melanoleuca*) population was more than double the ecological estimate.

When using a capture-recapture analysis for census purposes, Arandjelovic *et al.* (2010) recommended that three times more samples should be collected than the predicted population size for apes when assuming a closed population model. Therefore, molecular surveys are a complementary method to more traditional census approaches.

"Evolutionary Significant Units" (ESUs) and "Management Units" (MUs)

ESUs and MUs are two types of conservation units, described using genetic information: ESUs have been defined as needing to be reciprocally monophyletic mitochondrial lineages (i.e. occupying different branches in a phylogenetic tree) and requires long-term historical population differentiation, whereas MUs are identified

based on current demographic isolation (i.e. no current or recent gene-flow), evidenced by differences in allele frequency distributions and significantly different frequencies for both mitochondrial and nuclear *loci* (Moritz, 1994). Although the criteria to identify these units have been subject to debate (for example Paektau 1999), such definitions can be key indicators to preserve genetic distinctiveness (evolutionary heritage, genetic diversity and differentiation). For instance, Kanthaswamy *et al*. (2006), based on the analysis of mtDNA and microsatellite loci suggested that the Bornean and Sumatran orangutans should be considered two distinct MUs and consequently the authors discourage the inter-island translocation of animals.

Population and Habitat Viability Analysis

Population and habitat viability analysis (PHVA) evaluates the risk of extinction within a certain period of time (e.g. 100 or 200 years) and identifies which factors play a major role in the extinction process. PHVA relies on stochastic modelling by using simulation software, such as VORTEX (Miller & Lacy, 2005), and requires the input of parameters on the ecology and life history of the species (e.g. population size, mortality and birth rates, sex ratio, dispersal sex and rates and main threats to the habitat) to be able to simulate (by Monte Carlo iterations) species responses that are realistic. Molecular census and genetic data can also be very important parameters for PHVA. Moreover, it allows the introduction of different and combined management measures (e.g. ecological corridors, reintroduction, translocations, habitat rehabilitation) and simulates the evolution of the species under such interventions. This tool allows conservationists to detect the major threats for rare and endangered species and thereby help implement the most long-term viable conservation actions.

Bruford *et al*. (2010) incorporated the genetic data of 200 orang-utans from the Kinabatangan floodplain in Sabah, Malaysia to study the implications of non-intervention, translocation, corridor establishment and the combination of the latter two measures, on the future genetic diversity of this highly fragmented population. They found that non-intervention would result in the extinction of some of the subpopulations within five generations and that the exclusive translocation or corridor establishment would not be sufficient to prevent high levels of inbreeding. Instead, a combination of the two measures would retain the demographic stability even of the most isolated subpopulations and constrain localized inbreeding to a sustainable threshold.

Final Remarks

The extensive use of molecular techniques as tools has provided new opportunities to better understand the mechanisms underlying the evolution and adaptation of primates (Fig. 8). By integrating genetic and ecological data into simulation models, conservation predictions will be more accurate and long-term conservation strategies will be more effective.

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Appendix five: Characterization of the urban primate bushmeat trade in Guinea Bissau using molecular tools for species identification (*in prep,* preliminary Methods and Results sections)

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Figure 1 Morphological aspect of primates being sold in Bissau (Chapa market).

Methods

Bushmeat markets and species frequencies

During March 2010 we were able to locate two bushmeat markets in Bissau: "Chapa" and "Rampa". Chapa market is a permanent "butcher stalls" type of market, selling not only bushmeat (Fig 1) but also domestic meat (cow, goat and domestic pig). Rampa market is close to a harbour in Bissau and is the final destiny of shipments, including bushmeat, fish and meat derived from domestic animals. The goods, in most cases pre-ordered by the customers, arrive by boat to Bissau across the Geba River. All goods in Rampa would be sold just a few minutes after the daily boat arrived, and this market seems to be less permanent than Chapa.

We visited both markets in the dry season during 19 days, divided in three different periods: five days from 1st to 5th March; five days between 30th March and 10th April and nine days between 19th May and 6th June. During our daily visits that did not last more than 15 minutes, we counted the number of primate carcasses present

in the market and recorded the morphological identification provided by the traders (Table 1). We inquired the price and the original location of the individuals. We were unable to follow the trade along entire days since our presence was considered inconvenient for the normal function of the commerce. In these short visits, we were able to list the non-primate species being sold but were not capable to follow their trade. We also visited restaurants where primate bushmeat is cooked and consumed to obtain more information on how primate meat consumption takes place.

In order to estimate an error rate associated with the morphological identification, we collected 50 tissue samples for DNA analysis (see table 1), stored it in tubes with 99% ethanol and labelled with the morphological identification provided by the traders.

Molecular Identification

DNA Extraction, PCR amplification and sequencing

To improve DNA extraction success, we aimed to extract from unburned tissue. Whole genomic DNA was extracted using DNeasy Blood and Tissue kits (Qiagen, ©), following the manufacturer's protocol but allowing overnight lysis of tissues.

We amplified a mitochondrial DNA fragment of approximately 700 base pair (bp) long from the standard barcode region of the first half of *COI* (*Cytochrome c Oxidase subunit I*) using the three primer sets used by Lorenz *et al.* (2005). All samples were first tested with primer set "OWMCOI" since it was successful in the amplification of 8 out of the 9 primate genera tested by Lorenz *et al*. (2005), three of which analysed in this study (*Papio, Cercopithecus* and *Chlorocebus*). "VERTCOI" primers proved to be less successful in Lorenz *et al.* (2005) study, allowing the sequencing of only 6 out of the 13 primate genera tested. Since in Lorenz *et al*. (2005) study, the "VERTCOI" pair worked for some genera that were not tried with "OWMCOI", we used this primer pair for samples that did not amplify any PCR product after using the "OWMCOI" primer set. "FOLMER" primer set (designed by Folmer *et al.* 1994, also in Lorenz et al. 2005) was only used for samples that did not yield any PCR product with neither of the first two primer sets.

The PCR reaction mix contained 10µl Qiagen © Master Mix (containing HotStarTaq, DNA polymerase, Multiplex PCR buffer and dNTP mix), 6µl RNase free water and 2µl 10x primer mix (containing each primer at 2µM). 2µl of template DNA was added for a final volume of 20µl. The cycling protocol was optimised for the

primers and samples. The thermal cycling programme involved 15 min at 95°C (a Taq activation step), 40 cycles of 30 sec denaturing at 94°C, 90 sec annealing at 50°C and 90 sec extending at 72°C. Finally a final extension step of 10 min at 72°C was included. Thermal cycling was performed on an Applied Biosystems GeneAmp PCR System 9700. PCR products were visualised by agarose gel electrophoresis, with 1% agarose gels stained with ethidium bromide, and viewed under UV light. For sequencing, PCR products were purified with 10 units of Exonuclease I and 5 units of Antarctic Phosphatase (New England Biolabs), using a cycling programme of 37° for 30 mins, 80° for 20 mins and 12° for 5 mins. Samples were sequenced bidirectionally by Macrogen Europe's EZ-seq direct service.

Query sequence specific assignment

Using Bioedit Version 7.0.5.3 (Hall 1999), a consensus was made between the forward and reverse sequences for each sample by visual comparison. All sequences were manually aligned and then trimmed to the maximum same length of 623 base pairs.

Sequences were checked for the presence of NUMTS, which may be signified by frameshift mutations (insertions or deletions), ghost bands on gels, or unlikely phylogenetic placement in trees (following Bensasson *et al*. 2001 and Song 2008). No evidence of the presence of NUMTS was found in this study.

To assign each *COI* sequence obtained from the samples to a specific level (the "query" sequence) we used a sequential method (Frézal & Leblois 2008): 1) we searched in a complete database for the most similar sequences; then 2) we chose the most similar sequences as "voucher" sequences; and finally 3) we used a phylogenetic approach (using genetic distances and maximum likelihood algorithms) to correctly assign the samples to the species level.

Therefore, each query sequence was compared to all *COI* reference sequences on NCBI (http://www.ncbi.nlm.nih.gov/genbank/) using the Basic Local Alignment Search Tool for nucleotides (BLASTn within NCBI, Altschul *et al.* 1990). BLASTn optimises alignments based on a similarity (threshold distance) algorithm.

The most similar *COI* sequences for each of the alleged species were obtained from NCBI (http://www.ncbi.nlm.nih.gov/genbank/) and Inprimat databases (www.inprimat.org) to serve as voucher sequences. We included in the alignment sequences from *E. patas* (ERYTHROCEBUS PATAS 1, accession number EF568610.1 and ERYTHROCEBUS PATAS 2, accession number AY972702.1), *C. sabaeus* (CHLOROCEBUS SABAEUS 1, accession number EF597503.1 and CHLOROCEBUS

SABAEUS 2, accession number NC_008066.1) and *P. papio* (PAPIO PAPIO 1, accession number AY972684.1 and PAPIO PAPIO 2, accession number AY972678.1) found on both Inprimat and NCBI. We used two vouchers for *P. badius*: a sequence found only in NCBI/GENBANK (PROCOLOBUS BADIUS 1, accession number NC_008219.1) along with a faecal sample collected and extracted by T. Minhos from a visually confirmed *P. badius* (PROCOLOBUS BADIUS 2) and sequenced for the mtDNA region under study. Although we found the same degree of similarity between two query samples and two different species (*Colobus polykomos* and *Colobus guereza* samples, see results section) we only included in the alignment *C. polykomos* voucher samples (COLOBUS POLYKOMOS 1, accession number AB016731.1 and COLOBUS POLYKOMOS 2, accession number AY972692.1). According to Lorenz *et al.* (2005), this discrepancy might be related with the wrong labelling for this sequence in particular. Also, according to current knowledge (Gippoliti and Dell'Omo 2003) there is only one *Colobus* species present in Guinea-Bissau - *C. polykomos.* Currently there are no *Cercopithecus campbelli COI* sequences on databases and we were unable to obtain a voucher sample from a visually confirmed *C. campbelli*. Therefore, we sequenced a fragment of the 12S rRNA gene for all samples suspected to be *C. campbelli* and compared it with the sequences available on NCBI (including two *C.cambelli* sequences, accession numbers AY665618.1 and AY665619.1) to obtain the specific identification.

Finally, we constructed a Neighbour joining (NJ) tree to view clustering of sequences with bootstrap support (1000 replicates) of each node using Mega 5.01 (see Fig 4). We included all sequences obtained by this study, and voucher sequences obtained via INPRIMAT and NCBI/GENBANK databases, in this analysis. Intra and Inter cluster variation was computed using Arlequin.

Species morphological identification error and calculation of the specific identification correction factor

We calculated the error rate associated with the morphological identification for each species by dividing the number of samples in which the molecular identification did not correspond to the morphological identification by the total number of tissue samples collected.

For each species, we present the number of individuals traded as being within the range between a minimum number of individuals molecularly assigned to that species and a maximum number of individuals, obtained through a correction factor calculated after the specific molecular assignment.

To calculate the correction factor, we assumed that collection of tissue samples was representative of the number of individuals identified morphologically at the markets as more than 20% of the individuals observed all species were sampled (see table 1). To calculate the maximum number of individuals at the market we summed the true positive identifications frequency (TPF) with the false negatives frequency (FNF) in each species. Therefore for any species,

Maximum number of individuals for that species = TPF + FNF

$$TPF = NRM \times \frac{NPTS}{NTS}$$

NRM - the number of morphological records

NPTS – number of tissue samples labelled and assigned to that species

NTS – number of tissue samples collected for that species

$$FNF = ONS \times \frac{NES}{NSOS}$$

ONS: the total number of individuals observed at the market minus the number of individuals observed using morphological identification for each species

NES: samples assigned to the species but not initially labelled as that species

NSOS: total number of tissue samples collected minus tissue samples molecularly assigned to that species

Corrected frequencies of the bushmeat trade and projection of the trade for the dry season

We calculated the relative percentage of each traded species using the maximum values obtained per species using the correction factor. We then extrapolated the trade for the entire dry season (November to May, 212 days). Using the relative percentages of trade for each species, we estimated the amount of individuals per species traded during the dry season.

Results

Trade at Bissau Markets

During the 19 days of study, we observed 150 primate carcasses being sold in Bissau, with more primates being sold at Chapa market (113) than Rampa market (37). We found that six species of primates are sold at Bissau markets: *Procolobus*

badius (Western Red Colobus), *Colobus polykomos* (Western Black-and-White Colobus), *Cercopithecus campbelli* (Campbell's Mona), *Papio papio* (Guinea Baboon) *Erythrocebus patas* (Patas monkey) and *Chlorocebus sabaeus* (Green Monkey).

Non-primate specimens observed at the markets included a species locally called "farfana", which is likely to be the Greater Cane Rat, *Thryonomys swinderianus*, the Crested Porcupine (*Hystrix cristata*, locally called "porco ispinho"), pangolins (*Manidae sp.*, locally called "tu(r)cutacar"), Red River Hog (*Potamochoerus porcus*, locally called porco do mato) and duikers (*Cephalophus sp.*, local names including "cabra do mato" and "fritamba").

The price of primates at market seemed to be related to their size and not their weight. The traders did not have scales at the stall and based their prices in the size of the specimens. *P. papio* males are the biggest and therefore most expensive carcasses at the markets, being sold at between 10,000 and 15,000 CFA (approximately 17€ -25€). The price of baboon females and females and males of the other species varied between 4,000 and 8,000 CFA (approximately 6-12€). Carcasses were mostly sold whole and to restaurants, although some private customers were also observed.

The restaurants that specialise in serving bushmeat meals are locally called Abafatórios. Here, primate meat is most often consumed as a snack whilst drinking alcohol. It is cooked in a stew (see Fig. 2) and eaten with bread. The meal (4 pieces of primate meat, not including bread) cost around 1,250 CFA (approximately 2€). Primate hands, feet and heads were referred as greatly appreciated by the customers.

Daily frequency of trade in Bissau markets varied across the dry season (Fig 3). In the first period of the study (1st to 5th March) trade averaged 9.6 primates per day across both markets (7.4 in Chapa and 2.2 in Rampa). In the second period of the study (30th March to 10th April), the trade increased to 16.8 primates per day across both markets (11.6 and 5.2 primates per day in Chapa and Rampa respectively). In the third period of the study (19th May to 6th June), approaching the start of the rainy season, we found a sharp decrease in the commerce (only 2 primates per day in Chapa market and no trade at all in Rampa market). The average and standard deviation of trade across periods is 9.46 ± 7.4 primates per day.

In the majority of cases, the primates were attributed to coming from the south of Guinea-Bissau. "Cacine" (a village in the Tombali Administrative region) was the origin most frequently recalled/named, followed by Cossé and Xitole (in Bafatá). These locations follow the main road from Bissau to the south of the country, so may not represent the places where the primates were actually hunted, but instead the origin of transportation.

Figure 2 Preparation of primate meat stew at "Abafatórios" restaurant. Baboon and green monkey heads are visible.

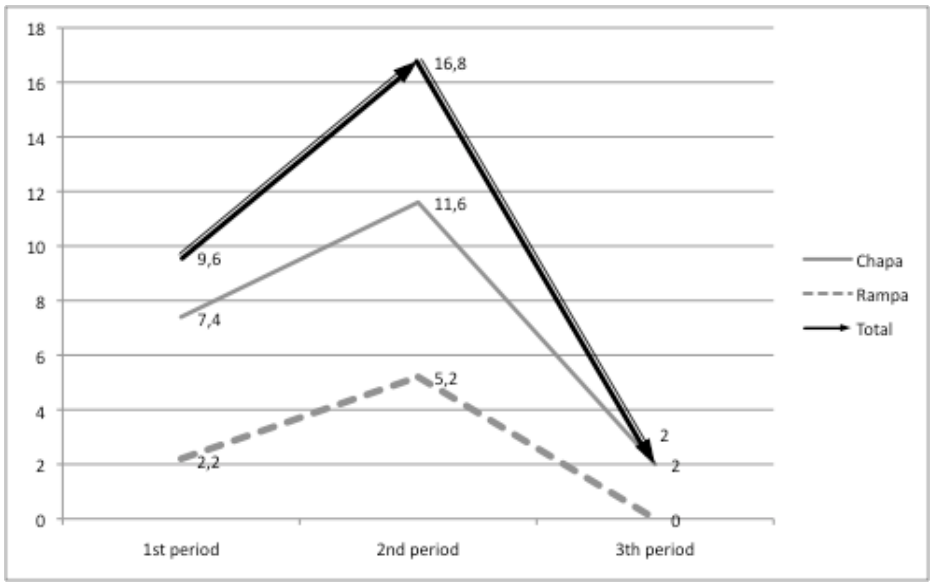

Figure 3 Daily frequency of trade across periods for Chapa and Rampa markets. The total values are also indicated.

Species assignment

All the tissue samples collected were successfully extracted and sequenced for a 623 bp fragment. We were able to sequence 46 samples with the OWMCOI primer set. Two samples (labelled as *Procolobus badius* and *Cercopithecus campbelli*) were sequenced using the VERTCOI primers and the two samples labelled as *Colobus polykomos* samples were sequenced using the FOLMER primer set.

All sequences were 'blasted' and compared with NCBI database *COI* sequences (using BLASTn, Altschul *et al.* 1990). Across species and discounting *Cercopithecus campbelli*, we obtained a 0-6% range of dissimilarity. Two samples showed a 95% similarity to *Colobus polykomos* but also with *Colobus guereza*; seven

samples were 99% similar with *Procolobus badius*; 12 samples showed 99-100% similarity with *Papio papio*; 15 samples were 99-100% similar to *Chlorocebus sabaeus* and two samples were 94% similar to *Erythrocebus patas* sequences. Twelve samples, suspected to be *Cercopithecus campbelli*, showed 89% similarity with both *Cercopithecus pogonias* and *Cercopithecus wolfi*. However, since there are no sequences identified as *Cercopithecus campbelli* in Genbank for the *COI* barcode region, we identified this species using a fragment of the 12srRNA gene (similarlity with 12sRNA gene).

All the sequences belonging to the same species are grouped together along with the respective voucher sequence in six clusters supported by 100% of the bootstrap replicates in the Neighbour-Joining tree constructed (Fig. 4). The six highly supported clusters corresponded to the six species sold at the bushmeat markets at Bissau: *C. campbelli, P. badius C. polykomos*, *P. papio, E. patas* and *C. sabaeus*, agreeing with the information given by the sellers based on morphological identification.

Morphological Identification error and corrected number of specimens per species

On average, the morphological identification error rate across species was of 23.4%. We found the greatest morphological identification error in *C. campbelli* (59.1%), followed by *P. badius* (40%), *C. polykomos* (33.33%) and *P. papio* (7.70%). We didn't find any error in the morphological identification of *E. patas* and *C. sabaeus*.

Most samples mislabelled in the morphological identification were molecularly assigned to *C. sabaeus*: 10 samples initially labelled as *C. campbelli* and 3 samples labelled as *P. badius*. Accordingly, after applying the correction factor to the total number of specimens observed at the markets, the total number of *C. campbelli* decreased in 35 specimens, while all other species increased in number, on average by 18 specimens. We found an increase of 13 specimens for *P. papio,* 5 specimens for *E. patas*, 1 for *P. badius,* 1 for *C. polykomos* and 57 for *C. sabaeus* (see Fig. 5). Therefore, our results suggest that at the Bissau markets two species are predominately traded, at similar frequencies: *C. sabaeus* (32.2 %) and *C. campbelli* (30.6 %). *P. papio* was the third most traded species (19.26%) followed by *E. patas* (4.9%), *P. badius* (11.5%) and *C. polykomos* (1.4%) (see Fig. 6).

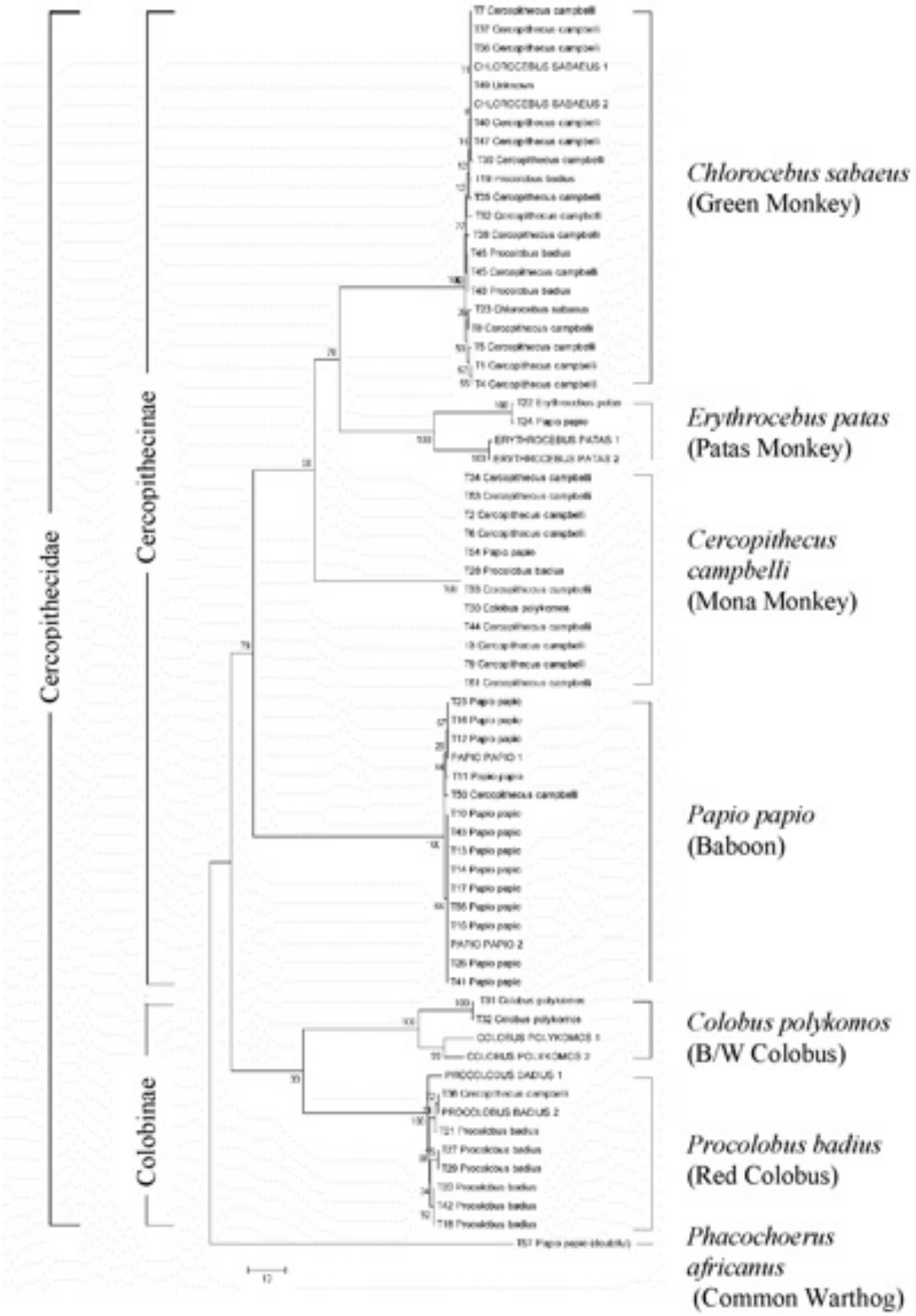

Figure 4 Neighbour-joining tree with bootstrap values supporting molecular identification

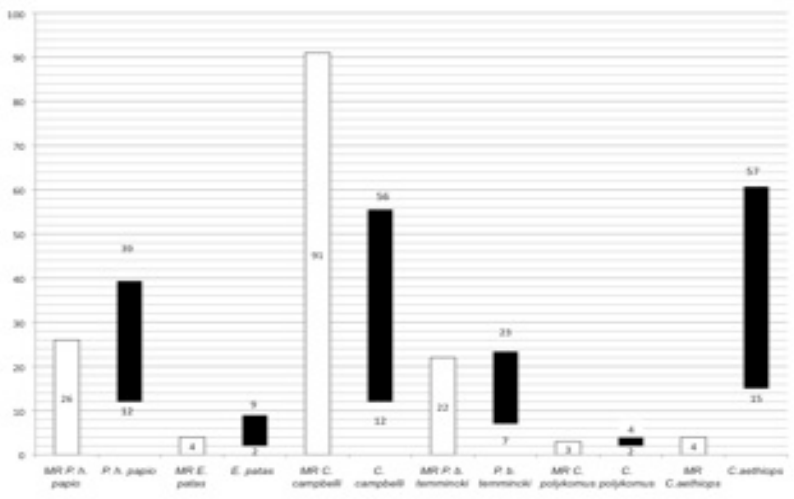

Figure 5 Total number of specimens per species. White bars refer to the morphological identification (MR). Numbers inside white bars represent the number of specimens identified morphologically at the markets. Black bars represent the potential range in frequency of specimens after the molecular identification correction. Numbers below black bars represent the minimum number of specimens per species (i.e.. samples molecularly assigned to that species). Numbers above black bars represent the estimated/potential maximum number of individuals at the markets.

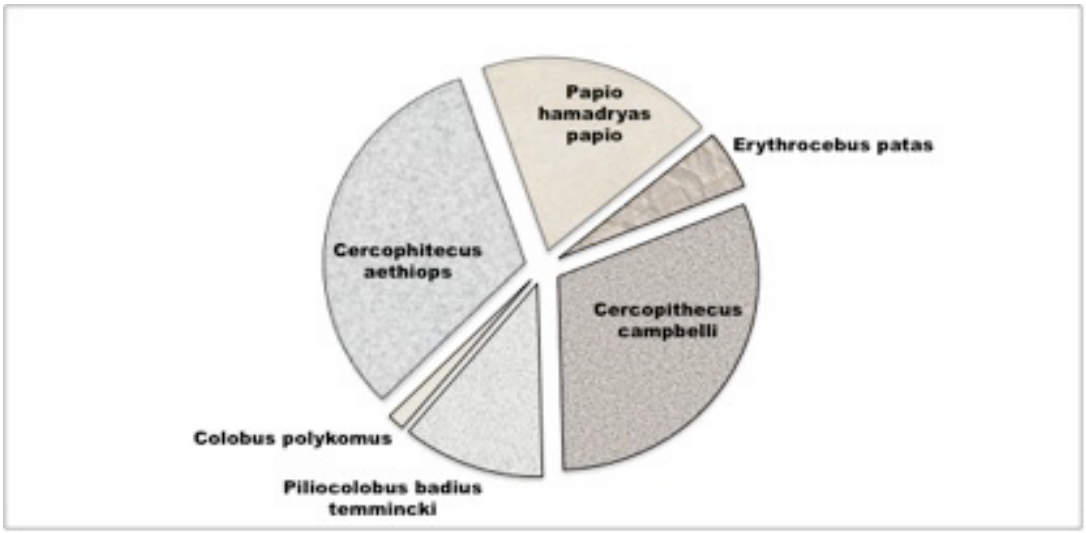

Figure 6 Relative percentage of species traded at Bissau markets.

Projection of the primate bushmeat trade in Guinea-Bissau

We extrapolated the number of primates traded in Bissau over the entire dry season (212 days). Using the average daily trade across the three periods, 2006.9 primates are traded every dry season. During this period, and assuming the relative percentages for the 19 days of study, we predict that 646 specimens of *C. sabaeus*, 616 of *C. campbelli*, 387 specimens of *P. papio*, 99 specimens of *E. patas*, 232 specimens of *P. badius* and 28 specimens of *C. polykomos* are traded in the bushmeat markets in Bissau.

Table 1: Data collection and molecular species assignment

Species (Scientific name/common name)	**N individuals observed**			**N tissue samples collected**			**% tissue samples collected per observed individual**	**N tissue samples labelled and assigned to the species**	**Error rate**	**Tissue samples assigned to species.**
	Chapa	Rampa	Total	Chapa	Rampa	Total				
Cercopithecus campbelli (Campbell's Mona)	70	21	91	19	3	22	22.7%	9	59,1%	12
Erythrocebus patas (Patas monkey)	1	3	4	1	0	1	25%	1	0	2
Procolobus badius (Western Red Colobus)	18	4	22	10	0	10	45.5%	6	40%	7
Colobus polykomos (Western Black-and-White Colobus)	3	0	3	3	0	3	100%	2	33,33 %	2
Papio papio (Guinea Baboon)	18	8	26	12	0	13	50%	12	7,7%	12
Chlorocebus sabaeus (Green monkey)	3	1	4	1	0	1	25%	1	0%	15
Total	113	37	150	46	3	50				