Phylogeography, conservation genetics and parasitology of chimpanzees (*Pan troglodytes verus*) in Guinea-Bissau, West Africa

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**This dissertation is submitted for the degree of Doctor of
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Thesis submitted for partial fulfillment of the requirements for the degree of Doctor of Philosophy in Anthropology, specialty of Biological Anthropology and Ethnoecology, held under the scientific supervision of Professor Cláudia Sousa (Universidade Nova de Lisboa) and Professor Michael W. Bruford (Cardiff University).

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With the institutional and logistical support of:

STATEMENTS

I hereby declare that this thesis is the result of my own personal and independent investigation. Its content is original and all sources are acknowledged by giving explicit references.

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A handwritten signature in black ink, appearing to read 'Ricardo Silva', written over a horizontal line.

Lisbon, 14th January 2013

I declare that this thesis is ready to be appreciated by a jury to be appointed.

The supervisor,

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Lisbon, 14th January 2013

I declare that this thesis is ready to be appreciated by a jury to be appointed.

The supervisor,

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Cardiff, 14th January 2013

DEDICATION

To my family for
their unconditional love and constant support.
Dedicated as well to my Guinean human friends for
showing me that I don't really need more than
what I have and to my chimpanzee ones for convincing me that
"convictions are hopes" (Bertolt Brecht).

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To all,

Muito Obrigado! Thank you so much! Diolch! Děkuji! Obrigadu!

**PHYLOGEOGRAPHY, CONSERVATION GENETICS AND PARASITOLOGY OF
CHIMPANZEES (*PAN TROGLODYTES VERUS*) IN GUINEA-BISSAU, WEST AFRICA
PHD DISSERTATION**

RUI MIGUEL MOUTINHO SÁ

ABSTRACT

KEYWORDS: Chimpanzees; Guinea-Bissau; Phylogeography; Symbionts; Conservation

Chimpanzees are disappearing at an alarming rate and it is imperative that strategies should be applied towards their conservation. The evolutionary history of West African chimpanzees remains ambiguous and controversial. Chimpanzees in Guinea-Bissau live at the most western limit of the species distribution and no studies so far have included individuals from this area. Little is known about their ecological, social and behavioural characteristics and their phylogeography and genetic structure has never been evaluated. Furthermore, little is known about their symbiotic fauna.

The aims of my Ph.D. research were to evaluate concomitant threat factors that may have a negative impact on chimpanzee persistence in Guinea-Bissau, and to test a set of hypothesis regarding their phylogeographic and genetic structure. First I report on the trade and ethnobiological use of chimpanzee body parts for traditional practices. Second, I investigate how Guinea-Bissau chimpanzees relate to other members of the *Pan troglodytes verus* subspecies in West Africa especially those from Guinea Conakry in order to uncover their evolutionary history. Third, I assess their genetic diversity and structure where I expected to find significant population genetic structure among isolated subpopulations. Finally, I investigate the gastrointestinal symbiont diversity of chimpanzees living in a disturbed habitat, especially focusing on infection from parasites with direct life cycles and the effects of increased intra and interspecific contact.

My research shows that in addition to habitat loss and fragmentation and the pet trade, transnational traffic and the use of chimpanzee body parts for traditional purposes constitute additional threats and must be taken into consideration for conservation measures. Second, I showed that Guinea-Bissau chimpanzees have experienced a complex paleodemographic history revealed by the phylogeographic

analyses suggesting that an historical bottleneck followed by several expansion events. Furthermore, a clear pattern of genetic structure was observed where isolation by distance and vicariance have affected patterns of genetic structure. Chimpanzee females were inferred to disperse in a stepping stone way. Moreover, the two main mitochondrial lineages emerged during the early Pleistocene (1-0.78 MYA) and the divergence time of the haplogroups dates back to middle Pleistocene (0.78-0.12 MYA) coincident with the Gunz (0.68-0.62 MYA) and Mindel (0.45-0.30 MYA) glaciations that caused the contraction of west African tropical forests but followed by an expansion afterwards during the interglacial periods that restored its connectivity.

Lastly, I identified at least 13 different symbiotic genera (*Troglodytella abressarti*, *Troglocorys cava*, *Blastocystis* spp., *Entamoeba coli*, *Iodamoeba buetschlii*, *Giardia intestinalis*, *Chilomastix mesnili*, *Bertiella* sp., *Probstmayria gombensis*, unidentified strongylids, *Strongyloides stercoralis*, *Strongyloides fuelleborni* and *Trichuris* sp.), which have colonized the Guinea-Bissau chimpanzee gastrointestinal tract. Symbiont richness was higher in chimpanzee subpopulations living in fragmented forests compared to the community inhabiting continuous forest area. In fragmented areas chimpanzee density and range-use intensity decreased, which might contribute to low prevalence/total absence of *Trichuris* sp. in samples from chimpanzees in these areas when compared with those inhabiting continuous forest.

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RESUMO

PALAVRAS-CHAVE: Chimpanzés; Guiné-Bissau; Filogeografia; Simbiontes; Conservação

Os chimpanzés estão a desaparecer do seu habitat natural a um ritmo alarmante e é imperativo que estratégias de mitigação sejam aplicadas na sua conservação. A história evolutiva dos chimpanzés na África Ocidental permanece ambígua e controversa. Os chimpanzés da Guiné-Bissau vivem no limite mais ocidental da distribuição da espécie e até agora nenhum estudo genético e parasitológico incluiu indivíduos pertencentes a esta área. Pouco se sabe sobre suas características ecológicas, sociais e comportamentais e a sua filogeografia e estrutura genética nunca foram avaliadas. Além disso, pouco se sabe sobre sua simbiose-fauna.

Os objectivos do meu trabalho de doutoramento foram: I) descrever concomitantes factores de ameaça que têm um impacto negativo na sobrevivência dos chimpanzés na Guiné-Bissau e, II) testar um conjunto de hipóteses sobre sua estrutura filogeográfica e genética. Em primeiro lugar, relatam o uso comercial e etnobiológico reportando o tráfico, de partes do corpo do chimpanzé para práticas de medicina animista tradicional. Em segundo lugar, investiga como os chimpanzés da Guiné-Bissau se relacionam com os outros membros da subespécie *Pan troglodytes verus* na África Ocidental e se existe uma relação haplotípica com os chimpanzés da vizinha Guiné-Conacry, a fim de elucidar sobre a sua história evolutiva. Em terceiro lugar, foi avaliada a sua diversidade genética e estrutura onde se esperava encontrar uma estrutura populacional genética significativa entre subpopulações isoladas e baixa diversidade genética entre as populações. Finalmente, no que diz respeito à sua riqueza e prevalência de simbiontes, onde se esperava encontrar uma diversidade elevada de simbiontes gastrointestinais, consequência de um habitat perturbado devido a efeitos de fragmentação, ou seja: uma maior infecção com parasitas com

ciclos de vida directos à medida que o contacto intra e interespecífico aumenta, e poucas diferenças na prevalência de simbioses entre subpopulações.

A minha pesquisa revelou que além, da alteração do habitat (por perda e fragmentação), e comércio de mascotes; o tráfico transnacional e a utilização de partes do corpo dos chimpanzés para fins tradicionais constitui uma ameaça real adicional e deve ser tido em consideração em futuras medidas de conservação. Em segundo lugar, demonstro que na Guiné-Bissau os chimpanzés tiveram uma história paleodemográfica complexa revelada pelas análises filogeográficas que sugerem um “bottleneck” seguido por vários eventos de expansão e que explica os altos níveis de diversidade haplotípica observados. Além disso, demonstra que existe um claro padrão de estrutura genética e que esta pode ser explicada tanto por um padrão de isolamento por distância mas também por vicariância e que afectam o fluxo de genes. As fêmeas chimpanzés tendem a dispersar seguindo o modelo “step and stone”. Além disso, as duas principais linhagens mitocondriais observadas surgiram durante o Pleistoceno Inferior (1-0,78 MYA) e o tempo de divergência entre haplogrupos remonta ao Pleistoceno Médio (0,78-0,12 MYA) em consonância, em termos paleoclimáticos, com as glaciações de Gunz (0,68 MYA -0,62) e Mindel (0,45-0,30 MYA). As glaciações causaram a contracção da floresta tropical na África Ocidental intercalando com diversos episódios de expansão, durante os períodos interglaciares e que restauraram a sua conectividade e enquadram o cenário filogenético promovendo um contacto secundário entre linhagens.

Por fim, identifico a presença de pelo menos 13 géneros de simbioses (*Troglodytella abrassarti*, *Troglocorys cava*, *Blastocystis* spp., *Entamoeba coli*, *Iodamoeba buetschlii*, *Giardia intestinalis*, *Chilomastix mesnili*, *Bertiella* sp., *Probstmayria gombensis*, de estrongilídeos não identificados, *Strongyloides stercoralis*, *Strongyloides fuelleborni* e *Trichuris* sp.), que colonizam o ecossistema gastrointestinal dos chimpanzés da Guiné-Bissau. A riqueza simbiótica foi maior em subpopulações de chimpanzés que vivem em florestas fragmentadas, em contraste com outras comunidades que habitam áreas mais contínuas de floresta. Surpreendentemente, em áreas fragmentadas: a alta densidade de chimpanzés e a sua utilização do território contribuem para uma baixa prevalência ou total ausência de *Trichuris* sp. em amostras de chimpanzés nestas áreas quando comparados com aqueles que habitam floresta contínua. Por fim, apresentam-se também algumas recomendações de conservação para que o seu futuro seja duradouro e se mantenha persistente.

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LIST OF ACRONYMS AND ABBREVIATIONS

AMOVA	Analysis of molecular variance
CITES	Convention on International Trade and in Endangered Species of Wild Fauna and Flora
CLNP	Cufada Lagoons Natural Park
CNP	Cantanhez National Park
CT	Total Haplotype Diversity
CRT	Total Haplotypic Richness
DEFRA	Department for Environment, Food and Rural Affairs
DNA	Deoxyribonucleic Acid
ESUs	Evolutionary Significant Units
F_{ST}	Fixation Indexes
H	Haplotye diversity
IBAP	Instituto da Biodiversidade e das Áreas Protegidas
IUCN	International Union for Conservation of Nature

k	Mean number of pairwise differences
mtDNA	Mitochondrial Deoxyribonucleic Acid
MUs	Management Units
PCR	Polymerase Chain Reaction
SAMOVA	Spatial Analysis of Molecular Variance
SD	Standard Deviation
STR	Short Tandem Repeats
θ_w	Watterson estimator of genetic diversity
Π	Nucleotide Diversity

CHAPTER 1

“Today I am proud to say that I am inhuman, that I belong not to men and governments, that I have nothing to do with creeds and principles. I have nothing to do with the creaking machinery of humanity – I belong to the Earth!”

— Henry Miller, Writer

CHAPTER 1

GENERAL INTRODUCTION

1.1- Chimpanzee conservation: does it matter?

Chimpanzees have an impressive cognitive ability, memory and extraordinary intelligence (Inoue and Matzuzawa 2007), a complex social organization (Goodall 1986) and unique cultures (McGrew 2004). Chimpanzees are man's closest relatives, in evolutionary terms (Jobling et al. 2004). In fact, 4-7 million years ago we shared the same common ancestor (Goodman 1999), and at least 98% of our DNA is identical (Consortium 2005). Since the early 1960', chimpanzee studies have become key for the understanding of human evolution and biology (Sayers and Lovejoy 2008).

West African chimpanzees are more threatened in any other region across the continent (Kormos and Boesch 2003). It is estimated that there are currently less than 38,000 western chimpanzees (Butynski 2003) and only between 600 to 1000 individuals (Gippoliti et al. 2003) in the Republic of Guinea-Bissau, according to the estimate of UNEP-GRASP (United Nations Environment Programme – Great Apes Survival Project, Caldecott and Miles 2005). In fact, they have even been considered extinct in this almost unknown country (Lee *et al.* 1988). The scientific community agree unanimously, they affirm that tropical forests are disappearing at an excessive speed and with them the last populations of great apes (Laurance et al. 2012). If we do nothing gorillas, chimpanzees and bonobos are likely to have disappeared from Africa by the middle of the 21st century. The situation for orangutans is even more dramatic. In 20 years time, they might only exist in zoos (Boesch et al. 2008).

Chimpanzees are disappearing at an alarming rate (Campbell et al. 2008). In Guinea-Bissau follows the continent-wide trend: chimpanzees are endangered and,

if nothing is done, their extinction from this country is certain. In this chapter I set out the conceptual framework for my study, including its scope and subject, objectives, concepts and hypotheses. A literature review is also presented as well as the thesis structure.

1.2- The genus *Pan*

1.2.1- Taxonomy

The most recent taxonomy, and the one adopted here, was proposed by Groves (2001) and Grubb et al. (2003) and is used by the IUCN-SSC Primate Specialist Group (<http://www.primate-sg.org>). It identifies two species in the genus *Pan*: bonobos (*Pan paniscus*) and common chimpanzees (*Pan troglodytes*). Chimpanzees are categorised in four subspecies: the east African chimpanzee (*Pan troglodytes schweinfurthii*), the central African chimpanzee (*Pan troglodytes troglodytes*), the west African chimpanzee (*Pan troglodytes verus*) and the Nigeria-Cameroon chimpanzee (*Pan troglodytes ellioti*, formerly classified as *vellerosus*).

1.2.2- Habitat and Distribution

Among the hominids (except for the genus *Homo*) common chimpanzees are the most generalist species, living in several kinds of habitat: humid evergreen forests, mosaic woodlands, mangroves, deciduous forests and woodland savannah (Kortlandt 1983). Chimps are distributed all over northern Senegal to the south of the Congo River and in the eastern area of Lake Tanganyika, Tanzania (Stumpf 2007) (Fig. 1.1).

The Eastern African chimpanzee (*P. troglodytes schweinfurthii*) possesses a geographical range from the north of the Central African Republic, to the southwest of Sudan, east with the Ubangi River and south in the Equator line in the Central Africa Republic. In this area there are many sites where important data have been

collected during recent decades (Inskipp 2005) including Gombe and Mahale (Tanzania) and Budongo (Uganda).

The Central African chimpanzee (*P. t. troglodytes*) is distributed south of the Sanaga River (Cameroon; Prescott et al. 1994). It extends from east to the western region of the Central African Republic, to the southern limit of Dzanga Ndoki National Park (Inskipp 2005). It is also widely spread throughout Gabon (Blom et al. 1992).

The chimpanzees from Nigeria-Cameroon are distributed all over southwest Nigeria in small and fragmented populations (Oba Hills Forest Reserve), to the southeast and east of the Niger River delta, throughout the boundaries of Cameroon in Gashaka Gumti National Park (Oates 2003).

West African chimpanzees exist in the southwest of Mount Assirik, Senegal, in the southwest of Mali and southeast in the Cantanhez Woodlands, in Guinea-Bissau. They are also present in Guinea, Sierra Leone, Liberia, Cote D'Ivoire and in the southwest of Ghana (Inskipp 2005). The western chimpanzee is considered extinct in Gambia, Togo and Benin (Brownell 2003). According to the estimations by Butynski (2003) this subspecies' geographic range is about 631 000 km².

Figure 1.1: *Pan troglodytes* ssp. geographic range (WWF 2009).

1.2.3- Diet, community structure and social organization

Chimpanzees are essentially omnivorous but show a preference for fruits (Wrangham et al. 1998). In times of food scarcity they increase the proportion of leaves and other less energy-rich food in their diet (Wrangham 1977 and Doran 1977). Hunting behaviour has also been documented in some long-term studies (see Goodall 1986; Boesch and Boesch 1989; Nishida 1979), with considerable inter-population differences in the sort of prey hunted, their habitat characteristics and hunting tactics.

Chimpanzees are polygynous living in multi-male/multi-female groups and their demographic resident number changes in each population. The social group core lies in males, which are generally related and bounded (male-bounded society) (Goodall 1986). They are characterized as fission-fusion communities, meaning that a chimpanzee community can be divided in parties or sub-units, as a function of the resource availability (Goodall 1986). Community is understood here as a group of individuals that share a range or territory. In the chimpanzee case, besides living in a specific geographic area, the community also demonstrates the sharing of information and bonding. Some food, such as fruits and meat, can also be shared among individuals (Casanova 2006). There are cultural elements that are shared by all the individuals and even a daily interaction concerning social and sexual life aspects (Reynolds 2005). Party composition can vary between one to several individuals and be led by males or females. A community of chimpanzees can include from two to eighty members (Casanova 2006). Their home range, i.e. part of the territory usually employed by community effectives, can vary from 6 to 20 km² (e.g. in Taï Forest and Budongo; Boesch and Boesch-Achermann 2000; Newton-Fisher 2002). Most of the females leave the original group when they reach adolescence, shifting to another group (Nishida et al. 2003; Boesch and Boesch-Achermann 2000). There are, however, populations where this fact has not been confirmed yet, like the

case of Bossou (Sugiyama 1999). Males are usually the philopatric sex (Nishida 1979).

At the same time some ecological factors, such as resource abundance, the presence of estrous females or even predation pressure can modify chimpanzee social dynamics and patterns (Wrangham 1980; van Schaik 1983; Symington 1988; Strier 1989; Chapman et al. 1995; Newton-Fisher et al. 2000).

Strong male dominance and male-male alliances characterize chimpanzees at all sites (Goodall 1986; Wrangham 1986; Nishida and Hiraiwa-Hasegawa 1987; Nishida and Hosaka 1996; Boesch and Boesch-Achermann 2000; Watts 2000b; Arnold and Whithen 2003). Chimpanzees perform grooming with those whom they create alliances (Mitani et al. 2000), in a reciprocal way (Watts 2000a; Arnold and Whiten 2003) and according to their status hierarchy, in which the closest individuals to the top are groomed most (Mitani, Merriwether et al. 2000; Takahata 1990; Newton-Fisher 2002). Contrasting with males, female chimpanzee relationships are traditionally ranked as egalitarian and weak (Wrangham 1980; Sterck et al. 1997). Chimpanzees are territorial and the inter-community interactions are very hostile and sometimes deadly (Goodall 1986).

1.2.4- Behaviour and cultural aspects:

Chimpanzees are terrestrial, diurnal and can be arboreal (Inskipp 2005). They move mostly on the ground but they build their nests and feed in trees (Groves 2001). Chimpanzees have great mobility, being able to travel up to 12 km a day (Doran 1997), twice as far as western gorillas (Goldsmith 1999). When there is a lack of resources, for example during the dry season, chimpanzees from Tai have been observed to reduce their daily range, as well as party composition, spending more time feeding, several times a day in small quantities (Inskipp 2005).

Culture has always fascinated scientists through its role as a non-genetically determined evolutionary process (Mesoudi and Whiten 2004). Cultural transmission

offers a quicker method in which some characteristics can be diffused. It is, however, difficult to define 'culture' (McGrew 1998). Nevertheless several authors agree that cultural behaviours are those that: 1) – are specifically learned from members of the same group (cultural variation can occur in any social level) and 2) – are transmitted by a social apprentice mode. I will adopt this particular definition. For more detailed reviews see (Boesch and Tomasello 1998; Frigaszy and Perry 2003; Laland and Hoppit 2003; McGrew 1998; Russell and Russell 1990; McGrew 2004; Whiten et al. 1999, 2001, 2005 2007).

There are several examples of cultural use by chimpanzees: from the hunting of western red colobus (*Procolobus badius*) (Wrangham et al. 1994), to termite fishing in Tanzania (McGrew 1998), the use of medicinal plants (Huffman and Wrangham 1994) to nut cracking in western Africa (Carvalho et al. 2008). One other aspect that has also preoccupied scientists is chimpanzee intelligence. This aspect occurs about the origins and factors that could have led not only chimps, but also other primates and social animals to develop special cognitive abilities and to face social challenges (Casanova 2006).

Tomasello and Call (1997) demonstrated that chimpanzees have the ability to differentiate their own knowledge and the other's knowledge, that is, they can perceive other individuals' mental states. This formulation was named 'Mind Theory' (Tomasello 1999, Call et al. 2000).

In Table 1.1 the main traits of chimpanzees are summarised.

Table 1.1: Chimpanzees in a glance (after Stumpf 2007).

Activity pattern	Diurnal
Social organization	Fission-fusion
Community structure	Multimale/multifemale
Dispersing sex	Adolescent females
Locomotion	Knuckle walking
Substrate use	Arboreal/semiterrestrial
Sleep site	Arboreal nests
Diet	Primarily frugivorous
Sympatric gorillas?	Variable, only in some areas
Dominant sex	Male
Intercommunity interactions	Usually antagonistic
Male-male association	Very strong
Male-female association	Weak in eastern, strong in western
Female-female association	Very weak in eastern, strong in western
Resolve tension with	Aggression
Male-female aggression	Common
Female-female aggression	Rare
Pant-grunt submissive vocalization	Common
Grooming	Male-male most, female-female least
Infanticide	Rare but present at all sites
Tool use	Habitual
Consorts	Some but not all sites
Boundary patrolling	Habitual
Hunting	Occurs at most sites
Food sharing	Yes (mainly meat)
Vocalizations	Lower pitch
Sexual behaviour during nonswelling	Very rare
Copulation posture	Dorsoventral
Female-female sexual behaviour	Very rare

1.3- Major threats to chimpanzee *in situ* conservation

IUCN report chimpanzees as endangered since 1996 and its conservation status has not changed since then (Table 1.2). Chimpanzees have long inter-birth intervals; slow life stories, a high maternal care and a high infant mortality rate and are vulnerable to anthropogenic threats (Stumpf 2007). CITES – the Conservation on International Trade in Endangered Species of Wild Fauna and Flora (2008) classifies chimpanzees as most endangered and inscribe them into their Appendix I. Walsh et al. (2003) recommended that chimps should be part of the Critically Endangered Status list.

Table 1.2: Chimpanzee conservation status evolution (after IUCN 2011).

Year	Status
1986	Vulnerable
1988	Vulnerable
1988	Vulnerable
1990	Vulnerable
1994	Vulnerable
1996	Endangered
2000	Endangered
2003	Endangered (Butynski 2003)
2005	Endangered (Caldecott and Miles 2005)
2007	Endangered (IUCN 2007)
2009	Endangered (IUCN 2009)
2011	Endangered (IUCN 2011)

Recent density estimates point to a range of 0.1 to 6 individuals/km², with forested habitats having higher densities than savannah (Miles et al. 2005). The modal density seems to be 1/km² (Poulsen and Clark 2004). According to a prediction by Jones et al. (2007), 91% of the areas with resident chimpanzee communities are showing population declines. The viability of these populations depends on a wide variety of factors, including habitat preservation, anti-poaching measures and on the maintenance of their genetic diversity (Miles et al. 2005). Their main direct (short-term) conservation threats are: 1) – the destruction and loss of

the habitat; 2) – illegal pet trade; 3) – emergence of infectious and contagious diseases and 4) – political instability and economic constraints (IUCN 2007).

Habitat destruction is one of the major anthropogenic threats affecting chimpanzee populations (Nishida and Mwinuka 2005). Deforestation and agricultural practises that use 'slash and burn' techniques, in conjunction with intensive and sometimes illegal logging as well as mining, have a negative impact on chimp density because they modify, fragment and disturb their niches (Miles et al. 2005). Since 1900 western Africa had lost about 75% of its forest coverage (IUCN 1992). Associated to this there is also human population pressure that continues to expand at a rate of >3% annually (Figure 1.2).

Hunting, illegal bushmeat consumption, and the exotic pet trade have also contributed to the decline of chimpanzee communities. In some countries they are hunted and their meat is sold in local markets (Caspary et al. 2001; Wilkie and Carpenter 1999; Kormos and Boesch 2003). The infant chimpanzee pet trade is frequently associated with the death of the mother and of other members of the group (Miles et al. 2005). Human beliefs and biomedical investigation can, in an equal manner, contribute to their decline. Some beliefs and some ethnic traditions use parts of chimpanzee body parts for medical purposes (Caldecott and Miles 2005). Chimpanzees can be opportunist and take advantage of crops. In order to prevent crop raiding activity by chimpanzees, it is common for humans to set traps that can, deliberately or not, kill them (Sillero-Zubiri and Switzer 2001).

Infectious-contagious diseases are another main causes of death amongst chimpanzees (Goodall 1986). As human populations are expanding the risk of disease transmission to chimpanzees increases (Hanamura et al. 2008). Some epidemics like Ebola have been responsible for the death of a large number of chimpanzees (Walsh et al. 2003; Huijbregts et al. 2003; Kormos and Boesch 2003; Bermejo et al. 2006; Lahm et al. 2006).

Lastly, major political instability during the past few decades in countries such as Guinea Bissau, Sierra Leone, Liberia and Ivory Coast, in Western Africa, has

resulted in increasing poverty, increased availability of firearms and increasing number of refugees who hunt and destroy the forests for survival (Draulans and Van Krunkelsven 2002; Dudley et al. 2002; Miles et al. 2005). One of the direct consequences in this decrease in chimpanzee density (as well as other primates) is the impact on the local tropical flora and fauna (Chapman and Russo 2007). Some authors claim that chimpanzees have a major role in seed dispersal, since some large-seeded fruits are not accessible to smaller animals (Andresen 2000). If chimps are considered as flagship species they might have a major role in the preservation of their ecosystem (Sousa and Casanova 2004).

Figure 1.2: a)- Current scenario of human impact in Western Africa (2005), b)- Projection of human pressure for 2035 in the chimpanzee distribution area. The black, red and orange spots show a high, medium and low impact, respectively (after Caldecott and Miles 2005:236-237).

1.4- Consequences of habitat destruction

Deforestation in the tropics, where almost 90% of all primate species can be found, and the subsequent habitat loss and fragmentation affect directly primate populations in many ways (Lewis and Wu 2005). Included among these is that fact that the process of fragmentation may distribute primates randomly and non-adaptively among different forest patches (a stochastic sampling effect; Marsh 2003). In cases where the population size or demographic structure has changed, primate populations can become locally unstable or extinct (Marsh 2003). The fragmentation of continuous landscapes can: i) demographically isolate primate populations, inducing local extinction (Lovejoy et al. 1986); ii) modify group size,

population density and abundance (Tutin 1999); and iii) change dietary and behavioural habits, sometimes in an unfavourable way (Tutin 1999).

Additionally, fragmentation may also cause genetic changes, e.g. extinction of genetically differentiated populations and changes to the inbreeding level (Sherwin and Moritz 2000). These changes are likely to have a negative impact on population viability and the reduction of genetic diversity within and increased differentiation among populations reduces the chance for adaptation to environmental constraints and therefore, may affect the fitness and viability of the populations (Beebee and Rowe 2004). A reduction of heterozygosity affects the fitness of species and this change can be tracked by analysing the inbreeding coefficient (F_{IS}). Inbreeding depression often results in elevated homozygosity, although this can also be due to genetic drift, which may or may not exhibit an analogous pattern to inbreeding depression (Sherwin and Moritz 2000). Small populations will lose genetic diversity more quickly through drift and inbreeding, and these effects will increase when such populations are also fragmented (Bergl et al. 2008).

Contact and conflict between humans and non-human primates is another effect of habitat destruction (Hockings and Humble 2009). An increasing number of great ape populations are being forced into proximity with humans (Hockings and Humble 2009). The mechanisms of forest disturbance vary, but subsistence and small- and large-scale commercial agriculture, crop plantations and extractive industries such as logging and mining are significant threats to tropical forests (Caldecott and Miles 2005). The drive for economic growth through unsustainable exploitation of natural resources and/or the establishment of large-scale agricultural projects exacerbate the consequences of the human-primate contact (Hockings and Humble 2009). Increased rates of contact with great apes and human activities may lead as well to increased levels of crop-raiding (Ancrenaz et al. 2007; Hockings and Humble 2009); natural resource competition; domestic animal predation (McLennan 2008). The limitation of human mobility or attacks on humans by great apes could potentially intensify situations of conflict (Hockings and Humble 2009). One

immediate consequence of such conflicts is that humans sometimes develop negative perceptions towards great apes, retaliating by injuring or killing them to protect their resources (Costa et al. 2007) .

The study by Reynolds et al. (2000) is very instructive on how the development of the sugarcane industry in Western Uganda changed the landscape, the chimpanzee dynamics and the local human local perceptions about them.

Every aspect of chimpanzee and other primate's livelihood can be affected by habitat destruction and many authors have advocated the importance of research on fragmentation effects in order to better understand species responses (Marsh 2003). The chimpanzees of Guinea-Bissau are very good candidates to investigate this deleterious effect in order to develop suitable conservation measures.

1.5- Parasitism and its effects on hosts

Anthropogenic habitat change brings humans and primates into closer and more common contact promoting the risk of interspecific disease transmission (Daszak et al. 2001; Dobson and Foufopoulos 2001). Parasitic agents shared with nonhuman primates include several protozoans such as: malarias (*Plasmodium* spp.) *Trypanosoma cruzi*, *Giardia intestinalis*, *Cryptosporidium* sp. and a variety of gastrointestinal helminths (Chapman et al. 2005). Gastrointestinal parasites and other pathogen agents pose significant conservation risks to nonhuman primate populations, many of which are already threatened or endangered by habitat destruction and hunting (Chapman et al. 2005). Evidence indicates that between 1983 and 2000, Ebola virus outbreaks have contributed to the reduction of gorilla and chimpanzee populations by more than 50% over a broad geographic scale (Walsh et al. 2003; Bermejo et al. 2006; Walsh et al. 2007). Gastrointestinal and respiratory parasites transmitted between mountain gorillas, trackers, and ecotourists could threaten the long-term viability of gorilla populations in Virunga, as well as the economic sustainability of associated ecotourism (Wallis 2000).

Quantifying patterns of disease prevalence in nonhuman primate populations might be difficult since, for many parasites it is necessary to obtain clinical samples from individuals to determine their infection status (Chapman et al. 2005). Gastrointestinal parasites are notable exceptions, in that it is possible to diagnose animals by analysing their fecal material (Stuart and Strier 1995). Among gastrointestinal parasites, helminths and protozoans are most easily characterized in wild non-human primates (Stuart and Strier 1995). These parasites can affect host survival and reproduction directly through pathological effects and indirectly by reducing host condition (Dobson and Hudson 1992; Coop and Holmes 1996). Severe parasitosis can lead to blood loss, tissue damage, spontaneous abortion, congenital malformations, and death (Despommier et al. 1995). Less severe infections are more common and may affect nutrition, increasing energy expenditure, and impairing travel, feeding, predator escape, and competition for resources or mates (Dobson and Hudson 1992). Parasitosis emergence frequently results from a change in the ecology of the host, the parasite or both (Fa et al. 1995). Dobson and Foufopoulos (2001) conducted a survey of emerging pathogens of wildlife in North America and found that human involvement facilitated 55% of pathogen outbreaks. Similarly, Chapman et al. (2005) have documented that certain disturbance-related features of forest fragments are excellent predictors of infection prevalence in primates. In a five-year study, they compared patterns of gastrointestinal parasite infection and infection risk among populations of black-and-white (*Colobus guereza*) and red colobus (*Piliocolobus tephrosceles*) inhabiting undisturbed habitats and forest fragments (Chapman et al. 2005). Their results suggest that forest fragmentation alters prevalence and infection risk and that these factors are influenced by host density. They also examined the relationships between forest-fragment attributes and infection patterns. Inter-fragment comparisons were looked for nine potential factors and demonstrated that tree-stump density (i.e a proxy of degradation) had a strongly positive influence on the prevalence of parasitic nematodes. Both fragment size (negative relationship) and primate population density (positive relationship)

also predicted prevalence of some parasites. These results suggest that transmission dynamics of gastrointestinal parasites are affected by the degree and nature of anthropogenic disturbance of forest fragments (Chapman et al. 2005).

Given the framework of the effects of parasitism on nonhuman primate populations in a context of a very high anthropogenic change it seems important to survey the gastrointestinal parasites of the chimpanzees in Guinea-Bissau.

1.6- Paleoclimate and Biogeography

The evolutionary history of chimpanzees is intimately related with the history of their native forests (Gonder et al. 2006). This approach can be inserted into a biogeographic perspective, through the study of the distribution and diversity of organisms in time and space (Cox and Moore 2005). According to Lomolino et al. (2005) there are two ways to test hypotheses about species distribution and biodiversity: ecological and historical biogeography. Ecological biogeography is applied to the understanding of species distribution and diversity patterns, based on the interactions between organisms and their physical and biotic environment (Lomolino et al. 2005). Historical biogeography determines the series of events and processes that led to their speciation (i.e. origin, dispersal and species extinction) (Lehman and Fleagle 2006). The evolutionary history of chimpanzees has been clearly influenced by the biogeographic history of West Africa (Gonder and Disotell 2006). During the last 2.8 MYA (Plio-Pleistocene) the planet's climate has substantially changed (deMenocal 2004) (Fig. 1.3). According to Milankovitch's Astronomical Theory the main cause for these variations is the fluctuation in Earth's orbital parameters that caused expansion and cyclic contractions in the polar ice layers (Imbrie 1985; Imbrie et al. 1992; Livingstone 1993; Maley 1996; deMenocal 2004). Paleoclimatic recordings show that, approximately 800.000 years ago, in the Pleistocene, the amplitude of those cycles increased provoking significant changes in the Arctic and Antarctic ice layers (Fischer et al. 1999; Gibbard and van Kolfschoten

2004). According to this theory the gases on the atmosphere could have interacted with the oceanic currents and provoked changes in the polar ice volume, which, began expanding (Fischer et al. 1999; Zachos et al. 2001; deMenocal 2004). According to fossil records, during this period, several transitions in human evolutionary history also occurred.

Some recent isotopic and atmospheric dust measurement studies show that, as the glacial periods were intensifying, the average temperature of the planet was decreasing, resulting in significant changes in average sea and lake water levels (deMenocal 2004). Consequently atmospheric humidity was locked into the expanding ice, making those periods colder and more barren (deMenocal 2004). However, as the polar sheets decreased, water was again released to the atmosphere, making the interglacial periods milder and more humid (Imbrie 1985; Imbrie et al. 1992; deMenocal 2004).

These climate changes also had their effects on the African continent (Gonder 2000). Evidences strongly suggest that during the maximum glaciations periods, Africa's climate was colder and drier than nowadays (Maley 1996 and Cowling et al. 2008). This climate change is also the result of the variation of the cold sea currents, from northwest and southwest of Africa that were pushed towards the equator (Maley 1996). This chain of events contributed to the reduction of equatorial Africa's temperature and to the decrease of rain levels (Maley 1996). These changes had a direct effect on the landscape: wider habitats appeared, as well as a great abundance of herbaceous species, truly adapted to a more arid climate (Cowling et al. 2008). According to Nichol (1991) atmospheric dust levels had also increased, allowing the creation of dunes in humid areas where this couldn't happen before. On the other hand during interglacial periods there was an increase of pollen levels of species adapted to a humid forest environment, with forest recolonization of wider and more arid habitats (Cowling et al. 2008). From the last glacial period onwards, and in the following interglacial period (Holocene) it was possible for the tropical forest to reach their maximum extension (Maley 1996; Cowling et al. 2008).

Palinological studies conducted in Lake Bosumtwi (Ghana) have confirmed this prairie into forest species transition (Maley 1996).

Several authors claim that some tropical areas have more diversity and endemism than others (Gonder et al. 2006). This hypothesis deduces that, during the maximum glaciation periods, some original fragments from tropical forests prevailed in favourable climate conditions. It also suggests that as forests were contracting some animals were becoming isolated in those refuges. This 'Refugia Theory' (Haffer 1982; Endler 1982; Mayr and O'Hara 1986) states that forest lineages that persisted could have differentiated due to the isolation of populations in the refuges by vicariance) and/or followed by dispersal during the forests expansion in the holocene interglacial period (Grubb 1982; 1990). In this way, both forces, vicariance and dispersal, could have influenced African faunal distribution, as well as chimpanzees, even though these distribution patterns are more complex than can be explained only by the 'Refugia Theory' itself (Gagneux 1998).

The geographic determination of these refuges is not universally accepted (Gonder et al. 1997; 2006; Anthony et al. 2007). Areas that today have a bigger biodiversity and higher endemism are strong candidates for being refuges in the past (Grubb 1990; Hamilton 1982; Grubb et al. 2003). Hamilton (1982) and Maley (1996) point out five key-areas as being possible refuges: Guinea, Sierra Leone and Liberia; eastern Ivory Coast's and west of Ghana, Nigeria, Cameroon and Gabon; Congo Basin and eastern Tanzania, Uganda, Rwanda and Burundi, even though some smaller refuges could have existed outside these nuclear areas (Cowling et al. 2008). On another hand, vicariance and dispersal models acknowledge the existence of obstacles or barriers that affect gene flow (Cox and Moore 2005). Subsequent investigations have confirmed rivers and mountain chains as barriers to the tropical fauna dispersal (Eriksson et al. 2003; 2004; Anthony et al. 2007).

One of the barriers that promote the discontinuity of the landscape in Western Africa is the Dahomey Gap (extending from Ghana to the northern frontier of Nigeria and East of Benin). It is 400 km wide but it is believed that during maximum glacial

periods it could have been 1400 km. Nowadays it characterizes by the presence of savannah and a dry climate (Gonder et al. 2006). Some authors noticed that more remote areas from the Dahomey Gap have a higher biodiversity, such as the case of Guinea-Congolian vegetal formation, which goes from northern Ghana to the northern coast of Guinea-Bissau (White 1983; Caldecott and Kapos 2005). Other major barriers that block species dispersal and cause genetic structuring are rivers (for instance the Niger River, Volta, Sanaga, Cross River, Comoé, Sassandra, etc.) as some recent studies on chimpanzees, bonobos and gorillas have shown (Eriksson et al. 2004; Gonder et al. 2006; Anthony et al. 2007).

Most likely, this is the scenario in which chimpanzee evolutionary history developed: in several refuges, deprived of contact with other communities due to geographic barriers, with fluctuating vegetation and with the presence of other equally competitive species (Gagneux et al. 2001).

Figure 1.3: Plio-Pleistocene glacial and interglacial periods and atmospheric CO₂ (after Kukla 2005:1578).

1.7- The contribution of Phylogeography

The term 'Phylogeography' was coined for the first time in 1987 by Avise and can be considered as a subdiscipline of biogeography. Phylogeography articulates concepts and techniques from a broad range of areas: Molecular Genetics, Population Genetics, Demography, Phylogenetic Systematics, Ethology and Paleontology (Avise et al. 1987). Phylogeography is concerned with the spatial distribution of genetic lineages within and its comparison among species (Avise 1998; 2009). Due to the availability of intraspecific variation data in the form of DNA sequences and the incorporation of coalescent theory to infer demographic and evolutionary processes it is possible to assess hypotheses on species evolutionary history (Avise 2000).

The statistical phylogeography approach allows a better understanding of the order of events and mechanisms associated with colonization, diversity and extinction of genetic lineages in particular geographic regions (Avice 2000). With this analysis it is possible to address hypotheses about possible vicariance and/or dispersal and to identify the geological, ecological or ethological causes that might have had some influence in the patterns observed (Avice et al. 1987). The discipline has in vertebrates historically relied mainly on the analysis of mitochondrial DNA (mtDNA) (Avice 1995). Since the haplotypes register a matrilinear history of mutational events it is possible to connect them through a phylogenetic network, which can be overlaid on the past and present geographical distribution of the species (Avice 2000).

One central concept in phylogeography is that although many distinct genotypes may currently be present in a population, they all trace back to particular common ancestor at some point in history. The statistical expectation for the ways in which genotypes trace back to a common ancestor, given a particular mutation rate and effective population size is estimated using '*Coalescent Theory*' (Hudson 1990; Avice 2009). Coalescence is basically a lineage divergence model that considers genetic drift, and that goes back in time until a most recent common ancestor (MRCA) (Avice 2000). Coalescent theory established a conceptual framework to the study the processes that historically gave shape to the populations and origin to the current distribution of genetic variation. Of the countless contributions and applications of phylogeographic analysis it is possible to: i) point out studies whose purpose was to determine the place of origin and dispersion routes of species considered as pests or that colonized landscapes; ii) explain the population structure of a species; iii) identify the causes of the population variation observed in genetic and chromosomal traits and its correlation with geographic or ecological gradients, iv) uncover speciation modes (sympatric or allopatric) that explain the co-occurrence of distinct morphotypes in one species, v) enhance the detection of endangered populations facing imminent extinction, and finally, v) carry out studies of

comparative phylogeography that enable to consider the dispersion or vicariant factors on the evolution of codistributed species (Avice and Hamrick 1996; Avice 1995; 1998; 2000; 2004; 2009).

Phylogeographic data have supported the identification of ESUs (Evolutionary Significant Units) and MUs (Management Units) in threatened species, and therefore may improve conservation strategies for Guinea Bissau's chimpanzees (Moritz 1994).

1.8- Non invasive genetics and molecular markers

With the advent of PCR it was possible to amplify DNA from new sources such as feathers, hair, and feces (Goossens et al. 1998; Goossens et al. 2003). The application of non-invasive genotyping techniques appeared to be a solution for the problem of handling animals, which most of the time involved trapping, hunting, bleeding and taking biopsies to facilitate genetic sampling (Taberlet et al. 1999).

Morin and Woodruff (1992) pioneered this field by using non-invasive shed hairs and fecal DNA, mostly from wild chimpanzee communities (Morin et al. 1993; Morin et al. 1994a; Morin 1994b). From that point on many non-invasive studies were applied to different species, such as, the Iberian lynx (*Lynx pardinus*) (Pires and Fernandes 2003; Palomares et al. 2002); bears (Wasser et al. 1997); birds (*Otis tarda*) (Idaghdour et al. 2003); sea lions (*Eumetopias jubatus*) (Deagle et al. 2005), mustelids (Riddle et al. 2003; Gómez-Moliner et al. 2004; Hedmark et al. 2004) foxes (*Vulpes vulpes*) (Dalén et al. 2004), African elephants (*Loxodonta cyclotis*) (Eggert et al. 2003), etc.

Genetic diversity, besides being a key indicator of biodiversity, allows species, individuals or populations to be compared (Frankham et al. 2005). Molecular markers allow us to infer the distinctiveness of each individual (Parker et al. 1998); clarify their population structure and their differentiation in time and space (Avice 1998; 2000; 2004), and inform about the densities, abundances and effective sizes of the population (Kohn and Wayne 1997; Beebee and Rowe 2004).

Molecular techniques have a myriad of applications, but are especially useful if conservationists need to ensure the maintenance of genetic diversity and clarify behavioural, demographic and ecological aspects of endangered species, as well as for forensic analysis (Haig 1998). Each genetic marker has its own advantages and disadvantages, according to each research objective (Coote and Bruford 1996). Markers with a faster evolutionary rate have their applicability to the study of the individual, communities and populations, while those which have a slower evolutionary rate, are useful for the phylogenetic studies (Frankham, Ballou et al. 2005). The use of genetic markers is dependent on the studies problematic (Coote and Bruford 1996). This research used the Hyper Variable Region I (HVR1) of the D loop fragment from mtDNA as a preferred genetic marker.

1.8.1- Mitochondrial DNA

Mitochondria are fundamental organelles in each cellular unit. Found in all eukaryotic organisms they generate sufficient chemical energy to the cell in the form of adenosine triphosphate so that it can fulfil its basic functions. They could have allied to the eukaryotic cell through an evolutionary process of *endosymbiosis* (Margulis 1992), seeing that its genesis could be a bacteria that found the benefit of “allying” to another cellular type. Mitochondria also have the particularity of possessing a genome with functional genes, and that, in almost all mammals is transmitted matrilineally. They are also inherited clonally and do not undergo recombination (Avice 1998; Avice 2000).

MtDNA analysis is one of the most direct methods of studying genetic variation and has been extensively used in primate and other vertebrate populations; it gives direct information about the female population structure and even about the hierarchy of a determined sex “dominated” population (Di Fiori and Gagneux 2007). It can also supply information about founder effects, hybridization and introgression (Avice 2000; Avice 2004).

mtDNA analysis has been used in two major ways: 1)- to estimate genetic variation within populations, and 2)- to recognize evolutionary divergent sets of populations, including the resolution of evolutionary significant units and management units in order to evaluate conservation value of populations or areas from an evolutionary or phylogeographic perspective (Moritz 1994).

MtDNA genotypes are described as haplotypes (since they are effectively haploid), which differ from one another by particular mutations accumulated since female ancestors were last shared. Due to the rapid evolutionary rate many different haplotypes often coexist within a species, and it is possible to use these sequences to assess the matrilineal histories of individuals and populations (Avice 2009). The mtDNA control region is a unique non-coding region, which is typically approximately 1kb long and contains two hypervariable regions (HVR1 and HVR2). It is involved in the initiation and regulation of mtDNA replication and transcription (Moritz *et al.* 1987). These non-coding regions of mtDNA have a higher nucleotide substitution rate than both coding mtDNA and nuclear DNA (Morovvati *et al.* 2007). For this reason hypervariable areas of the mtDNA genome are considered 'mutational hotspots' (Stoneking 2000). This section of mtDNA is thus suitable for intra-population studies as the high mutation rate means that polymorphic sequences, i.e. different haplotypes, are more likely in unrelated individuals (Morovvati *et al.* 2007). Moreover, the hypervariable regions are flanked by conserved regions, allowing primer design for PCR (Galtier *et al.* 2009). This study made use of HVR1, which is considered more polymorphic than HVR2 (Lutz *et al.* 1998. Morovvati *et al.* (2007) showed that HVR1 was more discriminating than HVR2 in humans due to increased nucleotide differences. This makes the HVR1 region of the mtDNA the most suitable for studying population structure.

1.8.2- Microsatellites

Microsatellites are stretches of DNA sequence consisting of short tandem repeats of mono, di, tri, tetra, penta and hexa nucleotides (Tautz and Schlotterer 1994). Microsatellites have been found in all prokaryotic and eukaryotic genomes analysed to date (Toth et al. 2000). Microsatellite perfect repeats are without interruptions, imperfect repeats are interrupted by non-repeat nucleotides and compound repeats are cases where two or more microsatellites are found adjacent to one another. There may also be combination of these, for example imperfect compound repeats (Toth et al. 2000). Microsatellites are powerful genetic markers, due to their codominance, abundance, dispersal throughout the genome, multi-allelic variation, reproducibility and high level of polymorphism (Bruford and Wayne 1993). This high level of polymorphism is due to mutations affecting the number of repeat units. STR's provide a number of advantages over other molecular markers, namely that multiple STR alleles may be detected at a single locus using a simple PCR based screen, very small quantities of DNA are required for screening, and analysis is possible to automated allele detection and sizing (Schlotterer 2000). Microsatellites demonstrate a relatively high degree of transferability between species, as PCR primers designed to a microsatellite within one species frequently amplify a corresponding locus in related species, enabling comparative genetic and genomic analysis (Coote and Bruford 1996).

Studies of the potential biological function and evolutionary relevance are leading to a greater understanding of genomes and genomics. STR's were initially considered to be evolutionary neutral, however more recent evidence suggests they may play an important role in genome evolution and provide hotspots of recombination. Functional roles have been attributed to some microsatellites. They are thought to be involved in gene expression, regulation and function as transcriptional activating elements (Duran et al. 2009).

Microsatellites have many uses in genetics, such as the detection of alleles associated with disease, genome mapping, association studies, genetic diversity, paternity assessment, forensics and inferences of population history and conservation (Brumfield et al. 2003)

1.9- Chimpanzee phylogeographic and parasitological studies: an overview

This section follows a chronological approach in order to demonstrate the close relationship between the technical advances of molecular biology and the chimpanzee noninvasive genetic studies. The first noninvasive molecular studies with non-human primates had only appeared in the beginning of the 1990'. According to Goossens et al. (2003), this fact is due to, contrarily to other animal orders, primate populations have complex social systems, long generations and the obtainance of genetic samples before non-invasive genotyping was logistically and ethically impossible. The molecular studies with non-human primates can be grouped in what is currently called 'Molecular Primatology'.

Molecular Primatology can be defined as the application of techniques from Molecular Genetics to Primatology (Di Fiori and Gagneux 2007).

This recent denomination emerged with the need to group a series of ecologic, genetic, behavioural or population studies where nonhuman primates were used as systems. Its denomination was proposed by Morris Goodman (Wildman et al. 2003). In 2006 Anthony Di Fiori organized an international conference (i.e. Molecular Primatology: Progress and Promise, 2006), under the auspices of the Centre for the Study of Human Origins from New York University, where he gathered the support of the scientific community for the designation of this subdiscipline of Primatology.

Despite the proliferation of these studies in the last decades, there are some classical works that announce the emergence of Molecular Primatology. Nuttal

(1904), in his serologic monography: "Blood Immunity and Blood Relationship", suggested that human beings are intimately related to chimpanzees and gorillas because, besides owning the same blood type/groups (ABO), they produce the same antigens.

In the 1960's, the idea that it could be possible to rebuild organism's evolutionary history started to gain strength. This research path started, therefore to be applied to non-human primates (Buettner-Janush et al. 1961; Goodman 1962; Zuckerkandl 1963). In the 1970's appeared the first studies on the amino acid sequences and this novel technique was applied to studies of non-human primates phylogenies, and to unravel their molecular socio-ecology (Duggleby 1977). It is, however, in the 1980's and 1990's that the first intensive studies on DNA sequencing in non-human primates were initiated, with the development of several molecular techniques.

According to Di Fiori (Di Fiori 2003; Di Fiori and Gagneux 2007) the main scientific areas of the Molecular Primatology study can be classified as:

Molecular Ecology: the use of molecular techniques that allow the examination of consanguinity, dispersal patterns, social organization in primates and phylogeography (*vide* Avise 1995; 1998; 2000; 2004; Avise and Hamrick 1996; Beebee and Rowe 2004; Melnick et al. 1992; Rosemblum et al. 1997; Shimada 2000; Goldberg and Ruvolo 1997; Morin et al. 1994a; Goldberg and Wrangham 1997; Idani 1991; White 1996; Gerloff et al. 1999).

Conservation Genetics: the application of molecular techniques to primate conservation biology. Most of the non-human primate species inhabit tropical regions that are threatened due to various endogenous and exogenous factors. By this reason it's important to alert all the "Global Communities" that there are less than 200.000 chimpanzees worldwide. In this sense, conservation genetics becomes relevant because it helps to the characterization of the genetic diversity of some species, or even the way it is geographically structured, estimating the populations' effective size or the information on captivity colonies mating (see for more details:

Loeschcke et al. 1994; Frankham et al. 2005; Avise and Hamrick 1996; Smith and Wayne 1996; and more recently a new journal “Conservation Genetics”¹, that provides excellent perspectives on the molecular applicabilities to conservation).

Molecular Diversity and Adaptation: the adaptive meaning of non-human primates’ Intra and Inter molecular diversity rate. Also in here the molecular techniques offer an alternative method that allows the direct estimation of the consanguinity degree and the individuals’ kinship, basing in the genetic markers sharing (Pamilo and Crozier 1982; Queller and Goodnight 1989; Lynch and Ritland 1999).

Behaviour Genetics: Genetic and behavioural variation among individuals. That is, the attempt to understand what is the genetic basis that leads one individual to have more agonistic behaviours than other individuals, the reason for the existence, in the same sympatric species, of individuals that migrate later than others, or even, why do some individuals show greater cognitive ability than others (Fairbanks 2001; Plomin et al. 2001; Lesch et al. 1996; Bennet et al. 2002).

Comparative Genomics: analysis and comparison of different species genomes. This term was coined by Thomas Roderick (1986) and, in the following year, the current director of the OMIM Project (Online Mendelian Inheritance in Man) Victor McKusick launched the credited scientific Journal, Genomics (Di Fiori and Gagneux 2007). It is also in this sense that the chimps genome sequencing and comparisons to humans are included (Consortium 2005).

In 1969, Hill revises Schwartz’s taxonomy and classified chimpanzees into two species: *Pan troglodytes* and *Pan paniscus*. He also recognised that there are four subspecies in the common chimpanzees and a new subspecies of hybrid individuals between gorillas and chimps, which he called *Pan troglodytes koolakamba*. However, no study has, so far, confirmed the existence of this hypothethic subspecies (Hill 1969).

¹ <http://www.springer.com/life+sciences/ecology/journal/10592>

Excluding this last subspecies Hill's classification remained identical till 1997, when Gonder and colleagues (1999), by recurring to non-invasive genetic techniques, conducted a mitochondrial diversity survey among the chimpanzees from Nigeria and Cameroon. The study of these reserachers showed the existence of a fourth subspecies, endemic of that area. Based on Gray's attribution (1862) this subspecies was named *Pan troglodytes vellerosus* (Gonder et al. 1997).

This controversy around the number of chimpanzee subspecies demonstrates that there is a great genetic, morphologic, behavioural and ecological diversity among different chimpanzee communities (Gonder et al. 2006). In this sense, it's not surprising that many of the genetic studies that have been conducted in chimpanzees were focused on their systematics, phylogeography, evolutionary history and sociogenetics.

Morin and Woodruff (1992) used shed hairs as an innovative DNA source, mostly from wild chimpanzee communities. Their results suggested that there were 3 great lineages and that chimpanzees' evolutionary history should be revised (Morin et al. 1993; 1994a; 1994b; 2001; Morin and Woodruff 1992; Woodruff 1993).

Takenaka and colleagues (1993) successively applied paternity tests in captivity, recurring to non-invasive samples, opening the way, not only to future studies on wild communities but also to a better understanding of their social dynamics.

In 1994, based on mitochondrial DNA samples, Ruvolo and colleagues (1994) carried out a study on the hominoid' phylogeny and conclude that the genus *Homo* and the genus *Pan* form a separate lineage from gorillas. This study also revealed that both intra-population variability is higher to the one observed between the species of chimpanzees which, consequently, can have important effects in the genetic management of these species in captivity and also, that the little mitochondrial diversity in humans can be put in perspective when compared to other humanoids' diversity.

In order to maximise the genetic variability of a chimpanzee colony in Taronga Zoo, Houlden and colleagues (1997) performed a maternity and paternity analyses of several chimpanzees members and proved that, effectively, the alpha male had the highest reproductive success, confirming the idea of a correlation between hierarchy and reproductive success. They also claim that microsatellites are good markers to the distinction of the individuals' origin and provenience and, consequently, an election marker for management measures and genetic maintenance in captivity (Houlden, et al. 1997).

Additionally, Goldberg and Ruvolo (1997) conducted an extensive study on the phylogeography and sociogenetics of western African chimpanzees. This study demonstrated that they didn't show a classic phylogeographic pattern, with a low overall variability and little clustering similar to the studies done with human populations. In biogeographic terms these scientists verified that rivers didn't prevent the genes' flow between eastern chimpanzees, since no discontinuities were observed in the haplotypal trees, confirming *P. troglodytes schweinfurthii* as a subspecies with a great mobility, capable of moving in wide corridors and forest fragments (Goldberg and Ruvolo 1997; Goldberg and Wrangham 1997).

Gagneux and colleagues (1997) refer the technical difficulties intrinsically bounded to non-invasive methods, mostly referring false alleles and allelic dropout. They also warn that, if circumstances are more favourable, it is preferable to collect plucked hair in detriment of shed hair.

Gagneux and colleagues (1999a), in a study reflecting African hominoids' evolutionary history, presented a new date for the split between humans and chimps situated around 2.5 million years ago. In the same way, by comparing mtDNA phylogenetic sequences, they verified that there are profound subclades in gorillas and chimps and that chimpanzee' clades show a higher number of ramifications, suggesting that gorillas and chimpanzees had a very different evolutionary history from humans.

Another study, from a sociogenetics perspective, revealed that female chimpanzees in Taï Forest furtively seek males that don't belong to their social unit, increasing the genic flow of males among communities, even though they are phylopatric. This reproductive strategy allows females to choose a larger number of males, without losing the resources previously owned, as well as the protection conferred to them by their mates (Gagneux et al. 1999b).

In the same year, Taberlet et al. (1999) published an article about the advantages and disadvantages of the application of non-invasive methods, granting a major theoretical contribution through their model to the reliability in these kind of studies (Taberlet et al. 1999).

In 2000, Katherine Gonder presented a new extensive phylogeographic study about chimpanzees from Nigeria and Cameroon (Gonder 2000). New technical improvements appear in the microsatellites use (Goossens et al. 2000), and Meier et al. (2000) reconfirmed the relevance of microsatellites application in the genetic characterization of captivity chimpanzee communities. They also verified that there was not a correlation amid the position of males in the social hierarchy and the short-term reproductive success (Meier et al. 2000). A year later, Linda Vigilant (2001) published an article about "Paternity and relatedness in wild chimpanzee communities", in agreement with the study performed by Constable et al (2001) about the paternity of Gombe's Chimpanzees: "Noninvasive paternity assignment in Gombe chimpanzees" (Constable et al. 2001). An important phylogeographic study also emerged in this year, assembling four of the researchers that contributed the most to the comprehension of the chimpanzees' evolutionary history: "Gene flow in wild chimpanzee populations: what genetic data tell us about chimpanzee movement over space and time" (Gagneux et al. 2001).

Another relevant contribution was the publication of a study by Stone et al. (2002) based on the non-codificant region of Y chromosome. This study concluded that chimpanzees and bonobos have, from six to seven times, more variation than

humans and that their population effective size was big or relatively constant when compared to the former (Stone et al. 2002).

Nevertheless, Yu et al (2003) launched, once again, the controversy on the dating of the chimps and human beings' common ancestor by declaring that there is "Low nucleotide diversity in chimpanzees and bonobos". These authors pointed to a later dating of chimps and bonobos split (ca. 1,8 MYA), advancing the formation of the Congo River as hypothesis (Yu et al. 2003). Expectadly, in the following year, new studies appeared showing alternative results, like the case of Fischer et al. (2004), that stated that chimpanzee evolutionary history might have been more complex than previously thought, that in spite of a clear decrease trend in the number of chimpanzee wild populations, they exhibit a high genetic diversity. The authors also advance that this observation can be due to a sudden population reduction followed by a fast recovering. They also attributed a *split time* amid central and western chimps of about 430.000 and 650.000 years (Fischer et al. 2004). McGrew et al. in the same year (2004), demonstrated, in the Mont Assirik chimpanzees communities, Senegal, that non-invasive techniques could effectively be of a great contribution to the deep socioecologic knowledge and biology os the species (McGrew et al. 2004). Moreover, a phylogeographic study on the Bossou chimps' mitochondrial diversity was published, demonstrating that there is little genic flow among the communities of chimpanzees from the south of Guinea (Shimada et al. 2004).

2005 was certainly the "International Year of the Chimpanzee", given the fact that the chimpanzee genome was sequenced by one international consortium, allowing an effective "Comparative Genomics" (Consortium 2005) and the publication of a vast number of papers (see volume 437 of Nature).

An opinion article about the challenges placed to population genetics' research in a "post genomic era" is presented by Stone and Verrelli (2006). In this same year, Gonder et al. (2006) grant another important contribution to the understanding of the biogeography of chimpanzees from Nigeria-Cameroon, gradually strengthening the existence of the fourth species (Gonder and Disotell

2006, Gonder et al. 2006). Fischer et al. (2006) in the other hand, pointed out the isolation by distance model as a possible explanation for the complex demographic and evolutionary chimpanzee histories and disagree with the notion that subspecies are genetically distinct entities (Fischer et al. 2006). See as well Mitani's work about the demographic influences on the behavior of chimpanzees (Mitani 2006).

More recently, Becquet et al. (2007) by analysing 310 autosomatic microsatellites from 78 chimpanzees (41 western, 16 central, 7 eastern, 3 hybrids and 11 unknown) and 6 bonobos, obtained the following results:

(i) The high mutation rate from the microsatellite data allowed more information about historical/demographical events by locus. In terms of cluster analysis, the authors resorted to the program STRUCTURE that demonstrated the existence of four major clusters (i.e. discontinuous populations). This result, together with the map of haplotypes of the Y chromosome and the mtDNA, confirmed that there are few evidences for the existence of a fourth subspecies.

(ii) Concerning the principal components analysis (PCA), it also demonstrated a high level of population structure giving a statistical meaning to the chimpanzee populations' subdivisions: four discontinuous populations, four very significant eigenvectors.

(iii) The authors also tested for possible additional populations admitting, however, that the sample size is limited, i.e. there is little variation in the *P. t. vellerosus* samples and that a more systematic survey is necessary.

(iv) In terms of inbreeding evidence the authors found a reduced number of heterozygous genotypes, especially in western and central chimpanzees, and in terms of allele variation the difference between bonobos and western chimpanzees is bigger than central or eastern chimps. This fact derives on the effects of the genetic drift (i.e. bottleneck) on western chimpanzees. The first diverging population was *P. t. verus*, pointing out as a possible result, a diminished population effective size.

The main conclusions of this study were:

1. The three different chimpanzee subspecies denominations match to individuals clusters with similar allelic frequencies that can be defined from genetic data without considering the geographic "label";
2. There are low evidence of mixture between wild groups. Eastern and central populations are more related in time among themselves than western chimpanzee populations;
3. The PCA suggests something about the western population. Still, more data, more samples, more genetic markers and information about the geographic origin is required in order to clearly understand this structure;
4. No support for the existence of a fourth common chimpanzee population was found.
5. Despite of trying to include the biggest number possible of samples from the African continent, the geographic sample selection procedure wasn't random.
6. Chimpanzees from other regions, including those regions where chimpanzees are extinct, may give us the impression of discontinuity. Future studies that include chimps through more African populations may, in principle, identify intermediate populations and demonstrate more variation patterns (Becquet et al. 2007).

Caswell et al. (2008) presented new dating for the splits among chimpanzees and bonobos populations: 1.290.000 years ago for bonobos and chimpanzees; 510.000 for western and central chimps and at least 50.000 between eastern and central chimpanzees. They also estimate that the effective size of the central chimpanzee population is of 100.000 individuals, more than once thought, and that the River Congo formation may have been in the origin of the chimps and bonobos split, seeing that this date is consistent with this hypothesis (Caswell et al. 2008) (Fig. 1.4).

Figure 1.4: *Split* dates between chimpanzees, bonobos and humans (from Caswell et al. 2008:10).

Another recent study suggests that chimps and humans speciation process was complex, with the possibility of gene flow among both species after an initial period of separation (Patterson et al. 2006). Finally, Gonder et al (2011), by using genotypes of 94 chimpanzees, demonstrated that the species fall into three major populations: (i) Upper Guinea in western Africa (*P. t. verus*); (ii) the Gulf of Guinea region (*P. t. ellioti*); and (iii) equatorial Africa (*P. t. troglodytes* and *P. t. schweinfurthii*). This study also suggests that the chimpanzees from Nigeria-Cameroon occasionally hybridize (Gonder et al. 2011). Chimpanzee evolutionary history remains open and covered in controversy. Research in this area will surely contribute to shed light in certain chimpanzee phylogeographic hypothesis and time estimations.

In another perspective, the interaction between parasites and hosts can play a very important role in natural and wild populations' diversity (Nunn et al. 2004). According to Esch and Fernandez (1993) parasites can affect ecology and the hosts' evolution, as well as their growth and regulation (Hochachka and Dhondt 2000). They can also affect the whole community's biodiversity (Hudson et al. 1998; 2002), besides having a direct impact in the hosts' survival and reproduction and an indirect one by affecting their health conditions (Boyce 1990; Chandra and Newberne 1977;

Coop and Holmes 1996; Dobson and Hudson 1992; Hudson et al. 1992). A severe parasitosis can cause haemorrhages, tissue and organs damage, spontaneous abortions, congenital malformations and, in some cases, even the host's death (Chandra and Newberne 1977).

The very close phylogenetic relation amid humans and chimpanzees can also result in the parasite exchange (Ott-Joslin 1993; Wolfe et al. 1998). Recent studies have shown that many of the emerging infectious diseases (HIV/AIDS, Ebola, etc) have chimpanzees as a reservoir (Gao et al. 1999; Leroy et al. 2004). In the same way, many human diseases (poliomyelitis, respiratory diseases, etc) had their impact on chimpanzee and gorilla populations (Hill et al. 2001; Kalema-Zikusoka et al. 2002). Even though some studies about intestinal parasites have been performed in primate populations, such as the baboons (Appleton et al. 1986; Eley et al. 1989; Müller-Graf et al. 1997) and howler monkeys (Stoner 1996), the parasitological studies on chimpanzees are still insufficient (Ashford et al. 2000; Lilly et al. 2002; McGrew et al. 1989). As the chimpanzee's habitat is being fragmented, contact with humans is also being accented, increasing the risk of parasite transmission (Gillespie 2004). For this very reason it's important to maintain a current and updated record on their health conditions.

1.10- Research questions, hypothesis and predictions

The most recent genetic studies suggest the evolutionary history of West African chimpanzees as a very complex matter (Gagneux et al. 2001; Fischer et al. 2004; Fischer et al. 2006; Gonder and Disotell 2006; Gonder et al. 2006; Becquet et al. 2007; Caswell et al. 2008; Gonder 2011). Thus I set the question: Which were the demographic events that could have influenced the current mitochondrial genetic diversity in Guinea-Bissau's chimpanzees?

How do Guinea-Bissau chimpanzees relate with other members of the *Pan troglodytes* versus subspecies? Studies by Gonder (2006) suggest that there is a

different lineage in Nigeria and Cameroon and that there was not any significant gene flow exchange with other western chimpanzees. More up-to-date studies conducted with western chimpanzees can confirm this idea. In the same way, what's the relation that these chimpanzees have with the neighbouring communities of Guinea? Considering the study by Shimada et al. (2004) I hypothesize that chimpanzees in Guinea-Bissau share haplotypes with Guinea.

From another point of view, what sorts of symbionts colonize the chimpanzee gastrointestinal tract ecosystem in Guinea-Bissau?

I would expect to find a high level of gastrointestinal symbiont diversity consequence of a disturbed habitat, a higher infection of parasites with direct cycles as contact intra and interspecific increases, and no differences in parasite prevalence between subpopulations.

1.11- Aims and objectives

This research addresses a gap in the current knowledge about the evolutionary and demographic history of chimpanzees in Guinea-Bissau. Similarly, it establishes baseline ecological information regarding their symbiontism patterns and how they are affected by habitat fragmentation.

To accomplish these aims I conducted extensive non-invasive surveys across the species distribution in the country. My objectives were:

- 1- To report 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”).
- 2- To revisit the chimpanzee West African phylogeography;
- 3- To analyse and interpret their genetic diversity and differentiation;
- 4- To assess their genetic structure;
- 5- To reflect about their demographic history and to infer their lineage split divergences in a paleoclimate context;

- 6- To monitor their gastrointestinal symbiont fauna and health status;
- 7- To contribute to their conservation by aporing genetic and parasitological recommendations.

1.12- Thesis structure

This thesis is organized into self-contained chapters. The data chapters of this thesis (Chapters 3-5) were written as separate manuscripts. Each one of these chapters includes an abstract, introduction, specific questions and hypotheses, methods, results, discussion, acknowledgements, and references. Formatting follows the rules and guidance for the particular journal.

Chapter 2 describes the study areas, the non-invasive sampling strategy, laboratory procedures (i.e genetic and parasitological) and general data analyses.

Chapter 3 reports 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 ethnographic interviews were conducted and observations made with a view to providing insight into how these human traditions and myths might pose an additional threat. This chapter has been submitted to **Traffic Bulletin** as: Rui M. Sá, Maria Ferreira da Silva, Fernando Sousa and Tânia Minhós. “The trade and ethnobiological use of Chimpanzee body parts in Guinea-Bissau: implications for conservation”. After peer review was published and is included in Appendix L.

Chapter 4 revisits the chimpanzee West African phylogeography with special focus on chimpanzees from Guinea-Bissau. It investigates their evolutionary history and unveils their demographic history. Based on a 261bp from the HVR1 mtDNA, their genetic structure was assessed and conservation genetics implications discussed. This chapter is *in preparation* for submission to **Evolutionary Anthropology** as: Rui M. Sá, Kathljine Koops, Annaria Marra, Tânia Minhós, Maria Ferreira da Silva, Joost Van Schinjdell, Cláudia Sousa and Michael W. Bruford.

“Inferring the evolutionary history of a peripheral and endangered ape population: the chimpanzees of Guinea Bissau”.

In Chapter 5 I present the results of the parasitological survey of the chimpanzee population inhabiting Cantanhez National Park, in the south of Guinea-Bissau. I compared three chimpanzee subpopulations living within the Park in the areas with different levels of anthropogenic fragmentation. This chapter was submitted and is currently *under review* to the **American Journal of Primatology** as: Rui M. Sá, Jana Petrášová, Kateřina Pomajbíková, Ilona Profousová, Klára J. Petrželková, Cláudia Sousa, Joanne Cable, Michael W. Bruford and David Modrý. “Gastrointestinal symbionts of chimpanzees in Cantanhez National Park, Guinea-Bissau with respect to habitat fragmentation” and is currently under revision.

Chapter 6 summarizes the major findings of this research, its limitations and future directions. It also provides recommendations and considerations for chimpanzee conservation in Guinea-Bissau.

Finally, from Appendix I to M, I attached five manuscripts that are either in preparation (Appendixes I and J) or published (Appendix K-M). These manuscripts were produced during the course of my PhD and they fall under the Molecular Primatology research scope. I share first authorship with the first four (Appendix I-L) and the last one I am co-author (Appendix M).

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CHAPTER 2

"O meu grito de revolta fez vibrar os peitos de todos os Homens..."

"My scream of revolt thrilled the hearts of men..."

— Amílcar Cabral, Liberation Africanist leader

CHAPTER 2

GENERAL MATERIAL AND METHODS

2.1- Fieldwork

I performed the fieldwork in two missions to the Republic of Guinea-Bissau. The first field mission was during September-November of 2008, during the wet season in Cantanhez National Park, and the second mission was from January-June 2010, in different surveys during the dry season across the country.

2.2- Republic of Guinea-Bissau

The Republic of Guinea-Bissau is located in the Western coast of Africa, between Senegal, in the north, and the Republic of Guinea in the south and east. In georeferencial terms, this country is located between the latitude $10^{\circ}55'$ - $12^{\circ}40'N$ and the longitude $13^{\circ}38'$ - $16^{\circ}43'W$ (Fig. 2.1).

Figure 2.1: Guinea-Bissau location (BBC 2011).

A continental and an insular part, the Bijago Archipelago, compose Guinea-Bissau's territory. The total surface of the country is 36125 km². (PNUD 2010).

In administrative terms, Guinea-Bissau is organised into 8 regions and one autonomous sector.

Guinea-Bissau's climate is, generally, tropical humid with two clear distinct seasons. The wet season, starts from May to November, and the dry season occurs from December to April. The mean annual temperature, under the influence of the trade winds, varies from 24 to 27 °C. The average relative humidity level is accented (about 70%) and is, essentially, influenced by the winds, rainfall and the coastal proximity.

The relief comprehends rather little accented altitude zones, being the maximum reached values of about 300 m (PNUD 2010).

Of all West African countries, Guinea-Bissau is the one that possesses the highest surface of mangrove areas (Sousa et al. 2005). Mangrove vegetation shows a special adaptation due to its tolerance to the influence of salt seawater and to the settling sediments (PNUD 2010). Besides its physical function (e.g. anti-erosion, protection from storms, etc.), mangroves or 'tarrafes' [Creoule] have an ecological function of utmost importance to this country's economy. These areas become preferable for reproduction for the marine, terrestrial, and bird species. Human communities use frequently mangrove molluscs and crustaceans on their diet (PNUD 2010).

On the other hand, sub-humid forests show a heterogenic vegetal coverage with a great diversity and density, with vegetal stratum composed of large and medium trees and bushes (Cassamá 2006). Deep inside the forest we can find a fresh microclimate with low temperature oscillations. In function of the climate conditions, especially of a higher rainfall levels, recorded there, the main and representative spots of this formation are located in the south of Guinea-Bissau, occupying vast areas in the south of Quinara and in the region of Tombali (Cassamá 2006).

There is a clear reduction of the vegetal formations, in quantitative and qualitative terms, in almost all regions of the country, probably because of “slash and burn”, itinerant agriculture, lumber exploration with trading purposes, the creation of big and significant crop fields (monocultures), mostly of cashew nuts, the appearing of new populations in dense forest density areas searching for new arable lands and the occupation of mangrove areas in order to cultivate rice, groundnuts, sweet potatoes among others crops (Catarino 2004; Cassamá 2006).

The deforestation is caused by itinerant agriculture, also called as “pam-pam” [Creoule]. In the limits of the sub-humid forest there is a transition forest with a surface of 24000 ha in the region of Gabú and of 24418, 59 ha (0,99%) in the coastal area (Catarino 2004).

Several studies confirm that, biogeographically, Guinea-Bissau is located in a Guinean-Congolese and Sudanian transition zone (Catarino et al. 2001). Zaire Guinean floristic elements, reach their most northern extension limits in Cantanhez (Malaisse 1996). These elements are in the origin of the formations of the Guinean and sub-Guinean humid sectors, where dense, dry and semi-dry forests prevail, as well as sub-humid (deciduous) forests and natural palm trees. On the contrary, the Sudan-Sahelian zone, where wide forests and arboreal savannahs constitute the main formations (Catarino 2004). Its location in this transition zone also influences its fauna elements (PNUD 2010). Guinea-Bissau is no exception in relation to the greater radiations that characterise the African fauna, especially concerning primate presence (Gippoliti and Dell’Omo 2003).

According to Casanova and Sousa (2007) there are, at least, twelve families of mammals (18 Artiodactyla, including the *Hippopotamus amphibious*, 20 species of Carnivores, 25 Cetacean, 24 Chiropteran, 5 Insectivore, 1 Lagomorphs, 1 Pholidota, 11 Primates, 1 Proboscidean, 19 Rodents, 1 Sirenia, and 1 Tubulidentata).

Eleven species of primates are present in the country, and nine of them are inscribed in the Appendix II of CITES (please see table 5) (Casanova and Sousa 2007, Gippoliti and Dell’Omo 2003, Gippoliti et al. 2003) (Table 2.1).

Table 2.1: Non-human primates of Guinea-Bissau (from Casanova and Sousa 2007:9)

Common Name	Scientific Name (Groves 2001)	IUCN Conservation Status
Northern Lesser Bush Baby	<i>Galago senegalensis</i> <i>senegalensis</i>	Lower Risk
Sooty Mangabey	<i>Cercocebus torquatus atys</i>	Endangered
Patas Monkey	<i>Erythrocebus patas</i>	Endangered
Vervet Monkey	<i>Chlorocebus sabaues</i>	Lower Risk
Mona Monkey	<i>Cercopithecus campbelli</i>	Lower Risk
Diana Monkey	<i>Cercopithecus diana</i>	Endangered
Lesser Spot-nosed Guenon	<i>Cercopithecus petaurista</i> <i>buettikoferi</i>	Lower Risk
Guinea Baboon	<i>Papio papio</i>	Lower Risk
Western Red Colobus	<i>Procolobus badius temmincki</i>	Endangered
Western Black and White Colobus	<i>Colobus polykomos</i>	Lower Risk
Chimpanzee	<i>Pan troglodytes verus</i>	Endangered

Guinea-Bissau has a population of 1520830 (INE 2009). It is a multi ethnic and multi religious society (Table 2.2) (INE 2009). It has a density, per Km² of 37.5 and, 64% of its population live in rural areas. It has a demographic growth rate of 2%, an average life expectancy of 44.7 years and an infant mortality rate of 138 per 1000 children (INE 2009).

If we consider the projections made to the year 2050, the Guinean population will be of 2,900,000 individuals (INE 2009). For a more detailed description about the main socio-demographic indicators, please see Table 2.3).

In economical terms, this country has a gross domestic product of 301 million US dollars; its main wealth comes from the primary sector with 85%, where the cashew nuts (*Anacardium occidentale*) are the main exporting product (INE 2009).

Among the main exporting countries there's India, Thailand, Uruguay, Portugal and South Korea. It's a dependent country relating the funds for international aid, like the United Nations, European Union, World Bank, FMI and frequently depends on the international food assistance (CIA 2008).

The Republic of Guinea-Bissau was formerly a Portuguese colony. Thirteen years after the struggle against Portuguese colonialism, Guinea-Bissau proclaimed, in 1973, its independence. In 1974 it was recognised the right to self-determination. The first presidential elections happened in 1994. However, the transition to democracy was difficult, especially after the end of the civil war, that took place from 1998 to 1999, and the following political instability established, that the country has been feeling until today (PNUD 2010).

In environmental terms Guinea-Bissau has signed several conventions favouring the protection of natural resources, forests and biodiversity. However, the absence of effective judicial and law enforcement systems doesn't allow the application of the commitments that once had assumed (PNUD 2010; Casanova and Sousa 2007).

According to the last report from the United Nations Development Plan (PNUD 2010), the Republic of Guinea-Bissau has a poverty index of 44.8 and a human development index of 0.374, making it the third poorest country in the world, engaging the 175th position worldwide (PNUD 2010). Corruption and bureaucracy, human rights violation, disrespect for the sovereignty organs, political instability, its major external debt and its weak production ability strongly weaken human empowerment (PNUD 2010).

Table 2.2: Major ethnic groups and beliefs of Guinea-Bissau (INEC 2007).

Ethnic groups	%
Balante	27
Fulani	23
Mandinka	13
Mandyako	11
Pepel	10
Beafada	3
Mancanha	3
Bijago	3
Nalu	1,5
Others	3
Religious affiliations	%
Indigenous beliefs/ Animism	45
Muslim	40
Christian	13
Other	2

Table 2.3: Major socio-economical and demographic indicators (INEC 2007).

Indicator	Value
Population	1,520,830
Population density	37,5 %
Life expectancy	44,7 (Total)
Infant mortality rate	138/1000
Literacy rate	44.8 %
Armed forces	9,250
Agriculture, forestry, fishing	85 %
Industry	2 %
Number of telephones	7/ 1000
Paved road as a share of total roads	10

2.2- Study Areas

2.2.1- Study Area 1: Tombali Region

Surveys were conducted in Cantanhez and Catió Peninsulas (2008 and 2010 missions) from the Tombali region. Cantanhez National Park (CNP) is located in this region (Fig. 2.2) and comprises a total area of 1068 km². The Cacine and Cumbidjã Rivers act as natural boundaries and form the Cubucaré Peninsula. The park was formally established in 2008 but since the mid-1980's was considered a forest hunting reserve (IBAP 2008). The climate is tropical humid with two annual seasons: the rainy season from June to October and the dry season from November to May with little or no rainfall. Mean annual rainfall is 2000 mm (SD $\pm$ 500) and the temperature varies between 28° and 31° C (Simão 1997).

CNP is $\pm$ 70m above sea level and consists of a patchy environment of small rivers; mangroves, arborous and grassland savannahs; agriculture fields, human settlements and sub-humid forests characterize the landscape. Forest fragments consist of a mixture of evergreen and semi deciduous vegetation cover (Catarino 2004). Native fauna include seven species of non-human primates: the lesser bush baby (*Galago senegalensis*), the western red colobus (*Procolobus badius temmincki*), the king colobus (*Colobus polykomus*), the vervet monkey (*Chlorocebus aethiops sabaues*), the Campbell's monkey (*Cercopithecus campbelli*), the Guinean baboon (*Papio papio*) and the western chimpanzee (*Pan troglodytes verus*) (Gippoliti and Dell'Omo 2003; Casanova and Sousa 2007).

The World Wide Fund for Nature (WWF) has recognized the Cantanhez forests as one of the 200 most important ecoregions in the world, with the last vestigial segments of humid forest in West Africa. However, through satellite images, Oom et al. (2009) identified a trend of forest loss with a conversion from closed humid forests to savanna-woodlands. The amount of land for crops is continually expanding due to increasing numbers of people (Hockings and Sousa 2011) (Fig. 2.2).

The name itself “Cantanhez” comes from, according to personal information, a herbaceous plant that grows in the high regions of the Cubucaré [specifically in the Amidara and São Francisco woods] used by men during the three years that they had to spend in the woods for the animist ceremony of “fanado”².

According to an overall tradition, recognized by all the others, the first ethnicity that settled and occupied the lands were the Nalús and they are considered, by this fact, as the “owners of the land” (Frazão-Moreira 2001).

The Cantanhez Forests are an archetype of the Guinea-Bissau’s collective consciousness, that is, they are perceived as the place of independence and resistance and as the cradle of the foundation of the main movement and political party of Guinea-Bissau – The PAIGC (African Party for the Independence of Guinea and Cape Verde).

Due to that, Cantanhez was a reference site in the armed rebellion against the Portuguese colonization and, from the natives’ point of view, an icon on the “Liberation War”³. The guerilla’s success, as well as the PAIGC’s, is intimately associated to these woodlands. This fact is a result of two main reasons: one of real or material order and other of spiritual and symbolic character. On the one hand the isolation, their forest resources diversity and their dense and closed surroundings allowed local communities to renew unique knowledges, acquired by ancestral experience. In the other hand, the conviction and hope on the spiritual support and on the protection of the “Nhinte Camatchol”⁴ or of the “Irãs”⁵ strengthened their

² “Fanado” [Creole] is an animist religious ceremony that is practised by several ethnicities (Nalús, Balanta, Fulas, Sosso, etc) and generally involves a crossing ritual to adulthood, culminating with circumcision. This cultural expression is part of the Guineans culture and it is still practised nowadays, involving however a more reduced presence in the “Sacred Woods” (about three months). It’s a collective ceremony and aims at the transmission of reserved knowledge, fortifying, as well, the cohesion and community unity (Campredon 1997).

³ The armed conflict begun in the south, in January of 1963 (Simpósio 2008).

⁴ “Nhinte Camatchol” is a Nalu sculpture used at fanado. It represents a bird’s head with a human face, being the main message of this ritual to the participants that they must consider true brotherhood, a collective interest affirmation, above individual or familiar interest. This mask couldn’t be seen by the non-initiated, under the penalty of death (Campredon 1997).

self-determination, self-esteem and identity, inciting the rebellion’s progression and concretization (Brito 2007).

This park also sheltered not only some important headquarters to the Portuguese troops, but also several guerrilla “barrakas”⁶.

This entire area had become known among the Portuguese troops as the “Death Corridor”, due to the intensity of the attacks (Simpósio 2008). These were key moments of war that are inserted in an environmental frame of dense forest.

It is still possible to find today traces of that conflict, namely land mines, inactivated bombs, and many buildings totally ruined, in several places of Cantanhez (Rui Sá pers. observation 2008).

Figure 2.2: Map of land use in Cantanhez National Park, Guinea-Bissau (INEP 2010).

⁵ “Irãs” means spirits of the forests.

⁶ “Barrakas” [Creole] military lodgings from the PAIGC’s guerrilla.

2.2.2- Study Area 2: Quínara Region

Surveys were performed in Buba, Fulacunda and Empada sectors, which belong to the Quínara administrative region. Cufada Lagoons Natural Park (CLNP) is located in this region, and was created by presidential decree on 2000. This park has long been considered as an area of high importance for biodiversity. CLNP covers an area of 700 Km². Is bounded by the Rio Corubal at north, and the road to Fulacunda at northwest, and the Buba River at south. The soils are most common brownish, yellow and red (Catarino 2006).

The dominant vegetation type is semi-dry forest and dry forest areas with patches of sub-humid and moist savannas. The park area is recognized as 'wetland of international importance' and is therefore recognized by the Ramsar Convention. In addition to chimpanzees (*Pan troglodytes verus*), baboons (*Papio papio*) and patas monkeys (*Erythrocebus patas*), it is possible to observe white pelicans (*Pelecanus onocrotalus*) and other attractive birds: *Phalacrocorax africanus*, *Platalea alba*, *Phalacrocorax africanus*, *Glareola pratincola*, *Egretta garzetta*, *Sarkidiomis melanotos*, etc. (Araújo 1994).

The resident human population is spread over 33 villages concentrated mostly in the north of the park along the river Corubal (Fig. 2.3).

Figure 2.3: Map of land use in Cufada Lagoons Natural Park, Guinea-Bissau (INEP 2010).

2.2.3- Study Area 3: Gabú Region

A chimpanzee survey was performed in the Boé sector during April-May 2010. This sector is located in the Gabú region at approximately 12° N by 14° W. The Northern border of the Boé sector is the Corubal River, while to the South and East lays the border with Guinea. The totally area of the sector is 3287.8 km² (Reintjes and Wit 1986). The sector has a population of approximately 12,000 largely subsistence agriculturalists and pastoralists. The majority of the population is of the Fulani ethnicity. There are no recent meteorological data for the Boé sector. The annual rainy season is typically from June until September, with little to no rainfall in the dry season. The Féfinê River is permanent and crosses the Boé from South to North, forming a tributary to the Corubal. There are a large number of small permanent and seasonal streams in the sector. The lake at Vendu Cham is an important site for avifauna. The sector is dominated by savannah vegetation on poor laterite soils, where *Parkia biglobosa* is a common tree species. The local population frequently burns the savannah. Where the laterite cap is broken, species rich gallery forests can be found. Many forest patches are being cleared by slash-and-burn methods to be replaced with subsistence agriculture and seasonal settlements. This land use results in potentially frequent contact between chimpanzees and humans or domestic animals and their faeces.

The chimpanzee population in the Boé is very poorly studied. Serra et al. (2006) estimated a population of 710 individuals for the sector, based mainly on interviews with villagers. Other than chimpanzees, seven other species of primates are present in the Boé: *Galago senegalensis senegalensis*, *Papio papio*, *Cercopithecus aethiops sabeus*, *Erythrocebus patas*, *Cercocebus atys*, *Colobus polykomos* and *Piliocolobus badius*. Other large mammals include *Hippopotamus amphibius*, *Tragelaphus scriptus scriptus*, *Syncerus caffer nanus*, *Phacochoerus africanus*, *Kobus ellipsiprymnus defassa*, *Crocuta crocuta* and *Panthera pardus*.

The World Bank and the United Nations Environment Program are in the process of creating the new protected area: Dulombi-Boé National Park (World Bank 2011) (Fig. 2.4).

Figure 2.5: Guinea-Bissau Protected Areas and future National Park of Dulombi-Boé (World Bank 2011).

2.3- Non-invasive sampling strategy

Data were collected from wild populations of *P. troglodytes verus* across Guinea-Bissau. Approximately 500 fecal samples (Table 2.4) were collected from: Tombali, Quínara and Gabú regions (Fig. 2.5). These samples were taken from free ranging chimpanzees. Cantanhez region was sampled extensively. Communities ranged in size from 2 to 23 animals when it was possible to see and count them. For each sample collected, and individual code, region, place, GPS coordinates, status, sex and age (i.e. if known) and morphometric measures were recorded. Approximately 5g of the outer layer of fecal material were stored at room temperature in absolute ethanol for 24h and then transferred to a second tube with silica gel type III (Sigma Alrich) and maintained at room temperature until DNA was extracted according with “Two steps” protocol suggested by Roeder et al. (2004).

For the parasitological survey, each sample was immediately fixed in PROTOfix™ CLR (Alpha-tech-Systems, Inc.) according with the fabricant instructions and stored at room temperature until futher analysis.

Permits to transport samples were obtained near the official institutions of Guinea-Bissau, Portugal and UK (IBAP, ICNB and DEFRA).

Table 2.4: Chimpanzee non-invasive sampling, Guinea-Bissau, for this study.

Regions	Areas	Sampling Site	N	Lat.	Lon.	Sample site Number
Tombali	Cantanhez	Madina	25	11°12'47.64"N	15° 2'12.72"W	1
Tombali	Cantanhez	Farim	20	11°12'31.02"N	15° 4'25.80"W	2
Tombali	Cantanhez	Caiquéne	25	11°13'30.93"N	15° 5'47.73"W	3
Tombali	Cantanhez	Canghode	27	11° 9'40.68"N	15° 7'35.46"W	4
Tombali	Cantanhez	Catomboi	15	11°11'38.28"N	15° 4'36.36"W	5
Tombali	Cantanhez	Amidara	15	11°16'52.38"N	14°58'34.02"W	6
Tombali	Cantanhez	Cambéque	10	11°11'30.18"N	15° 0'49.74"W	7
Tombali	Cantanhez	São Francisco	15	11°19'18.96"N	14°59'29.22"W	8
Tombali	Cantanhez	Ponta Nova	15	11°18'41.64"N	14°58'51.36"W	9
Tombali	Cantanhez	Cadique	60	11°14'19.92"N	15°6'51.30"W	10
Tombali	Cantanhez	Muna	25	11°10'2.46"N	15°8'36.00"W	11
Tombali	Cantanhez	Mejo	15	11°22'48.45"N	14°54'51.00"W	12
Tombali	Cantanhez	Quebo Sutuba	10	11°9'34.51"N	14°54'41.12"W	13
Tombali	Cantanhez	Lautchande	25	11°14'55.14"N	15°4'20.40"W	14
Tombali	Cantanhez	Cambafre	15	11°18'1.74"N	14°56'22.20"W	15
Tombali	Cantanhez	Cancira	11	11°22'48.45"N	14°54'51.00"W	16
Tombali	Cantanhez	Bagriel	4	11°20'22.44"N	15° 0'50.94"W	17
Quínara	Cufada	Buba Itchinque	27	11°45'11.58"N	15° 5'18.36"W	18
Quínara	Cufada	Tira Camisa	5	11°41'25.56"N	15° 4'2.94"W	19
Quínara	Cufada	Bani	5	11°43'42.54"N	15° 5'31.20"W	20
Quínara	Cufada	Injassane	5	11°44'29.88"N	14°59'4.74"W	21
Quínara	Cufada	Nhala-Molha Pé	10	11°37'45.72"N	14°53'55.08"W	22
Quínara	Cufada	Buba Tumbo	5	11°38'37.92"N	15° 0'19.80"W	23
Quínara	Empada	Dois Rios	20	11°37'56.22"N	15° 9'18.66"W	24
Tombali	Catió	Briame	18	11°17'19.92"N	15°14'28.86"W	25
Gabú	Boé	Dinguirai	12	11°45'18.72"N	13°51'55.38"W	26
Gabú	Boé	Pataque	8	11°53'04.9" N	13°57'32.5" W	27
Gabú	Boé	Aicum	6	11°56'29.22"N	13°52'37.38"W	28
Gabú	Boé	Béli North	18	11° 52'04.7" N	13° 57' 31.9" W	29
Gabú	Boé	Béli	14	11°51'10.80"N	13°54'39.66"W	30
Gabú	Boé	Vendu Leidi	10	11°44'33.00"N	13°43'22.44"W	31
Gabú	Boé	Munhini	5	11°45'9.84"N	13°55'27.66"W	32

Figure 2.5: Guinea-Bissau sampling sites for this study. Numbers represent sampling sites indicated in Table 2.4.

2.4- Ethnographic observations

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 men were found to be offering wild animal body parts for sale (e.g. skin, bones, teeth, horns and scales). When possible, morphological identification of the specimens viewed was made and photographs taken.

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 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).

2.5- Genetic procedures

All molecular procedures were performed in MWB laboratory (aka G10), at Cardiff School of Biosciences.

2.5.1- DNA extraction

To test the best preservation method to storage the samples, a pilot study was conducted during the 2008 mission in CNP. The samples were stored at room temperature in Silica Gel (SIGMA ALDRICH, type III), absolute ethanol, or according the 'Two Steps method' (Roeder et al. 2004). A set of 16 fresh chimpanzee samples was collected, and the same sample was divided and stored in those preservation methods. The "two steps" proved to be the most reliable to preserve the chimpanzee DNA and was therefore subsequently used in the next genetic surveys

(Fig. 2.6).

A separate and dedicated fume hood was used during all the faecal extraction process. Before the start of the process all the areas (hood, bench, and centrifuge) were decontaminated with bleach (20%) and DNA Away (DUTSCHER, France, catalogue number 038188). Tweezers, 2mL and 1,5mL Eppendorf tubes were previously autoclaved, and all the 5 dedicated equipment was exposed to UV-light overnight inside the hood. Each sample was extracted in duplicate and two negative controls were included to monitor possible contamination. The extraction procedure was divided in two consecutive days by using the QIAmp DNA Stool Mini Kit® (QIAGEN, Germany) according to the manufacturer instructions with the following modifications: Day 1- To each faecal sample (approximately 0.1g) 1.7mL ASL buffer was added, vortexed 10 minutes and then allowed to soak at room temperature in an orbital shaker overnight. Day 2- The centrifuge and incubating times were increased to 3-10 min and immediately before the precipitation step with 100% ethanol, 4 µL 1350 µg/mL poly-A Carrier RNA (QIAGEN, Germany, catalogue number 1017647) was added. The poly-A increases the efficiency of binding and elution when the expected DNA yield is ≤ 10 ng. The elution time with AE buffer was also increased to 30 min and the final volume reduced to 150 µL.

“Wash” protocol

I also tried one alternative faecal extraction protocol developed by (Flagstad et al. 1999) and (Palomares et al. 2002) with the following modifications: 0,70g of faecal material (desiccated in Silica Gel) was placed in a 50mL sterile tube and washed (10 to 30 X with a disposable Pasteur pipette) with 600µL of phosphate buffered saline (PBS) (pH 7.4). After the wash step all the solution was taken into a 2mL tube and centrifuged at full speed (approx. 13000 rpm) and the supernatant was poured off. 1mL of ASL buffer was added to the pellet and let soak overnight. The next steps were the same as above.

Two blank extraction negatives and several PCR's were included to monitor possible contaminations.

Figure 2.6: PCR results of the best preservation DNA reagent after electrophoresis. The “Two Steps” presented the most convincing results.

2.5.2- DNA amplification and sequencing

To test the extraction success, a mtDNA HVRI PCR was performed on a Applied Biosystems Gene Amp® PCR System 9700 using 200 µl PCR tubes in a total volume of 20 µL PCR reaction mixture consisting of: 9 µL of QIAGEN Multiplex PCR kit containing buffer, DNTP's, Taq polymerase and 3 mM MgCl₂ (final concentration), 0.1 µL 1x BSA, 0.1 µL 1x Q solution, 6.8 µL ultrapure water and 0.2 µM primers and 2 µL DNA template. PCR profile were as follows: 95_C for 15 min, then amplified for 45 cycles of 94_C for 30 s, 51_C for 60 s, and 72_C for 60 s, and finally extended at 72_C for 10 min. Primers L15926 (5'-TAC ACT GGT CTT GTA AAC C-3', corresponding to positions 15326-15344 of the complete chimpanzee mtDNA genome [EMBL/GenBank accession No. D38113]) and H16555 (5'-TGA TCC ATC GTG ATG TCT TA-3', corresponding to positions 15971-15990 of D38113) were used because they amplify the HVRI mtDNA region of chimpanzees. These primers have been reported to reliably amplify a 600 bp of the mitochondrial control region fragment (also known as D-loop) in chimpanzees (Shimada et al. 2004) (Table 2.5). Reactions were visualized on a 2% agarose gel stained with ethidium bromide and DNA was quantified based on the intensity of the band when compared to the ladder scale

intensity (Fig. 2.7). Samples with less than 5ng/ μ L were not used because they rarely work both for sequencing and microsatellite analyses.

Table 2.5: HVR1 chimpanzee primers.

Primer	Sequence	Reference
L15926	5'-TACACTGGTCTTGTAAC-3'	Shimada et al. 2004
H16555	5'-TGATCCATCGTGATGTCT-3'	Shimada et al. 2004

PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (SAP) enzymes (New England Biolabs, UK) at 37_C for 60 min and inactivated at 80_C for 15 min and send for sequencing to MACROGEN EUROPE using the same amplification primers (Table 2.5). Chromatograms were inspected and sequences were edited, assembled and aligned using the program SEQUENCHER (Gene Codes Corporation). The consensus sequences were BLASTed to confirm chimpanzee species.

Figure 2.7: MtDNA gel picture. Positive amplifications indicated by bright band around 700bp. + positive control; - DNA isolation controls; b blank PCR control.

2.5.3- Sequences authenticity

In order to effectively ensure that the sequences of this study were not contaminated with "numts" all PCR products were carefully inspected both in

agarose gels (for dual bands in the presence of the expected size of fragment), and for double peaks in the sequencing electropherograms. Furthermore, a great degree of variation was found in the dataset, and all the sequences aligned in accordance with the reference sequence of the mitochondrial genome of HVR1 *Pan troglodytes troglodytes* [GenBank Access No.: X93335] (Arnasson et al. 1996).

Complementary, the sequences produced by this study were also compared with the sequences deposited in chimpanzee numts database MITOMAP (<http://www.mitomap.org/MITOMAP>). No evidence was found for the presence of "numts" in the dataset of this study.

2.5.4- Microsatellites and molecular sexing

I tested 9 microsatellite loci initially developed for humans but that cross amplify great apes. All primers were previously used in chimpanzee population genetic studies by Gusmão et al (2002) and Roeder et al (2006). The 9 loci were designed, optimized and combined in two multiplexes by using the software Multiplex Manager 1.0 (Holleley and Geerts 2009): M1 (Amelogenin, D16S2624, D1S550, D10S1432, and D2S1326) and M2 (D5S1457, HUMFIBRA, D4S1627, and DYS439). Multiplex 1 includes the amelogenin sexing system that allows an accurate DNA-based sex identification of chimpanzees using non-invasive samples (Bradley et al. 2001). PCR amplification mixture was carried using a QIAGEN Multiplex PCR kit according to the manufacturer's instructions. Final reaction volume was 8 µL. Primers were 5'-fluorolabelled and their final concentration in the PCR mixture was 0.2 µM. PCR profile on a Applied Biosystems Gene Amp® PCR System 9700 were: 95_C for 15 min, 45 cycles of 94_C for 30 s, 57_C (M1) or 58_C (M2) for 1, 40 min, 72_C for 1, 30 min and a final extension of 72_C for 20 min. Loci used, ranges, dyes, primer sequences and multiplex combinations are shown in Table 2.6. Samples were sent to MACROGEN KOREA where they were electrophoresed on an automated genetic analyzer using 1 µL PCR product and the internal size standard ROX 400. Alleles were scored using GeneMapper v. 3.2 software (Applied Biosystems).

From these multiplexes (M1 and M2) two loci are not working properly specially the locus D1S550 and therefore a new multiplex combination (M3) with 5 additionally loci was created and optimized (Table 2.7).

Table 2.6: Multiplex composition, primer information and allelic size ranges.

Locus	Multiplex	Label	Allele size range (bp)	Primers
Amelogenin	M1	FAM	104-110	F-5'- CCTGGGCTCTGTAAAGAATAGTG-3' R-5'-ATCAGAGCTTAAACTGGGAAGCTG-3'
D16S2624		NED	119-139	F- 5'- TGAGGCAATTTGTTACAGAGC-3' R- 5'-TAATGTACCTGGTACCAAAAACA-3'
D1S550		HEX	147-177	F- 5'-CCTGTTGCCACCTACAAAAG-3' R-5'-TAAGTTAGTTCAAATTCATCAGTGC-3'
D10S1432		FAM	162-182	F-5'- CAGTGGACACTAAACACAATCC-3' R-5'-TAGATTATCTAAATGGTGGATTTC-3
D2S1326		FAM	210-282	F-5'-AGACAGTCAAGAATAACTGCCC-3' R-5'-CTGTGGCTCAAAAGCTGAAT-3'
D5S1457	M2	FAM	101-133	F- 5'-TAGGTTCTGGGCATGTCTGT-3' R- 5'-TGCTTGGCACACTTCAGG-3'
HUMFIBRA		HEX	171-203	F-5'-GCCCCATAGGTTTTGAACTCA-3' R-5'-TGATTTGTCTGTAATTGCCAGC-3'
D4S1627		FAM	214-250	F-5'AGCATTAGCATTTGTCCTGG-3' R-5'-GACTAACCTGACTCCCCCTC-3'
DYS439		HEX	230-258	F- 5'-TCCTGAATGGTACTTCCTAGGTTT-3' R- 5'-GCCTGGCTTGAATTCTTTT-3'

Table 2.7: Multiplex 3 composition, primer information and allelic size ranges.

Locus	Multiplex	Label	Allele size range (bp)	Primers
DQCAR	M3	FAM	99-119	F- 5'-GAAACATATATTAACAGAGACAGACAAA-3' R- 5'-CATTTCTCTTCCTTATCACTTCATA-3'
D1S207		HEX	128-160	F- 5'-CACTTCTCCTTGAATCGCTT-3' R- 5'-GCAAGTCCTGTTCCAAGTCT-3'
D13S159		TAMRA	154-190	F- 5'-AGGCTGTGACTTTTAGGCCA-3' R- 5'- CCAGGCCACTTTTGATCTGT-3'
D14S306		FAM	196-248	F- 5'- AAAGCTACATCCAAATTAGGTAGG-3' R- 5'- TGACAAAGAACTAAAATGTCCC-3'
D6S311		HEX	208-248	F-5'- ATGTCCTCATTGGTGTGTG-3' R- 5'- GATTGAGAGCCAGGAAGAT-3'

2.5.5- Data analysis

The haplotypes were defined by analyzing the sequences in the program DNASP v. 4.01 (Rozas et al. 2003). The haplotype diversity (h) nucleotide (π) and Watterson (θ_w) were estimated in the program ARLEQUIN v.3.01 (Excoffier et al. 2005) as well as the estimator F_{ST} in the analysis of their genetic differentiation. The haplotype richness (H_r) was calculated using the method implemented in the software CONTRIB v.1.02 (Petit et al. 1998). The analysis of molecular variance (AMOVA) using the pairwise differences were also held in the program ARLEQUIN and the most likely pattern of population subdivision was estimated using the spatial analysis of molecular variance in the program SAMOVA 1.0 (Dupanloup et al. 2002).

In order to evaluate a possible pattern of isolation by distance a Mantel test and a RMA were applied in the program IBDWS v 3.16 (Jensen et al. 2005). Similarly, to assess whether there are vicariant barriers that may prevent gene flow, the program BARRIER v. 2.2 was used (Manni et al. 2004).

The phylogenetic reconstruction of the Network was made in the program NETWORK v. 4.6, which uses the algorithm of median-joining (Bandelt et al. 1999).

For the phylogenetic analysis, the best model of evolution and replacement of nucleotides was assessed through the Akaike Information Criterion implemented in the program jMODELTEST v.0.1.1 (Posada 2008). Using the methods of neighbor joining and the Bayesian inference program MRBAYES v. 3.1 (Ronquist and Huelsenbeck 2003) for phylogenetic trees reconstruction. The posterior probabilities were inferred using the Metropolis-Coupled Markov Chain Monte Carlo algorithm (MC-MCMC) from one million generations, with sampling every 100, 4 chains, 2 runs and an initial burn-in 25%. Stationarity of the chains was established from $p < 0.01$ split frequencies. The convergence of the MCMC Bayesian inferences was evaluated in the program Tracer v.1.5 (Rambaut and Drummond 2007). All trees obtained were viewed and edited in Figtree software v.1.3.1 (Rambaut 2006) and in the program TREEGraph v. 2.0 (Stöver and Müller 2010).

With the aim of analyzing their divergence times, a relaxed molecular clock and a coalescent approach, implemented in the program BEAST v. 1.6.2, was used (Drummond and Rambaut 2007). Three independent runs were performed using the

same mutation rate; the same optimized parameters and the model suggested by jMODELTEST. The convergence of the runs was also evaluated in the program TRACER after the combining of logs and trees in LogCombiner and TreeAnnotator respectively (Drummond and Rambaut 2007). The analysis of demographic history and the chance of sudden expansion and space were tested using: mismatch distributions (Rogers and Harpending 1992) and tests of neutrality (Tajima D and Fu Fs) and the program ARLEQUIN; tests Fu and Li F * and D * in the program DNASP v.4.01.

For genotyping criteria the “multi-tubes” approach (Taberlet et al. 1996) was initially considered (up to seven positive PCR repeats to confirm homozygosity). However, due to the high number of extracted DNA samples, time and financial constraints prevented that each individual was genotyped as many times as recommended by the protocol. The number of replicates was therefore estimated by using a maximum likelihood approach implemented in the software GEMINI v. 1.4.1 (Valière et al. 2002) that uses a priori information about the error rates (i.e. allelic dropout and false alleles), which were determined in the software PEDANT v. 1.0 (Johnson and Haydon 2007, 2009). General parameters in the software GEMINI included 100 simulation replicates and the range of repetition number was set from 2-12. This test revealed that four PCR repetitions were appropriate to achieve a high probability of identity assuring a 95% confidence of the genotypes, and the per locus consensus threshold revealed that for a sample to be included in the final dataset must have at least two positive PCR results for nine of the 14 loci. For molecular sexing the result was considered as true when observed three times out of the four repetitions.

Allelic dropout, null alleles and stutter peaks were assessed using MICROCHECKER, v. 2.2.3 (van Oosterhout et al. 2004). Moreover, a quality index (QI) for all loci was determined according to Miquel et al. (2006) and samples with a QI below 0.50 were removed from the dataset, as they are genotypes with poor quality.

To detect repeated individuals the software GIMLET v. 1.3.3 (Valière 2002) was used, and duplicated genotypes were removed. This software was also used to calculate the Probability of Identity (PI) and the Probability of Identity between

siblings (PI_{sibs}) (Waits et al. 2001). Finally, GenAlEx v. 6.4.1 (Peakall and Smouse 2006) was used to conduct all standard population genetic analyses.

2.6- Parasitological screening

All parasitological analyses were performed in the Department of Parasitology of the Veterinary and Pharmaceutical Sciences University, Brno, Czech Republic.

2.6.1- Laboratory techniques

About half of each sample was kept in the original flask for possible further analyses/repetitions. The other half was diluted 1:1 with H₂O and filtered through standard sieves (~100µm) for macroscopic examination. The filtered material of each fecal sample was prepared for coprological examination by combining a modified Sheather's solution flotation (s.g. 1.33) and a MIF (Merthiolate Iodine Formaldehyde) sedimentation techniques and then stained with Lugol's iodine. Direct smears were performed during fieldwork. Several drops were examined for each technique from each sample in a compound microscope at 100x-1000x magnifications. Symbionts were identified on the basis of egg, cyst, trophozoite and larvae stages, color, shape, content and size by using identification keys. Measurements were made to the nearest 0.1µm ±SD, using a calibrated ocular micrometer. Representatives were photographed using Nomarski interference contrast. Mean egg/cysts sizes are based on measurements of 10 eggs/cysts from 10 different samples.

2.6.2- Statistical analyses

Chi square or Fisher's exact tests with subsequent Bonferroni correction were performed in order to compare sample prevalence in chimpanzees inhabiting North, Central and South CNP areas for each parasite taxa separately. Differences in symbiont richness among areas were investigated using Kruskal-Wallis ANOVA, with LSD post hoc tests used for pairwise comparisons. The value of $p < 0.05$ was set as

significant. SPSS, version 17 (SPSS Inc., Chicago, IL) and SISA (<http://www.quantitativeskills.com/sisa/>) were used for all statistical analyses.

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CHAPTER 3

“The purpose of anthropology is to make the world safe for human differences.”

— Ruth Benedict, Anthropologist

CHAPTER 3

THE TRADE AND ETHNOBIOLOGICAL USE OF CHIMPANZEE BODY PARTS IN GUINEA-BISSAU: IMPLICATIONS FOR CONSERVATION

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3.1- ABSTRACT

Anthropogenic disturbances such as habitat loss, fragmentation and hunting for pet trade are reported to be the main threats to chimpanzee survival in the wild. Here we report for the first time the use of chimpanzee body parts for traditional medicine purposes in Guinea Bissau as an additional anthropogenic threat and discuss the impact that this activity might have in its populations. Chimpanzee skins along with other mammal and reptile body parts were found in the main Bissau market as well as in a market in Dakar (Senegal). Traders and local people were interviewed using an ethnoprimateological approach in order to access the use and origin of the animals' body parts. All interviews confirmed the use of these animal by-products for folk medicine purposes. Moreover, evidences of a transnational trade were also found. This phenomenon seems to be a common practice in West African countries and might constitute an important additional factor contributing to the decline of wild populations of several threatened species. It is urgent to better understand the extent and impact of such practices, which can be accomplished by combining ethnography, and molecular approaches in order to suggest adequate conservation measures and law enforcement.

Keywords: Body parts, Chimpanzees, Conflict, Conservation, Ethnoprimateology, Folk medicine, Guinea-Bissau, Trade

3.2- 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/km² in a total area of 17.225 km² 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/km² in a total area of 36.513 km², 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.

3.3- 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 ethnoprimateological 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).

3.4- RESULTS AND DISCUSSION

3.4.1- 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 *Procolobus 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 Hyena *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. 3.1).

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).

3.4.2- Costs, origin and scale

Interviews with urban traders revealed that the cost of a piece of Chimpanzee skin (Fig.3.1) 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. 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”*.

3.4.3- 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).

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3.4.4- 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

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 (Robillard in litt., July 2011).

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

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.

3.4.5- 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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3.7- FIGURES

Figure 3.1. All animal-derived products for human traditional purposes in Bandim market, Bissau, Guinea-Bissau.

CHAPTER 4

“By comparing the human and chimp genomes, we can see the process of evolution clearly in the DNA changes since we diverged from our common ancestor.”

— Robert Waterston, Geneticist

CHAPTER 4

INFERRING THE EVOLUTIONARY HISTORY OF A PERIPHERAL AND ENDANGERED APE POPULATION: THE CHIMPANZEES OF GUINEA-BISSAU

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4.1- ABSTRACT

The phylogeography of West African chimpanzees has been intensively studied yet remarkably none have included the relict and peripheral population of Guinea-Bissau. Guinea-Bissau represents the most western limit of the species distribution, with known populations in the Quínara, Tombali and Gabú regions. We investigated the diversity, phylogeographic structure and demographic history of this region by analyzing it alongside existing *Pan troglodytes verus* data from the hypervariable mitochondrial region. We included 334 individuals from 28 locations, including chimpanzees from Guinea Bissau (n = 189) and the Nimba Mountains (Guinea, n = 145). From the 51 haplotypes detected, haplotype (*h*) and nucleotide diversity was high (94% and 0.05325 ± 0.00116) and phylogenetic analyses revealed the existence of two deep evolutionary lineages. In addition we describe eleven subclades, where subclade B1 presented a star shaped pattern that was observed in the median joining network suggesting a demographic expansion. The bimodal mismatch distribution and the neutrality tests imply that an expansion has occurred promoting secondary contact between the two lineages. Analysis of molecular variance revealed 74% of the genetic variation is partitioned within individuals ($\Phi_{ST}=0.25956$, $p<0.0001$). Barrier analysis identified three major genetic discontinuities and the estimated divergence times point to vicariance events in the Middle Pleistocene and the recurrence of past climatic oscillations in the African forests. We suggest that the two phylogeographic groups should be considered as distinct management units. We also indicate priority areas for conservation based on the genetic diversity of the populations.

Keywords

West Africa, evolutionary history, genetic diversity, chimpanzee, *Pan troglodytes verus*, Guinea-Bissau, Nimba, phylogeography

4.2- INTRODUCTION

The evolutionary history of the genus *Pan* (i.e. chimpanzees and bonobos) has been a permanent topic of interest due to their close affinity with humans¹. Studies on wild chimpanzees began in the 1960s and since then they have become reference models, providing unique insights for our understanding of human biology and evolution². Chimpanzees are frequently used for comparison in human genetic studies and they were among the first species when non-invasive molecular techniques were applied^{3,4}. Several efforts have been made to unravel their complex evolutionary history, but even now debate continues concerning the exact number of chimpanzee subspecies⁵⁻⁷.

Chimpanzees are usually divided into four subspecies¹¹⁻¹³, but this classification is contested^{1, 14}. The subspecies *P. t. troglodytes* has a range that extends throughout Central Africa. *P. t. schweinfurthii* is located essentially in Eastern Africa, and based on morphometric variation some taxonomists argue that this subspecies can be further divided in two: *P. t. schweinfurthii* and *P. t. marungensis*¹⁵. The chimpanzees from southern Nigeria and western Cameroon, previously known as *P. t. vellerosus*¹⁷, have been reclassified as *P. t. ellioti*^{16, 18-19}.

The fourth subspecies *P. t. verus* is located in the Upper Guinea regions of West Africa and spans the region from southern Senegal to Liberia and Sierra Leone¹¹. West African chimpanzees are extinct in the wild in Gambia, Togo and Benin and may be present in Burkina Faso^{11, 20-22}. They inhabit a broad range of habitats including wooded savannahs, savannah forest mosaics, mountain forests and moist lowland forests²³. Recent studies of chimpanzee genetic diversity suggest that *P. t. verus* shared a last common ancestor with *P. t. troglodytes* between 0.38 and 0.55 mya and the Niger River and the Dahomey Gap have been proposed as a geographic barrier that facilitated the split^{1, 5-7, 27-32}. Central and eastern chimpanzees share a more recent ancestry³³⁻³⁶. The contrast between low genetic diversity and lack of subdivision in humans, and the high diversity with geographical subdivision in chimpanzees has led to the hypothesis that the chimpanzee belongs to a single population with a continuous exchange of gene flow and some authors even propose that chimpanzees should not be classified into subspecies^{1, 3, 36}. However,

Morin et al. (1994) argue that the West African chimpanzee mtDNA sequences are more divergent than those from the other subspecies. The average genetic distance among *P. t. ssp. verus* and the other subspecies is two-thirds the genetic distance between chimpanzees and bonobos, suggesting that they could be elevated to species level⁴.

Evolution and environment interact to shape a species' biogeography, population structure, and the flow of genes within its current range³⁷. For chimpanzees four factors are recognized: i) geographical or physical barriers; ii) mating systems and social structure; iii) anthropogenic disruption, and; iv) paleoclimate and vegetation³⁸. Geographic barriers such as deserts, mountains and large rivers impact on the dispersal between populations³⁹. Chimpanzees do not seem to be affected in their movements by mountains or rock formations, however large watercourses do appear to form effective barriers, at least in the case of bonobos⁹ and in the Nigeria-Cameroon chimpanzees where the Sanaga River was identified as a barrier^{1,6}. The operational sex ratio (i.e. the number of breeding males per breeding females) and differential fitness have a large impact on the genetic constitution of chimpanzee populations, by potentially lowering the effective population size and rendering populations more prone to genetic drift⁴⁰⁻⁴¹. In the small chimpanzee community of Bossou for example, Shimada et al. (2004) demonstrated a skewed matrilineal genetic composition⁴².

Anthropogenic disruption caused by fragmentation and severe hunting is known to have a strong impact on the demography of wild primate populations and on their genetic constitution^{37, 43}. In the long term, fragmentation leads to a decline in genetic diversity, thus increasing genetic differentiation due to a decrease of gene flow between breeding groups combined with random genetic drift and inbreeding⁴⁴⁻⁴⁵. Lastly, the temperature oscillations during the Plio-Pleistocene (ca. 5 mya) have caused rapid changes in forest distribution, thereby fragmenting them into small refugia. During the drought periods the Upper Guinean forests in West Africa contracted and expanded substantially in interglacials⁴⁶. Based on current floristic and faunal biodiversity, four Pleistocene forest refuges have been proposed in Upper Guinea: the Grabo Hills region in Liberia, Cape Three Points in Ghana, the Nimba Mountains in the eastern part of the Guinean highlands, and the northern

limit of the Guinean sub-humid forests in Cantanhez, Guinea-Bissau⁴⁷⁻⁵⁰. It is still not clear to what extent western chimpanzee populations followed the distribution of forests and how their demography and distribution were affected by such historical changes^{1, 6, 38}. Yet, the extent to which such paleoclimatic changes caused subdivision in the region remains an open question, since any disconnections could have been followed by re-connections of the disrupted chimpanzee populations after expansion of the forests³.

The phylogeographic history of West African chimpanzees remains debated, yet remarkably no previous studies have included individuals from the peripheral relictual population in Guinea-Bissau. This country represents the most western limit of the species distribution where chimpanzee survival is considered very precarious⁵¹⁻⁵². Major threats to their survival include the pet trade, hunting for traditional medicine practices and habitat destruction caused by logging and agriculture⁵³⁻⁵⁴. Due to the absence of information regarding their status, chimpanzees have been erroneously considered extinct in Guinea-Bissau⁵⁵. However, in 1994 a survey reconfirmed their presence and made a preliminary evaluation of their conservation status⁵⁶⁻⁵⁷. The most recent national survey estimates 600-1000 chimpanzees in Guinea-Bissau^{51, 58} but the exact number of chimpanzee communities remains unclear. Little is known about their ecological, social and behavioural characteristics, and the extent of their genetic diversity at the population level and structure has never been evaluated.

Here, we reanalysed western chimpanzee phylogeography by further exploring their mitochondrial diversity, structure and demographic history in Guinea-Bissau and the Nimba Mountains (Guinea) using hypervariable control region mtDNA sequences. Specifically, we addressed the question: what influence did the Pleistocene historical events have on the current genetic diversity and structure of *P. t. verus* in Guinea and Guinea-Bissau? Population genetic theories predict that populations located on the periphery of a species' range should be smaller and more fragmented, displaying a greater genetic structure among populations and lower genetic diversities within populations due to genetic drift and inbreeding⁴³. These forces can drive peripheral populations close to extinction and a concern for conservation^{37, 44}. We expected that chimpanzees in Guinea-Bissau: 1)- experience a

higher genetic structure, and 2)- a lower genetic diversity, as they are located at the species' range edge. Furthermore, we compared our data with those from Bossou and other West African study sites to shed light on the evolutionary history of the subspecies. Finally, conservation insights are discussed in order to contribute towards a genetic management for this endangered and relatively unprotected ape in Guinea-Bissau.

4.3- MATERIALS AND METHODS

4.3.1- Study areas

Within Guinea-Bissau, chimpanzees are distributed south of the Corubal River. Their presence has been confirmed in two protected areas (i) Cantanhez National Park (CNP) and Cufada Lagoons Natural Park (CLNP) in the southwestern region, and (ii) the eastern region of Boé^{53, 59}. Extensive sampling surveys during 2008 and 2010 were performed at 27 sites in Tombali, Quínara and Gabú regions, and non-invasive samples were collected (see Table 4.1 and Fig. 4.1). The samples from Nimba region were collected by KK around the area of the coordinates 7°37'23.04"N Latitude and 8°26'10.62"W.

CNP is located in the Tombali region and comprises a total area of 1068 km² and comprises a patchy environment of small rivers; mangroves, wooded and grassland savannahs; agriculture fields, human settlements and sub-humid forests characterize the landscape⁶¹. The World Wide Fund for Nature has recognized the Cantanhez forests as one of the 200 most important ecoregions in the world, and one of the last segments of humid forest in West Africa⁶². CLPN is located in the Quínara region, created by presidential decree in 2000⁶³. It covers an area of 700 km² and is bounded in the North by the Corubal River in the Northwest by the road to Fulacunda, and in the South by the Buba River. The dominant vegetation type is semi-dry forest and dry forest areas with patches of sub-humid and moist savannas. The park area is recognized as 'wetland of international importance' and is therefore recognized by the Ramsar Convention⁶⁴.

The Boé sector is located in the Gabú region and is approximately 3288 km², having a population of approximately 12,000 largely subsistence agriculturalists and pastoralists. The sector is dominated by savannah vegetation on poor laterite soils, where *Parkia biglobosa* is a common tree species. The World Bank and the United Nations Development Program are in the process of creating a new protected area, the future: Dulombi-Boé National Park⁶⁵.

Lastly, the Nimba Mountains and particularly the Seringbara study site near the village with the same name, in south-eastern Guinea, which is only 6 km southeast of the Bossou research site (Fig. 4.1). The Nimba Mountains form a natural boundary between Guinea, Ivory Coast and Liberia and are protected as the Réserve Naturelle Intégrale du Mont-Nimba, in Guinea and Ivory Coast. On the Guinean side, the reserve extends over 12.7 km². The reserve forms a UNESCO World Heritage Site covering in total approximately 22,000 ha⁶⁶.

4.3.2- Non-invasive populations sampling strategy

Fecal samples from chimpanzees were collected at 28 sites (Table 4.1). Approximately 5g of the outer layer of faecal material was stored at room temperature in absolute ethanol for 24 h and then transferred to a second tube with silica gel type III (Sigma Alrich) and maintained at room temperature until DNA was extracted following the “Two Step” protocol suggested by Roeder et al.⁶⁷⁻⁶⁸. Permits to transport the Guinean-Bissau samples were obtained near the official institutions of Guinea-Bissau, Portugal and UK (IBAP, ICNB and DEFRA).

4.3.4- DNA extraction, amplification and sequencing

DNA was extracted using the QIAmp DNA Stool Mini Kit® (QIAGEN, Germany) according to the manufacturer’s instructions with the following modifications: Day 1 - 1.7 ml ASL buffer was added to each faecal sample (approximately 0.1g), vortexed for 10 min and then allowed to soak in an orbital shaker overnight at room temperature. Day 2 - The centrifuge and incubating times were increased to 3-10

min and 4 µl of 1350 µg/ml poly-A Carrier RNA (QIAGEN, Germany) was added immediately before the ethanol precipitation step with 100%. The elution time with AE buffer was also increased to 30 min and the final volume reduced to 150 µl. Each sample was extracted in duplicate and two negative controls were included to monitor possible contamination.

The mitochondrial (mt)DNA hypervariable region I was amplified in 20 µl volumes comprising: 9 µl of QIAGEN Multiplex PCR kit containing buffer, DNTP's, Taq polymerase and 3 mM MgCl₂ (final concentration), 0.1 µl 1x BSA, 0.1 µl 1x Q solution, 6.8 µl ultrapure water and 0.2 µM primers and 2 µl DNA template. The PCR profile was as follows: 95 °C for 15 min; 45 cycles of 94°C for 30 s, 51 °C for 60 s, and 72 °C for 60 s, and 72 °C for 10 min. Primers L15926 (5'-TAC ACT GGT CTT GTA AAC C-3', corresponding to positions 15326-15344 of the complete chimpanzee mtDNA genome [GenBank Accession No. D38113] and H16555 (5'-TGA TCC ATC GTG ATG TCT TA-3', corresponding to positions 15971-15990 of D38113) were used because they amplify the HVRI mtDNA region of chimpanzees which reliably amplify a 600 bp of the control region in chimpanzees⁷⁰. PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (SAP) (New England Biolabs, UK) at 37°C for 60 min, were inactivated at 80°C for 15 min and sequenced using MacroGen Europe's service using the same forward and reverse amplification primers. Sequences were edited, assembled and aligned using the program SEQUENCHER 3.1.2 (Gene Codes Corporation). The consensus sequences were submitted to a BLAST search to confirm chimpanzee origin.

4.3.4- Sequence authenticity

In order to ensure that the sequences of this study were not contaminated with '*Numts*' (Nuclear Copies of Mitochondrial DNA⁹⁸), which would provide non-homologous data, all PCR products were inspected both for dual bands in agarose gels and for double peaks in sequencing chromatograms. Furthermore, all sequences aligned with the reference *Pan troglodytes* HVR1 sequence [GenBank Access No.: X93335]⁷¹ and were compared with sequences reported to be chimpanzee *Numts*

from GenBank. No evidence was found for the presence of nuclear copies in this study.

To determine the individuality of each sample, those that exhibited the same haplotype and were of the same molecular sex and geographical location were genotyped using 6 nuclear microsatellite loci (D16S2624, D10S1432, D2S1326, D5S1457, HUMFIBRA, and D4S1627). When tested using a random subset of individuals (N=20) each microsatellite marker was found to be highly polymorphic based on the polymorphic information content (PIC) (D16S2624, PIC= 0.6979; D10S1432, PIC= 0.7127; D2S1326, PIC= 0.8096; D5S1457, PIC= 0.7273; HUMFIBRA, PIC= 0.5824; D4S1627, PIC= 0.7713). Sequences from specimens with the same haplotype and of the same sex from the same location but with differing genotypes, were considered to represent different individuals.

For genotyping criteria the number of replicates was estimated by using a maximum likelihood approach implemented in the software GEMINI v. 1.4.1⁷² that uses a priori information about the error rates (i.e. allelic dropout and false alleles), which were determined in the software PEDANT v. 1.0⁷³⁻⁷⁴. General parameters in the software GEMINI included 100 simulation replicates and the range of repetition number was set from 2-12. This test revealed that four PCR repetitions were appropriate to achieve a high probability of identity assuring a 95% confidence of the genotypes, and the per locus consensus threshold revealed that for a sample to be included in the final dataset must have at least two positive PCR results for nine of the 14 loci. For molecular sexing the result was considered as true when observed three times out of the four repetitions.

Allelic dropout, null alleles and stutter peaks were assessed using MICROCHECKER, v. 2.2.3⁷⁵. Moreover, a quality index (QI) for all loci was determined according to Miquel et al. (2006)⁷⁶ and samples with a QI below 0.50 were removed from the dataset, as they are genotypes with poor quality.

To detect repeated individuals the software GIMLET v. 1.3.3⁷⁷ was used, and duplicated genotypes were removed. This software was also used to calculate the Probability of Identity (PI) and the Probability of Identity between siblings (PI_{sibs})⁷⁸. Finally, GenAEx v. 6.4.1⁷⁹ was used to conduct all standard population genetic analyses.

4.3.5- Phylogenetic and population genetic analyses

The HVR1 haplotypes were defined by analyzing the sequences in the program DNASP v. 4.01⁷⁷. The haplotype diversity (h) nucleotide (π) and Watterson (θ_w) were estimated in the program ARLEQUIN v.3.01⁷⁸ as well as the estimator F_{ST} in the analysis of their genetic differentiation. θ_w summarizes the rate by which the evolutionary processes of mutation and genetic drift create and eliminate variation, and assumes neutrality. It is based on the number of polymorphic sites, but is independent of their frequency in contrast to π ⁹¹. The contributions of each population to the total haplotypic diversity (CT) and total haplotypic richness (CRT) were calculated using the software CONTRIB v.1.02 and partitioned into two components: the contribution due to a population's own level of diversity (CS and CRS), and its differentiation from other populations (CD and CRD), respectively⁷⁹.

For phylogenetic analyses, West African HVR1 chimpanzee sequences were included from the neighboring country of Guinea (i.e. Bossou, Yeale, Seringbara)^{42, 69, 72}, and from Nigeria and Cameroon⁷³. A median-joining haplotype network was constructed using the program NETWORK v. 4.6⁸³. For phylogenetic analysis, the best evolutionary model was the HKY+G substitution model with a gamma shape parameter of 0.2250 (-lnL=1566.0998), determined using the Akaike Information Criterion, implemented in the program jMODELTEST v.0.1.1⁸⁴. Phylogenetic trees were constructed using: (i) maximum parsimony (MP) with 1000 replicates of bootstraps; (ii) neighbor joining (NJ) with the Kimura 2-Parameter factor of correction and 10 000 bootstrap replacements in MEGA, and (iii) the Bayesian inference (BI) program MRBAYES v. 3.1⁸⁵. The BI posterior probabilities were inferred using the Metropolis-Coupled Markov Chain Monte Carlo algorithm (MC-MCMC) from six million generations, with sampling every 100, 4 chains, 2 runs and an initial burn-in of 25%. Stationarity of the chains was established from $p < 0.01$ split frequencies. The convergence of the MCMC BI chains was evaluated in the program Tracer v.1.5⁸⁶ where ESS>200 confirmed a good convergence. All trees obtained were viewed and edited in Figtree software v.1.3.1⁸⁷ and concatenated by the three

methods in a consensus tree in the program TREEGraph v. 2.0⁸⁸.

Analysis of molecular variance (AMOVA) using pairwise nucleotide differences were also calculated in the program ARLEQUIN and the most likely pattern of population subdivision was estimated using spatial analysis of molecular variance in SAMOVA 1.0⁸⁰ using 10,000 iterations from each of 100 random initial conditions, and testing all grouping options (predefined number of groups (K) ranging from 2 to 18).

In order to evaluate the relationship between genetic and geographic distance, a Mantel test and a linear regression by reduction of major axis (RMA) were applied in the program IBDWS v 3.16⁸¹. Euclidean geographic distances were estimated using the ruler tool in the software Google Earth. To evaluate the existence of vicariant barriers that may prevent gene flow between the geographical regions, the program BARRIER v. 2.2 was used⁸². This method employs a geometric network connecting all sampled populations. Delaunay triangulation was computed from the intersections of the medians of each triangle, and a Voronoi tessellation obtained. Genetic distances are calculated for all neighboring populations and associated to each network edge. We used F_{ST} pairwise distances as a measure of genetic differentiation among populations. Then a number of barriers were identified (defined *a priori*) through the Monmonier's algorithm associated with higher values of genetic distance between populations⁸¹. Only barriers showing values of pairwise genetic differentiation higher than the average for all populations were represented on a map.

The analysis of demographic history and the likelihood of sudden expansion were tested using: mismatch distributions⁹⁰ in ARLEQUIN; and tests of neutrality (Tajima D and Fu F_s) in the same software, and the tests Fu and Li F^* and D^* in the program DNASP v.4.01.

Finally, to analyze divergence times, a relaxed molecular clock and a coalescent approach was implemented in the program BEAST v. 1.6.2⁸⁹. The molecular clock was calibrated based on fossils from human-chimpanzee, human-Neanderthal and chimpanzee-bonobo divergence. Three independent runs were performed using the same mutation rate; optimized parameters and the substitution model suggested by jMODELTEST, and the Yule speciation process was used. The

convergence of the runs was also evaluated in the program TRACER after combining logs and trees in LogCombiner and TreeAnnotator, respectively⁸⁹.

4.4- RESULTS

4.4.1- mtDNA HVR1 diversity

We obtained a 261 bp sequence for the mitochondrial HVR1 from 334 west African chimpanzee fecal samples (189 from Guinea-Bissau, and 145 from the Nimba Mountains, Guinea). We found 55 polymorphic sites comprising 52 transitions (ti) and three transversions (tv) with an overall ti/tv ratio of 31.04 following the maximum likelihood estimate. The nucleotide composition was as follows: **A** (37.67%), **T** (17.10%), **C** (6.73%), and **G** (38.51%).

Fifty-one haplotypes were identified, found in 1 - 3 populations (Table 2) with 19 shared, including the haplotypes H1, H13 and H35 that were distributed between the two countries. Nimba possessed the highest number of exclusive haplotypes (n=12), followed by Cadique (n= 8) and Vendu Leidi (n=8) in Guinea-Bissau. H13 was the most common haplotype, present in 46 individuals. H3 and H4 occurred only in Guinea-Bissau and were widespread across all study areas. Cantanhez possessed the highest number of private haplotypes (n= 11) followed by Nimba and Boé (with 9 each), Cufada (n=5), Catió (n= 3) and Empada with one (H5). Three haplotypes are communal between CNP and CLNP (i.e. H2, H6, H9), and five between Boé and Cantanhez (i.e. H14, H18, H19, H21, H24). Within CNP, H15 is the most frequent haplotype occurring in seven localities (n= 21) (Table 4.2).

The haplotype diversity (h) for each study area ranged from 0.836 in Nimba to 0.929 in Gabú. The highest values of the nucleotide diversity (π) also occurred in Gabú (π = 0.05516) and the lowest in Nimba (π = 0.0429). The total haplotype and nucleotide diversities were h = 0.935 and π = 0.05325, respectively. An alternative measure to π is Watterson's theta estimator (θ_w). The average high θ_w for all population was 0.03058 with a variation between 0.02804 and 0.03620 in Nimba and Quínara, respectively. Estimates of the molecular diversity indexes are given in Table

4.3.

The *P. troglodytes verus* populations contributed differently to the total genetic diversity and differentiation inferred for the study areas. The contributions from the diversity (CS and CRS) and differentiation (CD and CRD) components of each population are shown in Fig. 4.2. The highest CS and CRS values were observed in Aicum, Béli, Béli North and Vendu Leidi populations, all belonging to the Gabú region. In contrast, the highest CD and CRD values were seen in Bani, and Injassane from Quínara region and in general from the Cantanhez populations. These contributions to genetic differentiation were probably due to exclusive haplotypes found in medium to high frequencies in this region (see haplotypes H15, H16, H17, H25, H26, H27, H28, H29, H40, H41, and H42 in Table 4.2).

4.4.2- Phylogenetic analysis

The chimpanzees from Nigeria Cameroon (*P. t. ellioti*) formed a monophyletic unit statistically well supported by posterior probabilities (BI= 1). The 51 *P. t. verus* haplotypes from Guinea-Bissau and Nimba Mountains clustered in two main clades, named Lineage A and B (Fig. 4.3).

The median-joining phylogenetic network confirmed the results of the phylogenetic tree analysis, but it provided more resolution on relationships among haplotypes and within subclades where it was possible to observe a clear star-shape pattern in Lineage B (Fig. 4.4). The haplotype distribution within Guinea-Bissau is presented in Figure 5.

4.4.3- Phylogeographic structure

Pairwise F_{ST} values calculated from haplotype frequencies of the 28 populations sampled indicate significant differentiation (Table 4.4). However, when the populations were grouped together the F_{ST} values varied from 0.00334 (Tombali-Quínara) to 0.21095 (Quínara-Nimba) and only the Nimba differed significantly from zero (Table 4.4). Pairwise Φ_{ST} calculated from Kimura's two parameter genetic

distances revealed similar results demonstrating that the three regions within Guinea-Bissau are not significantly differentiated.

In order to reveal the partitioning of genetic diversity a hierarchical AMOVA was conducted showing a strong differentiation among all chimpanzee populations ($\Phi_{ST} = 0.25956$, $p < 0.0001$). AMOVA revealed 74.04% of overall genetic variance partitioned among individuals within populations whereas differences among populations within regions accounted for 23.74% ($\Phi_{SC} = 0.24274$, $p < 0.0001$). Only 2.22% of the genetic variation is partitioned among regions and this was not significant (AMOVA $\Phi_{CT} = 0.2221$, $p > 0.05$) (Table 4.5).

The SAMOVA demonstrated that the strongest partitioning of genetic diversity was obtained when samples were assigned to 2 - 10 groups. The best grouping scheme divided the distribution range of *P. troglodytes verus* in Guinea-Bissau and Nimba into two main geographical groups ($K=2$; $F_{CT} = 0.34253$, $p < 0.0001$). When the number of groups exceeded three, SAMOVA showed lower F_{CT} values. With $K=2$ a partition between the south Cantanhez populations and all the others corresponded to the two main HVR1 haplotype lineages. The populations from south Cantanhez (i.e. Farim, Catombói, Cafatche and Canghóde) comprised only haplotypes from Lineage A while the rest of the populations in Guinea Bissau and Nimba have haplotypes from both lineages. Table 4.6 summarizes the SAMOVA analysis and the components of genetic variance explained for each cluster.

Mantel tests performed to assess isolation by distance revealed no correlation when all the populations were considered ($Z = 5.999555.85$, $r = -0.0822$, one sided $p = 0.8370$ from 30 000 permutations). However, when considering Cantanhez only (i.e. excluding Nimba and Gabú) to take into account the long-term historical divergence among populations as recommended by Telles and Diniz-Filho, (2005)⁹³ this correlation was significant ($Z = 159.8819$, $r = 0.5661$, one-sided $p = 0.0080$ from 30 000 permutations). The isolation by distance among Cantanhez populations was confirmed by the RMA ($y = -0.08010$ $x = 0.03709$, $r^2 = 0.320$).

The Monmonier algorithm (BARRIER software) suggested three main barriers (α , β , γ) to gene flow in the Guinea-Bissau chimpanzee distribution range: (α) the most significant barrier is located approximately in the south of the CNP in the

Cubucaré Peninsula, thus isolating the populations of Catombói, Cafatche and Canghóde (11°11'19.26"N- 15° 6'32.49"W); (β) another barrier located south of the Rio Grande de Buba in the Quínara region causing genetic discontinuity between the CLNP and the Empada and Catió chimpanzees (11°37'47.19"N- 15°10'23.11"W), and (γ) one barrier that separates Aicum and Vendu Leidi populations from the others in the Boé sector and in the Tombali region, located in the southeast of the country between the geographic coordinates (11°44'19.79"N- 13°44'3.80"W) and (11°56'39.19"N- 13°53'0.34"W). These barriers are represented in Figure 4.5.

4.4.4- Demographic history and divergence time

Historical demography was analysed by estimating parameters for neutrality tests and by conducting mismatch distribution analyses. Tajima's D tests were positive but not statistically significant for each region or for all populations grouped together. Similarly, the Fu's F_s values were not significant, although presenting a negative value for all populations (Fu's F_s = -93294). The test Fu and Li's and D' is based on the differences between the number of singletons and the total number of mutations, whereas the test Fu and Li's F' is based on differences between the number of singletons and the average number of differences between pairs of sequences⁹⁴. These two tests presented positive values and were statistical significant in each region and in all populations, with the exception of the test Fu and Li's D' for the Tombali region that was not significant suggesting a deviation from neutrality (see Table 4.3).

Considered altogether, the mismatch distributions for all populations were bimodal and they fitted with their distributions under the sudden and spatial expansion models and this may be due to their peripheral nature. The tests for the goodness-of-fit confirmed both demographic models (SSD= 0.00621, $p > 0.05$; SSD= 0.00710, $p > 0.05$ for the sudden and spatial expansion models, respectively). However, when considering only the sequences from Lineage A and Lineage B defined by the phylogenetic and network inferences, the patterns were unimodal fitting with the distributions expected under the sudden and spatial expansion

models. Furthermore, the model parameters Θ_0 and Θ_1 calculated for Lineage A and Lineage B separately, showed values expected under a model of rapid growth in both cases (Fig. 4.6). Tests for the goodness-of-fit for the observed data support similarly the expansion model revealed by the SSD values for both lineages (SSD=0.037, P= 0.046; and SSD= 0.0155, P= 0.192, respectively). Additionally, the small raggedness index values supported a smooth distribution (R-Index= 0.044; P= 0.237; R-Index= 0.0189, P= 0.055).

Considering a divergence rate of 22.5% per million years^{6, 95}, and a split time between humans and chimpanzees of 7-5 mya⁹⁶, and between chimpanzees and bonobos of 2.1-1.5 mya⁹⁶, the two major mitochondrial lineages are estimated to have diverged at 1 mya (95% HPD: 0.34-2.11). The most recent common ancestor (mrca) of Lineage A is estimated at 0.67 mya, while the TMRCA of Lineage B is estimated at 0.43 mya (95% HPD: 0.34 to 2.11 mya). The divergence time among the Lineage A haplogroups varied between 0.32 mya (95% HPD: 0.03 to 0.95) for haplogroup A3 and 0.47 mya (95% HPD: 0.9 to 1.22) and between 0.07 mya (95% HPD: 0-0.47-0.97) for haplogroup B2 and 0.29 mya (95% HPD: 0.04 to 0.81) for haplogroup B2.

4.5- DISCUSSION

This study is the first to investigate the mitochondrial genetic structure and demographic history of the Guinea-Bissau chimpanzees, and for that purpose Nimba chimpanzees were incorporated. It revisits West African chimpanzee phylogeography by including individuals from these areas. The high genetic diversity observed for all populations in the current study ($h= 94\%$, $\pi=5.3\%$ and $\theta_w=3.1\%$) is in agreement with previous reports for Upper Guinea chimpanzees^{5, 35, 38}. The nucleotide diversities fall within the range reported by Gagneux et al. (2009) for the subspecies ($\pi =0.07$, range 0-16%)⁵ and when compared with other populations³⁸: Tai, Ivory Coast ($\pi= 5.2\%$), Bossématié Forest, Liberia ($\pi= 4.81\%$), Tenkere, Sierra Leone ($\pi= 4.73\%$) and Solo, Mali ($\pi= 4.47\%$). The chimpanzees from the Tombali and Gabú regions in Guinea-Bissau exhibit a similar nucleotide diversity ($\pi= 5.4\%$ and $\pi= 5.5\%$, respectively). When compared with the eastern chimpanzees ($\pi =2.5\%$, range

0-9%)⁹⁷ the nucleotide diversities reported in this study are higher, but in the same range of central chimpanzees ($\pi = 5.6\%$)⁹⁸. Nevertheless, the genetic diversities were consistently greater in the peripheral chimpanzees of Guinea-Bissau when compared to Nimba.

Fifty-one haplotypes were detected from which 39 are exclusive to Guinea-Bissau. The Tombali region, where CNP lies, possessed the highest number of haplotypes ($n=26$) and five (H44, H46, H48, H49, H50) are newly reported for the Nimba Mountains population when compared to previous studies^{70, 72}. Three haplotypes were shared between the two countries (H1, H13, H35) over 800 km apart indicating that female gene flow must have been substantial until fairly recently. The Niger River has been proposed as a natural barrier to gene flow⁷³, but our findings suggest that this river has not been a substantial boundary between Guinea and Guinea-Bissau chimpanzees, or they have used the Liberia and Sierra Leone as a preferred route (Fig. 1).

Bayesian Inference phylogenetic reconstruction revealed two deep clades (Lineage A and Lineage B). The existence of these two evolutionary lineages in the western chimpanzees had been previously reported^{4, 6, 9}.

Our analyses indicated a high level of genetic structure ($F_{ST}=0.25956$, $p<0.0001$), with 74.04% of the total genetic diversity due to differences within populations among individuals (Table 5). The same pattern of genetic variation apportionment was found for the Nigeria-Cameroon chimpanzees²⁷ and bonobos⁹. In the current study, two major phylogeographical groups were identified by the SAMOVA algorithm ($K=2$): one restricted to the south of the CNP encompassing the populations of Farim, Catombói, Cafatche and Canghode, and a second cluster constituted by all the other populations (Table 7). The chimpanzees from south Cantanhez contain exclusively haplotypes from Lineage A, whereas the second group have haplotypes from both lineages.

The genetic structure of the Guinea-Bissau chimpanzees can be predicted either via isolation by distance or by vicariance. The Mantel test ($Z = 159.8819$, $r^2 = 0.5661$, $p < 0.05$) showed that there is a positive correlation between the genetic distance and the geographic distance. As the latter increases the former decreases causing a divergence between the haplotype frequencies promoted by the genetic

drift, thus establishing an isolation-by-distance pattern. This pattern explains that Cantanhez female chimpanzees prefer to migrate to a neighbouring population rather than travel long distances, hence following a stepping-stone model of dispersal. This pattern also occurs in other chimpanzee population's elsewhere^{14, 73}.

The genetic discontinuity analyses conducted in the BARRIER program, which combines fundamentals of statistical geometry (Voronoi Tessellation and Delaunay triangulations) with the maximum difference algorithm of Monmonnier⁸³ revealed the existence of three main barriers to gene flow. The most significant barrier (1) is located in the south of the CNP isolating the populations of Farim-Canghode. This barrier coincides with the best clustering of groups revealed by the SAMOVA analyses. This group is isolated in the very end of the Cubucaré Pensinsula limited by the Cumbijã and Cacine Rivers. Nowadays there are no apparent physical boundaries in the area. The second significant barrier (2) is located approximately at the 11° 37'N-15°10' causing a genetic discontinuity between the CLNP and the Empada and Catió chimpanzees. These Peninsulas are clearly separated by the Rio Grande de Buba at north and the Catió River at west, but its connectivity is linked with the CNP at southeast via the Nhala corridor. Lastly, a third genetic disjunction (3) was identified in the Gabú region, isolating the remote chimpanzees from Vendu Leidi and Aicum. The Féfine River could have been a natural barrier for these populations as they are located on the east side of the river.

Signatures of recent demographic and spatial expansion were revealed by: (i) the Fu and Li's D' and F' neutrality tests, (ii) the star shape pattern in the network and (iii) the bimodal pattern of the mismatch distributions. In addition, both nucleotide and haplotype diversities were high and according to Grant and Bowen (1998) this can reflect a secondary contact between different lineages⁹⁹. The bimodal pattern in both mismatch distributions suggests population subdivision, several demographic expansions in the past, various waves of migration among different populations or a combination of these¹⁰⁰. The same pattern was observed for the *P. t. ssp. ellioti*^{27, 73}. However, this pattern contrasts with the eastern and central chimpanzee subspecies characterized by a unimodal pattern^{5, 33}. Based on the mismatch analyses of the peripheral chimpanzees in Guinea-Bissau it appears that historical isolation and subsequent secondary contact has produced the

patterns of genetic diversity and mismatch distribution detected here, contrary to expectations.

The two mtDNA lineages emerged, according to this study around 1 mya after the divergence between the Central/Western chimpanzees around 1.6-1.3 mya⁴. Historically, they fall into the Upper Pleistocene (1-0.78 mya) and the haplogroup divergence within each lineage dates back to the Middle Pleistocene (0.78-0.12 mya). In paleoclimate terms these divergence estimates occurred during the Gunz (0.68-0.62) and Mindel (0.45-0.30) glaciations with a Last Glacial Maximum around 27 500-14 500 years ago. Several evidences and recent studies have demonstrated that during the glaciation periods the climate in Africa was cooler and drier than today⁴⁶. The recurrence of climatic changes during the Pleistocene periods in West Africa, with cycles of forest expansion and contraction¹⁰¹, likely led to repeated vicariance events that can explain the genetic structure and diversity observed for the chimpanzees in Guinea-Bissau and Nimba. Moreover, the concepts of endemism and diversity can be translated into genetic terms to make statements about the locations of the refuges and of lineage expansions out of those areas during the interglacial periods of the forest re-expansion⁷³. Populations with high levels of haplotypic diversity may have been protected within Pleistocene refuges during the periods of maximum glaciation^{1, 27, 33-34, 73, 79}. The distribution of the genetic diversity appears to follow that hypothesis.

4.5.1- Conservation considerations

Several studies have demonstrated that anthropogenic effects such as fragmentation and deforestation can promote changes in the diversity and genetic structure of species, usually decreasing the genetic diversity within populations and increasing differentiation among populations^{44-45, 102-103}. Further research is needed to uncover contemporary changes, because mtDNA is not a powerful marker to detect contemporary alterations in the genetic structure of fragmented populations as more polymorphic nuclear markers such as microsatellites, could result in a modification of the phylogeographical patterns obtained in this study. However,

taking into account that *P. t. verus* is an endangered hominid we considered relevant to use mtDNA to inform conservation strategies for this species in Guinea-Bissau. Petit et al. (1998) pointed out that the criteria for the selection of priority populations to be conserved must include its differentiation and its diversity levels⁷⁹. Additionally, Moritz (1994) propose the use of “management units (MU)” to recognize the management units with a significant divergence of alleles (at nuclear or organelle loci)¹⁰⁴. Therefore, the chimpanzee populations containing representative genetic diversity from the two major phylogeographic groups should be identified. The group in south Cantanhez constituted by the Farim-Canghode populations warrants special attention, as it is isolated in the very south of the CNP and under intense pressure from habitat fragmentation and loss (Rui Sá pers. observation). Hunting practices must be forbidden totally, with appropriate law enforcement in order to mitigate its deleterious effects. The guards of the CNP should be more present and active, supervising more frequently areas in the park where hunting is more frequent. Alternatives to chimpanzee crop-raiding attacks in this area must be developed in order to prevent locals to kill them during emergent conflicts. Canghode and Cafatche populations should be carefully protected, as they are the units that most contribute to the genetic diversity within this group. Among the second group the populations of the northern area of CNP (i.e. Amidara, São Francico, Mejo, Cancira), Buba Inthingue and Injassane in CLNP and the populations of Béli, Vendu Leidi, Dinguirai and Aicum in the Gabú region are recognized as the units that contribute most to maintaining genetic diversity. Special attention should be given to the populations of Vendu Leidi and Dinguirai as mining activities are planned for these areas. In addition, special attention should be also given to the population of Briame in the Catió Peninsula as they are living in an intense anthropogenic area and if the forest connectivity in the northern part between the Empada and Buba sectors is lost they will become isolated.

In conclusion, the results of the present study show that palaeoecological history and rivers have a role in shaping the genetic variability and structure of the chimpanzees in Guinea-Bissau and Nimba and they can be used for conservation purposes.

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4.8- TABLES

Table 4.1: Chimpanzee non-invasive sampling, in Guinea-Bissau and Guinea, for this study. Number of chimpanzees sequenced (N), geographic coordinates: latitude (Lat.), longitude (Lon.).

Region	Locality	N	Lat.	Lon.	Sample site Number
A Tombali-Cantanhez	Madina	1	11°12'47.64"N	15° 2'12.72"W	1
A Tombali-Cantanhez	Farim	4	11°12'31.02"N	15° 4'25.80"W	2
A Tombali-Cantanhez	Caiquéne	8	11°13'30.93"N	15° 5'47.73"W	3
A Tombali-Cantanhez	Canghode	12	11° 9'40.68"N	15° 7'35.46"W	4
A Tombali-Cantanhez	Cafatche	8	11°19'15.60"N	15° 6'31.98"W	5
A Tombali-Cantanhez	Catomboi	5	11°11'38.28"N	15° 4'36.36"W	6
A Tombali-Cantanhez	Amidara	10	11°16'52.38"N	14°58'34.02"W	7
A Tombali-Cantanhez	Cambéque	3	11°11'30.18"N	15° 0'49.74"W	8
A Tombali-Cantanhez	São Francisco	5	11°19'18.96"N	14°59'29.22"W	9
A Tombali-Cantanhez	Ponta Nova	5	11°18'41.64"N	14°58'51.36"W	10
A Tombali-Cantanhez	Cadique	16	11°14'19.92"N	15°6'51.30"W	11
A Tombali-Cantanhez	Mejo	10	11°22'48.45"N	14°54'51.00"W	12
A Tombali-Cantanhez	Quebo Sutuba	3	11°9'34.51"N	14°54'41.12"W	13
A Tombali-Cantanhez	Lautchande	16	11°14'55.14"N	15°4'20.40"W	14
A Tombali-Cantanhez	Cancira	8	11°22'48.45"N	14°54'51.00"W	15
B Quínara-Cufada	Buba Itchinque	10	11°45'11.58"N	15° 5'18.36"W	16
B Quínara-Cufada	Bani	2	11°43'42.54"N	15° 5'31.20"W	17
B Quínara-Cufada	Injassane	2	11°44'29.88"N	14°59'4.74"W	18
B Quínara-Cufada	Nhala-Molha Pé	3	11°37'45.72"N	14°53'55.08"W	19
B Quínara-Empada	Dois Rios	13	11°37'56.22"N	15° 9'18.66"W	20
A Tombali-Catió	Briame	10	11°17'19.92"N	15°14'28.86"W	21
C Gabú-Boé	Dinguirai	5	11°45'18.72"N	13°51'55.38"W	22
C Gabú-Boé	Pataque	4	11°53'04.9" N	13°57'32.5" W	23
C Gabú-Boé	Aicum	3	11°56'29.22"N	13°52'37.38"W	24
C Gabú-Boé	Béli North	7	11° 52'04.7" N	13° 57' 31.9" W	25
C Gabú-Boé	Béli	7	11°51'10.80"N	13°54'39.66"W	26
C Gabú-Boé	Vendu Leidi	9	11°44'33.00"N	13°43'22.44"W	27
D Nimba Mountains	Area of Seringbara	145	7°37'23.04"N	8°26'10.62"W	28

Table 4.2: Distribution of 51 HVR1 haplotypes found in 334 chimpanzees in Guinea-Bissau and Nimba (i.e. Guinea) from 28 populations (Sites 1-28)a. Haplotype number (H), total number of animals sequenced (NA), total number of haplotypes (NHT).

[illegible]

^aFor each site number and locality see Table 4.1.

Table 4.3: Summary statistics and demographic expansion tests observed in the study areas and in all populations, including the mean number of pairwise differences (k), and the theta *per site* from S (θ_w) according to Watterson (1975).

Parameters	Tombali	Quínara	Gabú	Nimba	All populations
Sample size	124	30	35	145	334
Number of populations	16	6	5	1	28
Number of polymorphic sites	45	39	42	44	55
Number of haplotypes	26	12	18	12	51
Haplotype diversity (SD)	0.9251 (± 0.100)	0.897 (± 0.031)	0.929 (± 0.021)	0.836 (± 0.012)	0.935 (± 0.005)
Nucleotide diversity (SD)	0.05355 (± 0.00129)	0.04839 (± 0.00322)	0.05516 (± 0.00311)	0.04082 (± 0.00307)	0.05325 (± 0.00116)
θ_w	0.02958	0.03620	0.03510	0.02804	0.03058
k	13.761	12.485	14.121	10.490	13.633
Tajima's D	2.36727	1.17622	1.89567	1.36985	1.93048
Fu and Li's F'	2.14021**	1.73893*	2.23514**	1.98212*	2.53128**
Fu and Li's D'	1.29726	1.59058**	1.81072**	1.84959**	2.08803**
Fu's F_s	3.62642	3.30846	0.64391	13.64252	-93294
Mismatch distribution	Bimodal	Bimodal	Unimodal	Multimodal	Bimodal

* $P < 0.05$; ** $P < 0.02$

Table 4.4: Pairwise F_{ST} values between chimpanzee study areas in Guinea-Bissau and Nimba Mountains.

	Tombali	Quínara	Gabú	Nimba
Tombali	—			
Quínara	0.00334	—		
Gabú	0.01178	0.00984	—	
Nimba	0.19211	0.21095	0.19992	—
P<0.05				

Table 4.5: Hierarchical analysis of molecular variance (AMOVA) for chimpanzee HVR1 mtDNA sequences from Guinea-Bissau and Nimba.

Variance component	Variance	%	d.f.	SSD	Φ statistics	P
Total						
Among regions σ_a^2	0.17410	2.22	5	298.867	$\Phi_{CT} = 0.02221$	0.52590
Among populations within regions σ_b^2	1.86041	23.74	22	394.057	$\Phi_{SC} = 0.24274$	<0.0001
Within populations σ_c^2	5.80365	74.04	307	1781.721	$\Phi_{ST} = 0.25956$	<0.0001

Table 4.6: Results from SAMOVA analysis with different clusters defined a priori. Number of groups (K), group composition and fixation indexes are reported.

K	Group composition	F _{CT} *	F _{SC} *	F _{ST} *
2	(2, 4, 5, 6) (1, 3, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28)	0.34253	0.17112	0.45503
3	(2, 4, 5, 6) (24) (1, 3, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 26, 27, 28)	0.33889	0.16451	0.44765
4	(1) (2, 4, 5, 6) (7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 27, 28) (24, 26)	0.33661	0.14285	0.43137
5	(7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 25, 27, 28) (1, 2, 3, 4, 5) (26) (24)	0.33159	0.14037	0.42542
6	(2, 3, 4, 5) (24) (1) (18) (7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, 23, 25, 27, 28) (26)	0.32997	0.14092	0.42439
7	(1) (5) (7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 19, 20, 21, 22, 23, 25, 27, 28) (16, 26) (18) (2, 3, 4) (24)	0.31821	0.12368	0.40253
8	(1) (16, 26) (5) (2, 3, 4) (24) (19) (18) (7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, 21, 22, 23, 25, 27, 28)	0.30858	0.12287	0.39353
9	(1) (16) (2, 3, 4) (7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 19, 20, 21, 22, 23, 25, 27, 28) (5) (24) (19) (18) (26)	0.29791	0.12851	0.38814
10	(8) (1) (24, 26) (2) (3, 4) (5) (21) (7, 9, 10, 11, 12, 13, 14, 15, 17, 19, 20, 22, 23, 25, 27, 28) (18) (19)	0.28449	0.11777	0.36876

***P<0.0001**

^aSee Table 4.1 for locality and sample site number.

4.9- FIGURES

Figure 4.1: Study areas: A (Guinea-Bissau, Tombali region including the peninsulas of Cantanhez and Catió); B (Guinea-Bissau, Quínara region including the Cufada Lagoons Natural Park and the Empada peninsula); C (Guinea-Bissau, Gabú region across the Boé sector) and D (Guinea, Seringbara and Nimba Mountains).

Figure 4.2: The contribution to the total haplotype diversity (CT) and haplotypic richness (CTR) of each population of chimpanzees using the HVR1 haplotypes. The blue and red bars represent the contribution of diversity (CS and CRS) and differentiation (CD and CRD), respectively.

Figure 4.3: Phylogenetic relationships among haplotypes of Guinea and Guinea-Bissau chimpanzees according to Bayesian inference. The numbers, in each node, refer to the posterior probabilities.

Figure 4.4: Haplotype network recovered by median joining analysis. Haplotypes are represented by circles with size proportional to their frequencies and coloured according to subclades recovered in the phylogenetic analyses. Numbers outside the circles represent the mutational steps, and inside the circles the number of individuals for each haplotype. Grey circles represent missing haplotypes, extinct or not sampled.

Figure 4.5: Map showing the geographic distribution of the HVR1 haplotypes in the *P. troglodytes verus* populations in Guinea-Bissau. The haplotypes are identified in the legend. The broken lines in red indicate the genetic barriers (α , β , γ) as defined by the Monmonier algorithm.

Figure 4.6: Mismatch distributions for the Guinea-Bissau and Nimba chimpanzees and respective associated parameters. Mismatch distribution according with the sudden expansion model and with the spatial expansion model for Lineage A (A) and Lineage B (B), respectively. Mean pairwise differences between pairs of haplotypes (k), mismatch derived parameters (Θ_0 , τ , Θ_1). Sum of squared differences (SSD) and Harpending Raggedness Index with respective P values. Number of female migrants (M).

CHAPTER 5

“Parasites are in general, actors that play in the shadows...”

— Claude Combes, Parasitologist

CHAPTER 5

GASTROINTESTINAL SYMBIONTS OF CHIMPANZEES IN CANTANHEZ NATIONAL PARK, GUINEA-BISSAU WITH RESPECT TO HABITAT FRAGMENTATION

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5.1- ABSTRACT

One of the major factors threatening chimpanzees (*Pan troglodytes verus*) in Guinea-Bissau is habitat fragmentation. Such fragmentation may cause changes in symbiont dynamics resulting in increased susceptibility to infection, changes in host specificity and virulence. We monitored gastrointestinal symbiotic fauna of three chimpanzee subpopulations living within in Cantanhez National Park (CNP) in Guinea Bissau in the areas with different levels of anthropogenic fragmentation. Using standard coproscopical methods (merthiolate-iodine formalin concentration and Sheather's flotation) we examined 102 fecal samples and identified at least 13 different symbiotic genera (*Troglodytella abrassarti*, *Troglocorys cava*, *Blastocystis* spp., *Entamoeba* spp., *Iodamoeba butschlii*, *Giardia intestinalis*, *Chilomastix mesnili*, *Bertiella* sp., *Probstmayria gombensis*, unidentified strongylids, *Strongyloides stercoralis*, *Strongyloides fuelleborni* and *Trichuris* sp.). The symbiotic fauna of the CNP chimpanzees is comparable to that reported for other wild chimpanzee populations, although CNP chimpanzees have a higher prevalence of *Trichuris* sp. Symbiont richness was higher in chimpanzee subpopulations living in fragmented forests compared to the community inhabiting continuous forest area. We reported significantly higher prevalence of *G. intestinalis* in chimpanzees from fragmented areas, which could be attributed to increased contact with humans and livestock.

Key Words: Cantanhez National Park; Fragmentation; *Pan troglodytes verus*; Parasites; Symbionts; *Trichuris* sp.

5.2- INTRODUCTION

The gastrointestinal tract of primates is colonized by broad range of organisms with a variety of relationships with their host. Although most of them are traditionally referred to as parasites, term “symbionts” is more appropriate to describe the community in its complexity [Combes, 2001; Douglas, 2010]. Then, symbionts can be further defined as (i) mutualists (in reciprocally positive interactions) (ii) commensals (neither benefit nor harm their facilitators) and (iii) parasites (occur in a win-lose relation when one species benefits while the other species is harmed) [Bronstein, 2009].

Some parasites can constitute a risk for nonhuman primate conservation by affecting health, behaviour and survival of their hosts, especially in cases when they are already threatened by other factors [Daszak et al., 2000; McCallum & Dobson, 2002; Goldberg et al., 2005]. Most primate populations nowadays live in more or less disturbed landscapes [*e.g.* Cowlshaw & Dunbar, 2000; Marsh, 2003; Gillespie et al., 2005a; Hopkins & Nunn, 2007]. Anthropogenic disruptions such as habitat loss and fragmentation, overexploitation and climate change are regularly listed as extinction drivers at global and local scales [Coop & Holmes, 1996; Caldecott & Miles, 2005; Smith et al., 2009]. These factors may modify symbiont host ranges, vector dynamics, parasite virulence and transmission rates and consequently may lead to changes in infection risks and clinical outcomes of the infections, finally leading to host population decline [Gillespie et al., 2005a; Pedersen et al., 2007].

Habitat loss reduces not only species movement and dispersal, but also resource availability and as a consequence animals suffer the effects of overcrowding [Gillespie & Chapman, 2008]. As the contact and conflict among individuals increases, their stress levels rise and immunological competence starts to weaken and therefore their infection resistance can decrease, eventually promoting the spread of diseases and symbiont transmission [Scott, 1988; Eley et al., 1989; Coe, 1993; Ashford et al., 1996; Sleeman et al., 2000; Nunn & Altizer, 2006]. Several studies on primate symbiont ecology reported higher prevalence and richness of gastrointestinal symbionts in non-human primates living in forest fragments assuming positive correlation between pathogen prevalence and richness and host density [Gillespie & Chapman, 2006; Gillespie & Chapman, 2008; Mbora et al., 2009; Kowalewski et al., 2011] while other studies recorded the opposite or no effect [Cristóbal-Azkarate et al., 2010; Lane et al., 2011].

Endangered species, such as chimpanzees, have usually limited geographic ranges and irregular distributions within their ranges [Fahrig, 2003; Chapman et al., 2005]. Many studies have reported the gastrointestinal symbionts of several populations of the common chimpanzee (*Pan troglodytes*) in different types of habitats [e.g. Huffman et al., 1997; Ashford et al., 2000; Muehlenbein, 2005; Howells et al., 2010; Petrášová et al., 2010]. However, there is a paucity of information about the symbionts of chimpanzees in Guinea-Bissau, West Africa, which represents the most western limit of the species distribution where its survival is considered very precarious [Sousa et al., 2005; Torres et al., 2010]. Major threats to their survival include the pet trade, hunting for

animistic/traditional medicine practices and importantly also habitat fragmentation caused by logging and agriculture [Casanova & Sousa, 2007; Sá et al., 2009].

We present the results of the parasitological survey of chimpanzee population inhabiting Cantanhez National Park (CNP), in the south of Guinea-Bissau. We compared three chimpanzee subpopulations living within the Park in the areas with different levels of anthropogenic fragmentation.

5.3- METHODS

5.3.1- Study Site

Cantanhez National Park (CNP) (Fig. 5.1) is located in Tombali Region, in southern Guinea-Bissau and comprises a total area of 1068 km². The Cacine and Cumbidjã Rivers act as natural boundaries and form the Cubucaré Peninsula. The park was formally established in 2008 but since the mid-1980's was considered a forest hunting reserve [IBAP, 2008]. The climate is tropical humid with two annual seasons: the rainy season from June to October and the dry season from November to May with little or no rainfall. Mean annual rainfall is 2000 mm (SD $\pm$ 500) and the temperature varies between 28° and 31° C [Simão, 1997].

CNP is approximately 70m above sea level and consists of a patchy environment of small rivers; mangroves, arborous and grassland savannahs; agriculture fields, human settlements and sub-humid forests characterize the landscape. Forest fragments consist of a mixture of evergreen and semi-deciduous vegetation cover [Catarino, 2004]. Native fauna include seven species of non-human primates: the lesser bush baby (*Galago senegalensis*), the western red colobus (*Procolobus badius temmincki*), the king colobus (*Colobus polykomus*), the vervet monkey (*Chlorocebus aethiops sabaeus*), the Campbell's monkey (*Cercopithecus campbelli*), the Guinean baboon (*Papio papio*) and the western

chimpanzee (*Pan troglodytes verus*) [Gippoliti & Dell’Omo, 2003; Casanova & Sousa, 2007].

The World Wide Fund for Nature (WWF) has recognized the Cantanhez forests as one of the 200 most important ecoregions in the world, and ones of the last vestigial segments of humid forest in West Africa. However, through satellite images Oom et al. [2009] identified a trend of forest loss with a conversion from closed humid forests to savanna-woodlands. This loss has been caused by ‘slash and burn’ agriculture. The amount of land for crops is continually expanding due to increasing numbers of people [Hockings & Sousa, 2011]. Consequently, the encounters of humans and domestic animals with wildlife occur with increased frequency [Hockings & Sousa, 2011].

Permission to carry out research in the CNP was conceded by the Instituto da Biodiversidade e das Áreas Protegidas as well as by the consent of the traditional leaders.

5.3.2- Study Population

Formerly, chimpanzees were erroneously considered extinct in Guinea-Bissau [Lee et al., 1988]. However a survey in 1994 reconfirmed chimpanzee presence in the study area and a preliminary evaluation of their conservation status was carried out [Gippoliti & Dell’Omo, 1995; Gippoliti & Dell’Omo, 1996]. According to the most recent national estimate there are 600-1000 chimpanzees in Guinea-Bissau with approximately 400 individuals occurring in CNP [Gippoliti & Dell’Omo, 2003; Sousa et al., 2005]. More recently, Torres et al. [2011] have demonstrated that suitable forest habitat for chimpanzees in the park declined by approximately 11% (270 km²) from 1986 to 2003, resulted in a decrease of chimpanzee population. However, the exact number of chimpanzee communities remains unclear.

Within CNP, chimpanzees live in three principal areas (*i.e.* North, Central and South) (Table 1, Fig. 2). Recent phylogeographic studies have shown that there are three chimpanzee genetic subpopulations in CNP corresponding to the areas. The three subpopulations are

significantly genetically different from each other. It was shown that 39.18% of the genetic variation occurs between subpopulations and the largest fraction of the variation (56.24%) is partitioned among individuals within populations [R. Sá, unpubl. data].

The Northern area has more available forested habitat (3.55%) than in the Central (1.24%) or South (1.84%) and can be perceived as a more continuous forest block. The central area is under a high anthropogenic pressure where chimpanzees are encroached at a very high density of 3 individuals/ km² [Hockings & Sousa, 2011]. The southern and central areas possess the higher number of forest fragments in comparison to the northern one (Table 5.1) and with continuing habitat degradation (Fig. 5.2).

5.3.3- Sampling Strategy

The current field study was conducted from September to November 2008, during the rainy season (mean annual rainfall ca. 2000 mm). Fresh faecal samples were collected opportunistically during the day, near the nests or on the trails in all three areas (North, Central and South). In total, 14 fragmented forest patches were surveyed from north, central and south CNP areas (Table 5.1). The samples could not be attributed to specific individuals and to avoid sampling the same individual only one sample was collected from the same nest. On the following days we moved to a different forest fragment where the connectivity with the previous nest site was limited or absent. However, we cannot exclude the possibility that the same individual was sampled more than once. Only fresh samples were collected (from their inner part to avoid soil contamination), any potentially rain- “washed” or old (>12h) samples were discarded. Approximately 5g of each sample was immediately fixed in a 13 ml single vial tube of PROTOfix™ CLR (Alpha-tech-Systems, Inc., Vancouver, WA) according with the manufacturer’s instructions [Jensen et al., 2000] and stored at room temperature until they were shipped under international permits to the Department of Pathology and Parasitology of the Veterinary and Pharmaceutical University, Brno, Czech Republic.

All samples were collected non-invasively in conformity with the ASP-Principles for the Ethical Treatment of non-human Primates.

5.3.4- Parasitological Screening

About half of each sample was stored in the original vial for possible further repetitions. The remainder was homogenized with water and filtered through a standard sieve (~100µm) for macroscopic examination. This filtered material was processed using combined a modified Sheather's solution flotation (s.g. 1.33) [Sheater, 1923] and a Merthiolate-Iodine-Formaldehyde Concentration (MIFC) sedimentation technique [Blagg et al., 1955]. A drop of Lugol's iodine was added to stain the preparations. Several drops were examined for each technique (Sheather's flotation and MIFC) from each sample in a compound microscope at 200x, 400x and 1000x magnification. Symbionts were identified on the basis of the morphology (*i.e.* size, wall structure, internal structures and shape), of their stages (*e.g.* eggs, cysts, trophozoites or larvae) using identification keys [Jessee et al., 1970; Ash & Orihel, 2007; Hasegawa et al., 2009]. Measurements were made to the nearest 0.1µm, using a calibrated ocular micrometer. Symbionts were micro-photographed using Nomarski interference contrast on an Olympus AX70.

5.3.5- Data analyses

The terminology were defined according to Stuart and Strier [1995], Bush et al., [1997] and Gillespie [2006]; therefore prevalence refers to the percentage of samples with a given symbiont taxa and sample richness is the number of unique symbiont taxa recovered from a sample. Chi square or Fisher's exact tests with subsequent Bonferroni correction were performed in order to compare sample prevalence in chimpanzees inhabiting North, Central and South CNP areas for each symbiont taxa separately. Differences in symbiont richness among areas were investigated using Kruskal-Wallis ANOVA, with LSD *post-hoc* tests used for pairwise comparisons. The value of $p < 0.05$ was

set as significant. SPSS, version 17 [SPSS Inc., Chicago, IL] and SISA [<http://www.quantitativeskills.com/sisa/>] were used for all statistical analyses.

5.4- RESULTS

A total of 13 symbiont taxa were identified including three symbionts specific for chimpanzees [entodinomorphid ciliates *Troglodytella abressarti* and *Troglocorys cava* and nematode *Probstmayria gombensis*]. Further we found ten symbionts with wider range of hosts and with more or less prominent zoonotic potential [*Blastocystis* spp., amoebas *Entamoeba* spp. and *Iodamoeba bueschtshlii*, flagellates *Giardia intestinalis* and *Chilomastix mesnili*; anoplocephalid tapeworm *Bertiella* sp., and several nematodes: unidentified strongylids, *Strongyloides stercoralis*, *Strongyloides fuelleborni* and *Trichuris* sp.] (Table 5.2). No flukes were observed. Neither tapeworm proglottids nor nematodes were macroscopically observed in fecal samples.

The median sample richness was 3 (range= 0-7, mean $\pm$ SD= 2.6 $\pm$ 1.6). Differences in prevalence among subpopulations were only detected for *G. intestinalis* (North, n=0; Central, n=4; South, n=2; Fisher exact test, $P= 0.012$), *Trichuris* sp. (North, n=13, Central, n=0, South, n= 2; Fisher exact test, $P= 0.001$) and *Bertiella* sp. (North, n= 0, Central, n= 1, South, n= 4; Fisher exact test, $P= 0.025$) (Table 5.2). There was a significant difference in sample richness among the three subpopulations (Kruskal-Wallis ANOVA, $H=6.636$, $P=0.036$) with LSD *post-hoc* tests revealing that the north community has significantly lower sample richness than the central community ($P=0.013$) but the differences were not

significant between north and south ($P=0.131$) and central and south ($P=0.301$) communities.

5.5- DISCUSSION

To the best of our knowledge, this is the first study assessing the gastrointestinal symbionts of chimpanzees in Guinea-Bissau. Based on our results we can conclude that the symbiont fauna of chimpanzees inhabiting CNP is similar to that of other wild chimpanzee populations living in primary and secondary forest habitats, *e.g.*: Lopé, Gabon [Landsoud-Soukate et al., 1995]; Dzanga-Ndoki, Central African Republic [Lilly et al., 2002]; Mahale, Uganda [Huffman et al., 1997; Huffman et al., 2009]; Gombe, Tanzania [File et al., 1976; Murray et al., 2000; Bakuza & Nkwengulila, 2009; Gillespie et al., 2010]; Kibale, Tanzania [Ashford et al., 2000; Krief et al., 2003]. We detected significant changes in symbiont fauna due to anthropogenic fragmentation. We demonstrate that fragmentation can have different impact on individual symbiont taxa and that both host ecology and symbiont characteristics must be taken in consideration to interpret the results.

Entodiniomorphid ciliate *T. abrasarti* was the most common symbiont species reaching the prevalence of 62 %; the other detected ciliate was *T. cava* found in 24.5% of samples. Both ciliates are currently considered chimpanzee specific commensals [Pomajbíková et al., 2010; Tokiwa et al., 2010; Profousová et al., 2011; Pomajbíková et al., 2012]. It was shown that at least *T. abrasarti* can actively participate in ape hindgut

digestion, however its contribution to overall hydrolytic activities is low [Profousová et al., 2011]. Although reported prevalence of both ciliates greatly varies among the studied populations, they are probably present in all individuals in wild chimpanzee populations [Pomajbíková et al., 2010; Pomajbíková et al., 2012].

Group of detected commensals further includes amoebas *Entamoeba* spp. and *I. buetschlii* and flagellate *C. mesnili*. All three species were determined based on the cyst morphology, which is consistent with those described in humans [Ash & Orihel, 2007]; however, molecular-phylogenetic analyses are necessary for precise identification and clarification of possible transmissions between humans and primates [Levecke, 2010]. Observed prevalence of both amoebas is similar to that reported e.g. in Gombe chimpanzees in Tanzania [Murray et al., 2000], but published data tend to vary significantly across study sites [Krief et al., 2005; Muehlebein, 2005]. Prevalence of *C. mesnili* (7%) is comparable to that reported by Landsoud-Soukate et al. [1995] in chimpanzee population inhabiting Lope, Gabon.

The *Giardia* cysts were recovered only from samples originating from central and southern chimpanzee subpopulations inhabiting more fragmented habitats. This finding is in accordance with previous studies relating *Giardia* infections in wild primates to increased contact with humans and livestock [Wolfe et al., 1998; Nizeyi et al., 1999; Graczyk et al., 2002; Salzer et al., 2007; Johnston et al., 2010; Kowalewski et al., 2010; Lane et al., 2011]. *G. intestinalis* is typical of cross-species transmissions, including zoonotic transmissions and determination of particular assemblages by molecular

techniques clarified this aspect of *G. intestinalis* ecology [Thompson, 2004; Hunter & Thompson, 2005; Sprong et al., 2009; Johnston et al., 2010]. Undoubtedly, chimpanzees living in fragmented CNP areas are exposed to high contact with local inhabitants and livestock. Humans create trails and enter frequently in the forest fragments as well as other livestock like pigs, goats and cows. Chimpanzees also leave the fragments to crop raid in villages. Thus, humans and livestock share water sources with chimpanzees, shedding the *Giardia* cysts into water and increasing the chance for chimpanzees to get infected. Future studies should focus on determination of *Giardia* assemblages using molecular tools in order to detect the exact reservoir of infection for chimpanzees, and to determine if cross-species transmission is occurring.

Cysts of *Blastocystis* spp. were detected in 49% of samples; such high prevalence was recorded only in introduced chimpanzees of Rubondo Island, Tanzania [Petrášová et al., 2010]. However, cysts of *Blastocystis* can be easily overlooked due to their small size and we assume that this protist is rather common in also in populations of wild great apes as reported in captive ones [Stensvold et al., 2009].

Both the strongylids and *Strongyloides* infections are common in free-ranging great apes with strongylids being reported as the most prevalent parasites in several populations of wild chimpanzees reaching prevalence between 70 – 100% [e.g. Gombe, Tanzania: File et al., 1976; Mahale, Tanzania: Huffman et al., 1997; Kibale, Uganda: Muehlenbein, 2005; Gillespie et al., 2010]. Therefore the prevalence of strongylid nematodes in CNP chimpanzees (38%) is lower in comparison with most studied sites,

with exception of Lope Reserve in Gabon, where the prevalence reached only 21% [Landsoud-Soukate et al., 1995]. Absence or very low prevalence of strongylid nematodes was recorded also in savanna chimpanzees [McGrew et al., 1989; Howells *et al.*, 2010] and introduced chimpanzees of Rubondo Island, Tanzania [Petrželková *et al.*, 2010; Petrašová et al., 2010]. No impact of fragmentation/increased human contact on these nematodes was recorded in CNP chimpanzees. Without developing larvae using coprocultures, strongylid eggs cannot be reliably determined even to genera [Greiner & McIntosh, 2009].

Both *Strongyloides fuelleborni* and *S. stercoralis* were found in CNP chimpanzees. Despite *S. fuelleborni* is known to predominantly infect chimpanzees whilst *S. stercoralis* is common in humans, mixed infections have been reported in both humans and chimpanzees [Hasegawa, 2010; Petrželková et al., 2010]. Future studies are warranted to clarify zoonotic potential of strongylids and *Strongyloides* occurring in chimpanzees and humans within CNP.

Probstmayria gombensis, enigmatic non-pathogenic nematode originally described from chimpanzee [File, 1976] was easily recognized by its characteristic L3 larvae. It has been found in several, but not all chimpanzee populations in similar or lower prevalence in comparison to CNP chimpanzees [*e.g.* Gombe NP, Tanzania: McGrew et al., 1989; Kibale NP, Uganda: Ashford et al., 2000; Muehlenbein, 2005].

Bertiella sp. (usually tentatively referred to as *B. studeri*) is so far the only tapeworm reported to occur in free-ranging chimpanzees, usually at low prevalence comparable to our study [Krief *et al.*, 2005; Howells et al., 2010] or even lower [Kawabata

& Nishida, 1991; Ashford et al., 2000]. Transmission occurs when an intermediate host, usually an oribatid mite, containing the cystercercoid is accidentally ingested by the primate. Surprisingly, prevalence of *Bertiella* sp. was higher in fragmented habitats. We speculate that fragmentation might have an effect on the intermediate hosts, oribatid mites; however, very little is known about life cycle and factors which affect this cestode and further research is warranted.

Probably the most striking difference in comparison to other free-ranging chimpanzee populations is a relatively high prevalence of *Trichuris* spp. (15%) in CNP chimpanzees with most cases recorded in the northern community. Whipworms are regularly found in many primate species, including humans [McGrew et al., 1989; Ashford et al., 2000; Murray et al., 2000; Mbona & Munene, 2006]. Most of the studies reported absence or low prevalence of *Trichuris* (less than 10%) in wild chimpanzees [e.g. Krief et al., 2005; Gillespie et al., 2010] with exception of the chimpanzee community in Mahale, Tanzania where prevalence of *Trichuris* sp. reached up to 46% in certain periods [Huffman et al., 2009]. Whipworms found in non-human primates are usually considered as *Trichuris trichiura* [Ooi et al., 1993; Reichard et al., 2008; Liu et al., 2012] and several authors implied the zoonotic cross-transmission between non-human primates and humans [e.g. Munene et al., 1998; Chapman et al., 2007]. However, direct evidence for transmission between wild great apes and humans is lacking and unusually broad host range of *T. trichiura* rather implies underestimated diversity of *Trichuris* in non-human primates. Also morphometric variation observed in eggs suggests that species found in chimpanzees is

probably divergent from human *T. trichura*; obviously, only molecular assessments can shed light on this uncertainty [Petrášová et al., 2010]. Significantly higher prevalence of *Trichuris* sp. in northern community in comparison to very low prevalence/total absence of *Trichuris* sp. in the central communities living in more fragmented habitats seems puzzling. In contrary to previous studies, habitat fragmentation does not result in higher prevalence of this nematode in CNP chimpanzees as it was shown *e.g.* in red colobus (*Procolobus rufomitratus* ssp. *tephrosceles*) and black-and-white colobus (*Colobus guereza*) in Kibale, Uganda [Gillespie & Chapman, 2006; Gillespie & Chapman, 2008]. High prevalence of *Trichuris* sp. is supposed to be associated with increased host density in the fragments [Gillespie & Chapman, 2006; Gillespie & Chapman, 2008]. To our knowledge there are no data on the effect of fragmentation on chimpanzee behavior and ecology. Based on personal observations [R. Sá, per. obs.] it seems that chimpanzees in fragmented areas tend to increase their travel distances by moving among the fragments probably in order to search for food. Thus the host density and range use intensity decreases leading to lower prevalence of *Trichuris* in fragmented biotopes in comparison to northern area with continuous forest. Additionally, we assume that crop raiding performed only by chimpanzees from fragmented habitats [R. Sá, pers. obs.] could improve their nutritional situation facilitating more effective immune response to parasites [Holmes, 1995] as suggested by Chapman et al. [2006], who reported less severe parasite infections in crop raiding black and white colobus in comparison to red colobus which did not crop raid. Similar situation was observed also in baboons [Eley et al., 1989; Hahn et al., 2003]

The symbiont richness observed in this study shows a classic host-symbiont over-dispersed distribution when most samples harbor few symbiont species while few others contain the largest proportion of multi-symbiont infections [Anderson and May, 1978; Shaw and Dobson, 1995; Morand & Poulin, 1998; Shaw et al. 1998]. The symbiont richness pattern is an important variable to understand the population dynamics of the host-symbiont relationship; it is assumed that the over dispersal pattern acts to increase density-dependent regulation of abundance in both hosts and symbionts, and to reduce the level of interspecific competition among symbionts [Tompkins et al., 2011]. Moreover, even individually benign infections can have a cumulative pathogenic effect [McCallum, 1994; McCallum & Dobson, 1995]. Compared to 7.83 symbiont species per individual in Gombe [Gillespie et al., 2010], the CNP chimpanzees have a reduced incidence of co-infections, however the data are in agreement with the ranges reported for chimpanzees at Rubondo Island, Tanzania [Petrášová et al., 2010] and in Kibale NP, Uganda [Muehlenbein, 2005], giving the average richness of 2.0 and 4.75 symbiotic species per individual, respectively.

Symbiont richness was higher in chimpanzee subpopulations living in fragmented forests compared to the north community, which live in a more continuous forest area; however pairwise comparisons revealed that only central community had significantly higher symbiont richness in comparison to northern one. Epidemiological theory predicts that symbiont species richness should positively correlate with host population density [*e.g.* Nunn et al., 2003] expecting higher host densities in fragmented biotopes. Gillespie

and Chapman [2008] recorded that the number of symbiont species infecting red colobus (*Procolobus rufomitratus*) in Kibale, Uganda was greater in forest fragments compared to unfragmented forest at higher densities in fragmented forests demonstrating a greater infection risk for those populations. Similarly, increased host density due to forest fragmentation and loss mediates increases in symbiont richness in Tana River red colobus (*Procolobus rufomitratus*) and mangabeys (*Cercocebus galerritus*) [Mbora et al., 2009]. The observed symbiont richness of this study is in agreement with the epidemiological theory suggesting that the forest fragmentation in the Central area is indicative of human disturbance, which is promoting a higher co-infection of symbionts in that chimpanzee subpopulation.

Oates [2006] argues that marginal populations are more prone to habitat destruction and vulnerable to demographic changes. Associated with these anthropogenic alterations, disease derived by parasite disturbance can enhance the extinction risk [Pedersen et al., 2007]. Under this complex scenario gastrointestinal symbiotic surveillance cannot be neglected for conservation purposes [Whiteman & Parker, 2005]. Currently, there are no apparent parasite illness problems on CNP chimpanzees, nonetheless, further monitoring and epidemiological surveys not only on chimpanzees but also, on other primate sympatric species in the area are necessary. The results of this study should be considered in the conservation and management of this endangered ape especially when there are ecotourist development plans for the park [IBAP, 2010; AD, 2010; Hockings et al., 2011].

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5.8- TABLES

TABLE 5.1: Characteristics of chimpanzee communities sampled in Cantanhez National Park, in Guinea-Bissau, West Africa.

Areas	Nº of Fragments	Size of Forested Area (km ²)	% of Forest cover	Distance to nearest village (m)	Nº samples collected
North	1	37.93	3.55	3,465	45
Central	5	13.20	1.24	67	25
South	8	19.67	1.84	650	32

TABLE 5.2: Symbiont prevalence (%) of western chimpanzees (*Pan troglodytes verus*) at Cantanhez National Park, Republic of Guinea-Bissau.

Symbiont Taxa	North (n=45)	Central (n=25)	South (n=32)	Total (n=102)
Protozoa				
<i>Entamoeba</i> spp.	2	20	34	18
<i>Iodamoeba buetschlii</i>	4	4	3	4
<i>Giardia duodenalis</i>	0	16	6	6
<i>Chilomastix mesnili</i>	4	16	3	7
<i>Troglodytella abrassarti</i>	42	80	69	62
<i>Troglocorys cava</i>	22	36	19	25
<i>Blastocystis</i> sp.	29	64	56	49
Cestoda				
<i>Bertiella</i> sp.	0	4	13	5
Nematoda				
Strongylida fam. gen. sp. (Hookworms)	27	48	16	38
<i>Strongyloides fuelleborni</i>	27	16	16	22
<i>Strongyloides stercoralis</i>	4	8	6	6
<i>Probstmayria gombensis</i>	7	4	0	4
<i>Trichuris</i> sp.	22	0	6	15
Overall %	87	100	91	91

5.9- FIGURES

Figure 5.1: Location of Cantanhez National Park, Republic of Guinea-Bissau, West Africa (after Hockings and Sousa 2011).

Figure 5.2: Chimpanzee distribution in three principal areas across Cantanhez National Park, Guinea-Bissau.

CHAPTER 6

“The other’s pain: the pain that hurts less.”

— Susan Sontag, Essayist

CHAPTER 6

GENERAL DISCUSSION

In this thesis I investigated concomitant threats that negatively impact on chimpanzee persistence in Guinea-Bissau. I also tested a set of hypotheses regarding their evolutionary history and examined the occurrence of gastrointestinal symbionts and how the observed parasitism patterns are influenced by habitat fragmentation. To achieve these goals I adopted a multidisciplinary approach that combined ethnography, non-invasive genetics and parasitology. Prior to this study, no research projects had assessed the Guinea-Bissau's chimpanzee mitochondrial diversity, structure and demographic history, and their gastrointestinal parasites were entirely unstudied. Overall, this set of studies has contributed to our understanding of the evolution and ecology of chimpanzees, specifically addressing a gap in knowledge regarding these apes in Guinea-Bissau. Moreover, this dissertation provides relevant considerations for the conservation of chimpanzees in Cantanhez, Quínara and Gabú regions.

6.1- Overview of major findings, limitations, and further research

Chapter 3, reports and examines one aspect of the illegal trade of chimpanzee and other non-human primate body parts in Guinea-Bissau. Such commerce can pose serious threats to wild populations. In this chapter I was interested to understand and place into context the social inclusion of chimpanzee and other animal body parts for human traditional practices. Morphologically identified chimpanzee skins were found in the market. Additionally, dried skins from Temminck's Red Colobus monkeys (*Procolobus badius temminckii*), Guinea baboons (*Papio papio*) and Olive baboons (*Papio anubis*) were identified, as well as from other mammals such as leopard (*Panthera pardus*) and lions (*Panthera leo*) among others. All the species are reported as occurring in Guinea-Bissau except for Olive baboons whose western limit of distribution is reported to be in Mali and the

republic of Guinea (IUCN 2010). Furthermore, in Colobane and Boucotte markets in Senegal (Dakar and Ziguinchor, respectively) several species of reptiles and mammals, including primate species were also found for use in traditional medicinal and/or magical ceremonies. Two of the Guinea-Bissau vendors mentioned that citizens from Senegal, Guinea and Gambia are involved in the trade. All vendors mentioned that the animal body parts come from the “south of the country”, particularly the regions of Cantanhez and Gabú. Most male informants in rural CNP and Boé villages associated the use of chimpanzee derived products with the female domain, and one informant admitted that he uses a stitched amulet made of chimpanzee body parts to help provide awareness and protect him. Although, not previously reported for Guinea-Bissau, the use of non-human primate body parts in traditional medicine is not unusual in Africa (Alves et al. 2010; Leypey and Fomine 2010). The suggested transnational trade of 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. In addition, ethnographic observations and informal conversations with Guinea-Bissau immigrants in diaspora in Lisbon informed that usually the “esoteric professionals” import frequently animal body parts (including those from endangered species and CITES regulated) from Guinea-Bissau for their use in their customers that can buy those products paying high prices (personal information). If on one hand ethnic ecological knowledge can be a driver of protection for some species, some traditional practices can have negative impact for other animal populations (Colding and Folk 1997).

This study was limited to the capital, Bissau; however further studies in different regions of the country are necessary. It is urgent to identify and analyse the practices and social mechanisms of traditional societies, such as taboos, involving non-human primates throughout the country. An ethnobiological approach, more ethnoprimatological focused can be particularly appropriate as this field (i.e. the study of human and non-human primate interactions) aims to understand the incorporation of non-human primates as pets, food, indigenous knowledge of non-human primate behaviour, etc. (Wolfe and Fuentes 2007; Fuentes and Hockings

2010). Further work must molecularly determine the origin of the skins and bushmeat in the markets in order to assess the frequency of the species traded (see Appendix I) and assign their geographical origin. Finally, cultural anthropological studies specifically centred on the use and symbolic incorporation and appropriation of non-human primate body parts by the Guinea-Bissau diaspora in Portugal will help to understand how such practices are disseminated.

In **Chapter 4**, I investigated for the first time the mitochondrial diversity, genetic structure and demographic history of the chimpanzees in Guinea-Bissau and Nimba Mountains, Guinea, thus revisiting the West African chimpanzee's phylogeography. Fifty-one haplotypes were detected and the genetic diversity was high. The phylogenetic analyses revealed the existence of two deep evolutionary lineages in consonance with previous reports (Morin et al. 1994, Gonder et al. 2006); and eleven haplogroups, some of them geographically associated. The bimodal mismatch distribution, the neutrality tests and the star shape pattern in the median joining network imply that a demographic expansion has occurred promoting secondary contact between the two lineages. The AMOVA revealed 74% of the genetic variation is partitioned within populations and a strong genetic structure ($F_{ST}=0.25956$, $p<0.0001$). Barrier analysis identified three major genetic discontinuities, two of them coinciding with the Rio Grande de Buba and the Féfine rivers and the estimates of divergence times pointed to vicariance events in the Middle Pleistocene where the recurrence of past climatic oscillations in the African forests, with cycles of forest expansion and contraction may have resulted in the genetic differentiation of the groups. Two phylogeographic clusters were identified based on the SAMOVA analyses and following the criteria pointed out by Petit et al. (1998) conservation units were identified in order to maintain the chimpanzee genetic diversity in Guinea-Bissau.

The dissimilar shapes of the mismatch distributions observed in this study and those from Nigeria-Cameroon (Gonder et al. 2006) suggest that these populations have experienced separate paleodemographic events and probably persisted in different refuges during the last glacial maximum. Gagneux et al. (1999) propose that the Upper Guinea chimpanzees may have persisted in a refuge around Liberia following the initial expansion of this subspecies into the forests of West

Africa given that similar HVR1 sequences were detected in populations of Guinea-Bissau and Nimba spanning 800 km. The population for the Upper Guinean chimpanzees may have occurred after the last glaciation or perhaps after an earlier glacial event. The mismatch patterns of this study clearly contrast with the unimodal mismatch distribution for the eastern chimpanzees suggesting that chimpanzees in East Africa have not undergone Pleistocene semi-isolated range of contractions and expansions and that these chimpanzees persisted during the glaciation periods by exploring more savanna like habitats (Goldberg and Ruvolo 1997).

Due to logistical constraints, during the sampling phase it was not possible to include specimens from the Dulombi and Cacine areas in Guinea-Bissau. Likewise, it would be important to include samples from Senegal, especially from the Niokolo-Koba National Park where chimpanzees populations also occur, which are almost adjacent with the Gabú region in order to expand the phylogeographical results into a broader context. Other molecular markers should also be used in order to confirm the mtDNA patterns. By using nuclear markers, such as microsatellites it will be possible to assess the effects of habitat fragmentation to chimpanzee gene flow in Guinea-Bissau where the connectivity of some forests are clearly compromised. This study is being conducted by me and still ongoing. It will use a landscape genetics approach applied to chimpanzee conservation. Recent studies in landscape genetics demonstrate that the characterization of the geographical structure of genetic variation needs no prior knowledge about the number of populations or the possible landscape effects (Manel et al. 2003; Holderegger and Wagner 2006). Several statistical models have been used on other species (e.g. Bayesian clustering, Monmonier's algorithm) indicating that habitat and landscape traits can determine and predict geographical patterns related with limited gene flow, beyond the traditional models of isolation-by-distance (i.e. Mantel tests) (Manel et al. 2003; Holderegger and Wagner 2006; Hobbs et al. 2006; Manel and Segelbacher 2009). Results can be combined with GIS data and integrated maps can establish genetic patterns connected with the landscape).

Precise population size estimates are difficult to calculate (Arandjelovic et al 2010), nevertheless a molecular chimpanzee censusing is being carried out in Guinea-Bissau. This research is being undertaken under the scope of the project:

“Guinea-Bissau endangered chimpanzee conservation genetics: biological anthropology contribution to biodiversity conservation” funded by FCT (PTDC/CS-ANT/099184/2008).

Finally the genetic findings of the different projects should be combined in order to conduct a PHVA (Population and Habitat Viability Assessment) to set up priorities, and to drive a positive conservation change in Guinea-Bissau (Carlsen et al. 2012). Such knowledge will allow the establishment of ecological corridors that will benefit not only the chimpanzees, but also the ecosystems and the Guinea-Bissau rich biodiversity.

Habitat fragmentation may cause changes in host-parasite dynamics resulting in increased susceptibility to infection, changes in host specificity and parasite virulence (Gillespie et al 2005; Pedersen et al. 2007). In **Chapter 5**, I report for the first time the symbiont-fauna of chimpanzees in Cantanhez National Park in areas with different levels of habitat fragmentation. Moreover, this is the first study to specifically address the effects of habitat fragmentation and gastrointestinal symbionts in chimpanzees, although previous studies have reported their prevalence and richness, none so far had specifically explored this association. Thirteen symbiotic genera (*Troglodytella abressarti*, *Troglocorys cava*, *Blastocystis* spp., *Entamoeba coli*, *Iodamoeba butschlii*, *Giardia intestinalis*, *Chilomastix mesnili*, *Bertiella* sp., *Probstmayria gombensis*, unidentified strongylids, *Strongyloides stercoralis*, *Strongyloides fuelleborni* and *Trichuris* sp.) were identified. The symbiotic fauna of the CNP chimpanzees is comparable to that reported for other wild chimpanzee populations, although CNP chimpanzees have a higher prevalence of *Trichuris* sp. Symbiont richness was higher in chimpanzee subpopulations living in fragmented forests compared to the community inhabiting continuous forest area. I reported significantly higher prevalence of *G. intestinalis* in chimpanzees from fragmented areas, which could be attributed to increased contact with humans and livestock confirming the hypothesis that CNP chimpanzees will exhibit a high level of gastrointestinal symbiont diversity consequence of a disturbed habitat, and a higher infection of parasites with direct cycles as contact intra and interspecific increases. Surprisingly, in fragmented areas chimpanzee density and range-use intensity decrease, which might contribute to low prevalence/total absence of *Trichuris* sp. in

samples from chimpanzees in these areas when compared with those inhabiting continuous forest.

One limitation of this study is the geographical limitation to Cantanhez. Further studies should include a national survey across the different regions during both dry and rainy seasons in order to evaluate fluctuations in chimpanzee gastrointestinal parasite prevalence. Furthermore, studies involving other primates (humans inclusive) must be included to understand the possible risks of zoonotic transmission. The comparative parasitological study of five sympatric primates is ongoing and the prevalence tables are included in Appendixes A-E. Molecular screening should be applied to detect water-borne (i.e. Microsporidia phylum. or *Cryptosporidium* spp.) or vector-borne agents (e.g. *Plasmodium* spp.) difficult to detect by regular coproscopy techniques and usually associated with immunocompromised individuals (Hunter and Nichols 2002). Preliminary molecular trials revealed the presence for *Encephalitozoon* (putatively *cuniculi*) and *Plasmodium* spp. (data not shown). Similarly, other agents such as virus (i.e. SIV) and bacteria should also be considered in further health surveys.

Special attention should be given to the whipworm *Trichuris* spp. One third of the world's human population is at risk of infection from soil-transmitted geohelminths principal among them whipworms from this genus (Starr and Montgomery 2011). Presently, trichuriasis is listed in the Global Network for Neglected Tropical Diseases (GNNTD: <http://globalnetwork.org>), yet few control programs have been consigned (Engels and Saviolli 2006; Hotez et al. 2007, 2009).

Aside from *T. trichiura*, *T. lemuris* and *T. cynocephalus* are the only *Trichuris* taxa described from primates (Kuntz and Meyers 1967). Notably, the low *Trichuris* spp. diversity observed in primates is inconsistent with the number of species described from other mammalian hosts. The vast majority of studies describe whipworm eggs as *T. trichiura*, as they are morphologically indistinguishable from *T. suis* (Ooi et al. 1993). Most likely, *Trichuris* spp. diversity and host specificity in nonhuman primates is underestimated. The taxonomic discrimination of species within the genus *Trichuris* remains controversial (Cutillas et al. 2009). Early studies based on the morphological species concept considered *T. suis* and *T. trichiura* as belonging to the same species (Soulsby 1982). More recently, phylogenetic studies

proved that *T. suis* and *T. trichiura* are genetically distinct, arguing that only molecular approaches can shed light and discriminate between these closely related congeners. In contrast, morphometric distinctiveness should be interpreted with caution as the exact species determination of *Trichuris* spp. based solely on the egg and larvae morphological features is unreliable (Huffman and Chapman 2009). Nevertheless, several authors automatically imply the zoonotic cross-transmission between humans and nonhuman primates (Chapman et al. 2006). Further studies are necessary to clarify the host specificity of these parasites in primates studies conducted in Guinea-Bissau will certainly shed light on this uncertainty.

Finally, attention is warranted to chimpanzee specific ciliates (i.e. *Troglodytella abrassarti* and *Troglocorys cava*) that occur at high prevalence. Usually these symbionts are considered as commensals, but little is known about their biology, transmission or function. *Troglocorys cava* for example was only fully described in 2010 and the distribution of this entodinomorphid ciliate was recently clarified (see Appendix M).

6.2 Reflection about the future of Guinea-Bissau forests

According to a recent estimate, West Africa experienced an annual rate of forest loss of 1.17% between 1990 and 2005 (Fao 2007). The same trend was observed in Guinea-Bissau where its open and closed-forest decreased by the same rate while savannah-woodland and mangroves increased 0.76% and 0.83% respectively, between 1990 and 2007 (Oom et al. 2009) (Fig. 6.1). This deforestation has been caused by 'slash and burn' agriculture and the amount of land for crops is continually expanding due to expanding human populations, particularly in Cantanhez (Hockings and Sousa 2011). Few trees appeared to be cut following the national legislation, and loggers seem to respect more the symbolic or traditional taboos instead. The numbers of licensed pitsawyers remain unknown as well as the timber permits conceded by the Forestry Department. Permits for clearing land are sometimes only obtained near traditional chiefs and this deregulation could generate conflicts between the guards in the parks, and loggers (pers. observation).

In the two years of my fieldwork study the forests of Canamina, Cafatche, Amidara in Cantanhez has been severely damaged. Similarly the forested area of the Cufada Lagoons Natural Park in the Quínara region were reduced by half during 2009-2010 (Cristina Schwarz pers. communication) and the bushfires for agroforestry in the Boé sector have increased dramatically (Joost van Schinjdell pers. communication). Moreover, the timber trade seems to be highly organized and due to local poverty few local forest owners are able to easily sell their land tenure. This scenario of forest depletion will have serious consequences for chimpanzees in the future.

Figure 6.1: Forest change in Guinea-Bissau between 1990 and 2007 (after Oom et al. 2009, CARBOVEG-GB PROJECT:364).

6.3 Conservation recommendations

The recommendations proposed by Casanova and Sousa (2007) should be addressed seriously by the Guinea-Bissau national agencies and local NGO's. 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 (Anon., 2011). However, limited resources and lack of awareness of management authorities and politicians is hindering law enforcement in the country. At the international level, conservation agencies should re-examine their strategies to mitigate the illegal chimpanzee body parts 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. The political instability in the country has neglecting CITES, that was never ratified, thus the government and political authorities need to rethink their position.

The chimpanzee pet trade within the country is frequent and usually the animals live in miserable conditions. The local authorities should not facilitate this kind of practice by confiscating the animals and releasing them into sanctuaries or rescue centers (Casanova and Sousa 2007). Although this kind of facilities do not exist in the country, with international help it will be possible to secure them abroad.

The creation of the national parks by IBAP was a good conservation strategy, as in principle they can protect the genetic diversity of the chimpanzee populations, however, in reality, the Parks are lacking, infrastructure, monitoring and seizure resources. The guards should be empowered with field equipment and adequate material, and they should receive, in a regular basis, adequate payments for their services either from the official agencies and local NGO's, or from research members as well as adequate training.

Based on the genetic approach two chimpanzee phylogeographical clusters were identified: one south of Cantanhez, and a second encompassing the remaining regions. The first group (constituted by Farim, Catomboi, Cafatche and Canghode) in South Cantanhez is in eminent risk of becoming extinct, as they are under intense anthropogenic pressure. Significant conservation efforts should be directed towards them. In the other group, three major priority areas should be taking into consideration: the northern area of Cantanhez constituted by: Cadique, Lautchande, Amidara and Mejo and the communities of Buba Intchingue and Injassane in Cufada (i.e. Quínara region); the area of Catió-Empada area where they will become isolated

if the corridor of Nhala disappear and the Boé region: especially the populations of Béli, Aicum, Vendu Leidi and Dinguirai.

Chimpanzee gastrointestinal surveys should be implemented on a regular basis, every two years in order to monitor their health status, pathogen status, and epidemiological surveillance. Baseline information was established with this study. However, it is necessary that further studies proceed the monitoring, as infectious diseases in wild great apes are one of the major threats to their survival. Research of chimpanzee infections is of importance for the understanding of the role and circulation of neglected and emergent infectious diseases, not only for the species, but also for other species, including humans.

Finally, local people must be empowered economically with sustainable activities (i.e. local handicraft, regional products: honey, jams, beverages, etc.) to sell at a national and eventually at an international level, where the producers should be paid adequately in a fair trade basis. Effective conservation projects must be implemented and developed with the full engagement of local and national stakeholders (Ancrenaz et al. 2007). Chimpanzees have great potential value for attracting ecotourism and this opportunity can be explored across Guinea-Bissau.

Evidence suggests that the western lesser spot-nosed guenons (*Cercopithecus petaurista buettikoferi*) seem to be already extinct in the continental part of Guinea-Bissau, only occurring in the Bijago Archipelago (see Appendix J), and if nothing is done chimpanzees might follow the same tendency.

6.4- Conclusion: Why should we conserve chimpanzees in Guinea-Bissau?

Chimpanzee numbers across Africa are fast declining and their conservation must be a bioethical imperative (Caldecott and Miles 2005; Oates et al. 2011). Each population is indispensable. Each chimpanzee community is unique in their ecological, behavioural and cultural characteristics (Whiten et al. 2001) and each study site contributes with important information for the understanding of their biology. The chimpanzees of Guinea-Bissau are located in the most western limit of the species distribution. They have a strong preference for constructing their nests in

palm trees (Sousa et al. 2011), and the genetic approach of this dissertation revealed important insights about gene flow patterns, genetic diversity and structure of this population. It also demonstrated that when compared to other study areas CNP chimpanzees have a higher prevalence of whipworms. Such information is crucial not only from a scientific point of view, but also for a future conservation framework. The establishment of long-term research sites in Guinea-Bissau will certainly provide more knowledge about this species. However, it is urgent to act now in order to prevent our closest living relatives from going extinct in this country. Pleading for the protection of chimpanzees seems futile considering the absence of compassion that humans show regularly to each others. I feel pessimistic about the future of chimpanzees in West Africa and I wonder how many species one ecosystem must lose before it collapses? How many chimpanzees must be killed before we humans are doomed too? In this sense, I position myself near the discourse of the multispecies anthropology, where multispecies ethnographers are studying contact zones where lines separating nature from culture have been broken down, where encounters between humans and other species generate mutual ecologies and shared niches (Kirksey and Helmreich 2010).

6.5- References

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Appendixes

“In the end we will conserve only what we love. We will love only what we understand. We will understand only what we are taught.”

— Baba Dioum, Senegalese Poet

APPENDIXES

Appendix A: Prevalence table of gastrointestinal symbionts of Chimpanzees in Cantanhez, Cufada, and Boé, Guinea-Bissau (2010)

Parasite	Samples (n =116)	Prevalence
Protozoa		
<i>Entamoeba</i> sp.	19	16.38%
<i>Entamoeba coli</i>	41	35.34%
<i>Iodamoeba buetschlii</i>	22	18.97%
<i>Endolimax nana</i>	4	3.45%
<i>Giardia duodenalis</i>	12	10.34%
<i>Chilomastix mesnili</i>	6	5.17%
<i>Blastocystis</i> sp.	46	39.66%
<i>Troglodytella abrassarti</i>	82	70.69%
<i>Troglocorys cava</i>	13	11.21%
Cestoda		
<i>Bertiella</i> sp.	13	11.21%
Nematoda		
<i>Strongylida</i> fam. gen. sp. (Hookworms)	36	31.03%
<i>Ascaris</i> sp.	2	1.72%
<i>Trichuris</i> sp.	9	7.76%
<i>Probstmayria gombensis</i>	1	0.86%
<i>Strongyloides stercoralis</i>	1	0.86%
<i>Strongyloides fulleborni</i>	4	3.45%
Overall	103	88.79%

**Appendix B: Prevalence table of gastrointestinal symbionts of babboons in
Cantanhez National Park, Guinea-Bissau (2010)**

Parasite	Samples (n =48)	Prevalence
Protozoa		
<i>Entamoeba</i> sp.	39	81.25%
<i>Entamoeba coli</i>	17	35.42%
<i>Iodamoeba buetschlii</i>	17	35.42%
<i>Endolimax nana</i>	3	6.25%
<i>Giardia duodenalis</i>	13	27.08%
<i>Chilomastix mesnili</i>	10	20.83%
<i>Blastocystis</i> sp.	31	64.58%
<i>Balantidium coli</i>	11	22.92%
Nematoda		
<i>Strongylida</i> fam. gen. sp. (Hookworms)	31	64.58%
<i>Strongyloides stercoralis</i>	7	14.58%
<i>Trichuris</i> sp.	37	77.08%
<i>Spirurida</i> fam. gen. sp.	3	6.25%
Overall	48	100%

Appendix C: Prevalence table of gastrointestinal symbionts of Western red colobus in Cantanhez National Park, Guinea-Bissau (2010)

Parasite	Samples (n =30)	Prevalence
Protozoa		
<i>Entamoeba</i> sp.	21	70%
<i>Entamoeba coli</i>	3	10%
<i>Iodamoeba buetschlii</i>	6	20%
<i>Endolimax nana</i>	1	3.33%
<i>Giardia duodenalis</i>	7	23.33%
<i>Chilomastix mesnili</i>	3	10%
<i>Blastocytis</i> sp.	2	6.66%
Cestoda		
<i>Bertiella</i> sp.	1	3.33%
Nematoda		
<i>Strongylida</i> fam. gen. sp. (Hookworms)	9	30%
<i>Trichuris</i> sp.	21	70%
Overall	29	96.6%

Appendix D: Prevalence table of gastrointestinal symbionts of Black and White colobus in Cantanhez National Park, Guinea-Bissau (2010)

Parasite	Samples (n =33)	Prevalence
Protozoa		
<i>Entamoeba</i> sp.	27	81.82%
<i>Entamoeba coli</i>	2	6.06%
<i>Eodamoeba buetschlii</i>	8	24.24%
<i>Endolimax nana</i>	1	3.03%
<i>Giardia duodenalis</i>	5	15.15%
<i>Chilomastix mesnili</i>	4	12.12%
<i>Blastocystis</i> sp.	7	21.21%
Nematoda		
<i>Strongylida</i> fam. gen. sp. (Hookworms)	8	24.24%
<i>Strongyloides stercoralis</i>	8	24.24%
<i>Trichuris</i> sp.	10	30.30%
Overall		90.91%

**Appendix E: Prevalence table of gastrointestinal symbionts of Humans in
Cantanhez National Park, Guinea-Bissau (2010)**

Parasite	Samples (n =72)	Prevalence
Protozoa		
<i>Entamoeba</i> sp.	14	19.44%
<i>Entamoeba coli</i>	58	80.56%
<i>Iodamoeba buetschlii</i>	16	22.22%
<i>Endolimax nana</i>	9	12.5%
<i>Giardia duodenalis</i>	13	18.06%
<i>Chilomastix mesnili</i>	13	18.06%
<i>Blastocystis</i> sp.	49	68.06%
Nematoda		
<i>Strongylida</i> fam. gen. sp. (Hookworms)	39	54.17%
<i>Ascaris lumbricoides</i>	13	19.06%
<i>Trichuris</i> sp.	7	9.72%
<i>Oesophagostomum</i> sp.	3	4.17%
<i>Spirurida</i> fam. gen.sp.	1	1.39%
<i>Enterobius vermicularis</i>	1	1.39%
<i>Strongyloides stercoralis</i>	6	8.33%
<i>Strongyloides fulleborni</i>	6	8.33%
Overall	70	97.22%

Appendix F: List of Guinea-Bissau chimpanzees HVR1 haplotypes, to be deposited in GenBank

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Appendix G: Chimpanzee symbiont identification microphotographs

Trophozoite of
Troglodytella abrassarti,
fecal sample *P. troglodytes*
verus, Cantanhez, Guinea-
Bissau.

Trophozoite of *Troglodytella*
cava, fecal sample *P.*
troglodytes verus,
Cantanhez, Guinea-Bissau.

Multinuclear and thick-walled cyst of *Entamoeba coli*, fecal sample *P. troglodytes verus*, Cantanhez, Guinea-Bissau; stained with Lugol's iodine.

Lemon-shaped cyst of *Chilomastix mesnili*, fecal sample *P. troglodytes verus*, Cantanhez, Guinea-Bissau, stained with Lugol's Iodine.

Vacuolar-shaped cyst of *Blastocystis* sp., fecal sample *P. troglodytes verus*, Boé, Guinea-Bissau.

Cyst of *Iodamoeba buetschlii*, with dark-staining glycogen mass readily seen, fecal sample *P. troglodytes verus*, Cantanhez, Guinea-Bissau; stained with Lugol's iodine.

Ellipsoidol cyst of *Giardia intestinalis*, fecal sample *P. troglodytes verus*, Cantanhez, Guinea-Bissau; stained with Lugol's iodine.

Tapeworm egg of *Bertiella* sp., fecal sample *troglodytes verus*, Cufada, Guinea-Bissau.

Larvae of *Probstmayria gombensis*, fecal sample *P. troglodytes verus*, Cantanhez, Guinea-Bissau.

Unidentified hookworm egg, *Strongylida* fam. gen. sp., fecal sample *P. troglodytes verus*, Cantanhez, Guinea-Bissau; stained with Lugol's iodine.

Larva of *Strongylloides stercoralis*, fecal sample *P. troglodytes verus*, Boé, Guinea-Bissau; stained with Lugol's iodine.

Thin-walled egg of
Strongylloides fuelleborni, fecal
sample *P. troglodytes verus*,
Cantanhez, Guinea-Bissau.

Typical barrel-shaped egg of
Trichuris sp., fecal sample *P.*
troglodytes verus, Cantanhez,
Guinea-Bissau.

Appendix H: CITES and DEFRA permits

1. Nome do(s) autor(es) Rui Miguel Monteiro, Dá Rua Jau, 11 - 4ºº Andar 1300-011 Lisboa		CERTIFICADO CERTIFICATE Para um depósito de conhecimento internacional no depósito de Patentes Europeias		2. Nº PT10-0000000
3. Nome do(s) inventor(es) e endereço, com o(s) endereço de correspondência, se diferente (Se o inventor não estiver no mesmo país que o inventor, indicar o país de residência de cada um dos inventores)		4. Nome do(s) inventor(es) e endereço, com o(s) endereço de correspondência, se diferente Instituto de Conservação da Natureza e do Ambiente, IP Rua de Santa Maria, 60 1180-000 Lisboa		
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Rua Miguel Buarque de Gusmão, 55 Rua Joo, 11 - Vila Antenor 13050-071 Lndia		Para ser entregue ao Conselho Nacional de Defesa do Meio Ambiente		PCD-28403010
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DEPARTMENT FOR ENVIRONMENT, FOOD AND RURAL AFFAIRS

European Communities Act 1972

**THE PRODUCTS OF ANIMAL ORIGIN (THIRD COUNTRY IMPORTS)
(ENGLAND) REGULATIONS 2006 (AS AMENDED)**

The Secretary of State for Environment, Food and Rural Affairs, by the authorisation issued under the terms of Regulation 4 of the Products of Animal Origin (Third Country Imports) (England) Regulations 2006 (as amended) authorises:

K12 3a 3a, Colson Road 12 Cardiff CF11 3EQ	Name and full postal address
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subject to and in accordance with the conditions set out below, the bringing in England of:

(Organisms) Faecal Samples, intended for particular studies or analyses.	Product
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from:

Republic of Guinea Bissau	Countries of origin
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at:

London Gatwick	Ports of entry
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until:

18 January 2011	Expiry Date
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Dated: 18 December 2010

 Official of the Department for
 Environment, Food and Rural Affairs

WELSH GOVERNMENT

European Communities Act 1972

THE PRODUCTS OF ANIMAL ORIGIN (THIRD COUNTRY IMPORTS)
(WALES) REGULATIONS 2007 (AS AMENDED)

The Welsh Ministers, by this authorisation issued under the terms of Regulation 4 of the Products of Animal Origin (Third Country Imports) (Wales) Regulations 2007 (as amended) authorise

R/L 14 24, Colum Road T2 Cardiff CF10 1BS	Name and full postal address
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subject(s) and in accordance with the conditions set out below, the bringing in from of

Chimpanzee Faecal Samples, intended for particular studies or analyses.	Product
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from

Republic of Guinea-Bissau (via Amsterdam)	Countries of origin
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at

Cardiff Airport	Ports of entry
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until

10 July 2011	Expiry Date
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Dated: 10 June 2011

Officer of the Department for
 Environment & Sustainable Development

Appendix I: Characterization of the urban primate bushmeat trade in Guinea Bissau using molecular tools for species identification [*in prep.*]

Minhós T, Wallace E, Ferreira da Silva M, Sá R, Carmo M, Barata A, Bruford MW

INTRODUCTION

Unsustainable overhunting is compelling large mammal and slow-reproducing species, such as nonhuman primates, to extinction (Ape Alliance 2006; Mainka & Trivedi 2002; Kümpel et al. 2010). In Africa, for example, this escalating threat is considered to have a much larger impact than habitat destruction (Linder & Oates 2011; Rovero 2012). Human consumption of hunted wildlife for meat (i.e. bushmeat) is today recognized as a world crisis and a great conservation concern (Milner-Gulland et al. 2003; Nielsen 2006; Davies & Brown 2007; Macdonald et al. 2012). Bushmeat is leading to severe primate population declines and local extirpations, particularly in West and Central Africa (Allebone-Webb et al. 2011). This practice is not only a strategy for alternative protein income and subsistence for the rural poor, but also for trade that has been fostered by an increased entrance in remote forests by loggers (Wilkie et al. 2000) and/or by the greater access to sophisticated weapons by hunters (Bowen-Jones & Pendry 1999; Macdonald et al. 2012). Other factors associated with bushmeat consumption are connected with cultural or taste reasons, even when people have available alternatives (Bennett 2002; Bennett et al. 2006). A significant part of the bushmeat trade is due to a luxury preference where prices of wild meat are higher than those of domestic livelihood meat (Wilkie & Godoy 2001; Brashares et al. 2004).

A recent study estimated that 273 tons of bushmeat is imported every year into Paris-Charles de Gaulle Airport via Air France carriers therefore demonstrating the transnational scale of this traffic (Chaber et al. 2010). Additionally, several authors recognize that primate bushmeat consumption is a source of zoonotic disease emergence such as many retrovirus implied for example, in the HIV global spread (Courgnaud et al. 2004; Switzer & Heneine 2011; Smith et al. 2012). Pourrut et al. (2011) also alert for the cross-species transmission of several parasites with

zoonotic potential between nonhuman primate consumption and humans, raising the food security concern aspect of this trade.

Collecting reliable information on bushmeat consumption is difficult because many species are protected by national and international laws and informants may be reluctant to discuss their involvement in such an undisclosed subject (Jenkins et al. 2011). Furthermore, other frequent problem concerning bushmeat assessment for conservation management is the inability to correctly identify the harvested species in order to determine their proportional consumption frequency, specially in cases where many of the animal body parts are seized already processed, smoked or powdered, and consequently, the morphological identification is almost impossible or very inaccurate (Fong et al. 2007; Rönn et al. 2009; Ntie et al. 2010). Hence, only molecular approaches can shed light on reliable species identification. Within this context, and as a result of the search for solutions to this problem, several authors have emphasized the use of forensic genetics and DNA as a tool for bushmeat mitigation (Malisa et al. 2005; Domingo-Roura et al. 2006; Herke et al. 2007). DNA barcoding derived from mtDNA sequences, for example, has been proposed as a suitable technique for the species identification (Hebert et al. 2004; Herke et al. 2007). The majority of nonhuman primate species are included in Appendix 2 of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), but remarkably few studies have used these molecular approaches. Lorenz et al. (2005), in the context of the Integrated Primate Biomaterials and Information Resource (www.IPBIR.org) used the Cytochrome C Oxidase Subunit I of the mtDNA to verify the identity of the samples and generated a database of DNA barcodes for nonhuman primates advocating its use for the effective prosecution of poachers involved in the bushmeat traffic. Similarly, Rönn et al. (2009) developed a genotyping system in a microarray format for identification of primate species used in the bushmeat trade. More recently, by combining DNA-typing techniques to bushmeat surveys and questionnaires in Nigerian urban markets, Olayemi et al. (2011) demonstrated that this integrative approach was successful to determine the trade of 17 mammalian orders including the white-throated monkey, *Cercopithecus erythrogaster* an endemic vulnerable species of Nigeria.

Much research of the bushmeat trade has focused on West and Central

African urban markets (Fa et al. 2006; Allebone-Webb et al. 2011; Kamins et al. 2011; Dupain et al. 2012; van Vliet et al. 2012). However, little is known about the bushmeat trade in Guinea-Bissau and its characteristics were never assessed although previous reports have claimed that large quantities of primates have been hunted to be sold in the capital (Bissau), or in other important urban areas (Cá 2008). There are currently 10 primate species in Guinea-Bissau. Two species are classified as endangered including the West African chimpanzee (*Pan troglodytes verus*) and the Temminck's red colobus (*Procolobus badius temminckii*) (Casanova & Sousa 2007).

Here, we assessed the contribution of DNA-typing identification of African nonhuman primates in Guinea-Bissau' bushmeat markets. We followed the trade at two markets in Bissau across four months and we used a DNA barcoding approach (mitochondrial DNA Cytochrome c Oxidase subunit I and 12S rRNA fragment) to test the accuracy of morphological identification; determine the diversity of nonhuman primates sold in those markets; and to estimate the quantities of carcasses sold.

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

MATERIAL AND METHODS

Bushmeat market

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. sabaues* (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. campbelli* 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

$$TPF = NRM \times \frac{NPTS}{\text{---}}$$

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

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

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 wolffi*. 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 (similarity 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. sabaesus*. Most samples mislabelled in the morphological identification were molecularly assigned to *C. sabaesus*: 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. sabaesus* (see Fig. 5). Therefore, our results suggest that at the Bissau markets two species are predominately traded, at similar frequencies: *C. sabaesus* (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 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. sabaues*, 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				
<i>Cercopithecus campbelli</i> (Campbell's Mona)	76	21	99	19	3	22	22.7%	9	58.1%	12
<i>Erythrocebus patas</i> (Patas monkey)	1	3	4	1	0	1	25%	1	0	2
<i>Procolobus badius</i> (Western Red Colobus)	18	4	22	10	0	10	45.5%	6	40%	7
<i>Colobus polykomos</i> (Western Black and-White Colobus)	3	0	3	3	0	3	100%	2	33.33 %	2
<i>Papio papio</i> (Guinea Baboon)	18	0	18	12	0	12	50%	12	7.7%	12
<i>Chlorocebus aethiops</i> (Green monkey)	3	1	4	1	0	1	25%	1	0%	15
Total	113	27	150	46	3	50				

Appendix J: Mitochondrial DNA variation of the lesser spot-nosed guenon (*Cercopithecus petaurista*) from the Canhabaque Island, Bijago Archipelago, West Africa [in prep.]

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Abstract

Little is known about the Western lesser spot-nosed guenon (*Cercopithecus petaurista buettikoferi*) with a paucity of studies on the species. The Bijago Archipelago in Guinea-Bissau, West Africa represents the westerly limit of the species. There has been a significant decline in population abundance and distribution over the past twenty years due to intensive hunting for bushmeat trade. Canhabaque Island represents one of the only four known extant populations in the country. Non-invasive sampling was undertaken over four sample sites (Menegue, Bane, Inhoda and Inorei) encompassing the entire island. Amplification of the HVR1 of the mtDNA was undertaken to determine the genetic variation of the island population as well as to infer past demographic events and some life history traits, such as dispersal sex. A total of 47 sequences of 520bp were obtained showing 12 haplotypes with 26 polymorphic sites. These included nine singleton variable sites and 17 parsimony informative sites, comprising 24 transitions and 2 transversions. Rare haplotypes dominated the dataset with 9 haplotypes having a frequency of less than three. The dataset showed a high haplotype diversity (H_d) of 0.809 and a low nucleotide diversity (π) of 0.00850 suggesting a post-bottleneck demographic expansion of the population. Neutrality tests, although negative were not significant. Mismatch distribution showed a unimodal pattern and a high raggedness statistic ($r = 0.1371$). The results of the AMOVA test indicated more variation within sub-populations than between sub-populations with the F_{ST} value of 0.21773 suggesting population substructure. The high H_d and low π values shown in the dataset are in agreement with those of other species in the Cercopithecidae family. The role that primates play in seed dispersal is discussed along with the potential for *C. petaurista* to be labelled as an 'umbrella species' under a taxon lead conservation strategy in Canhabaque Island.

Keywords: *Cercopithecus petaurista*, Canhabaque Island, mtDNA, genetic diversity.

Introduction

The guenons are the largest and most diverse primate group in Africa, where they are endemic to the sub-Saharan region (Groves 2001; Grubb *et al.* 2002). First described by Linnaeus in 1758, there is some debate over which species should be included in the Guenon taxon. Currently the most widely accepted and broad definition is that a guenon is a monkey from the tribe Cercopithecini (Butynski 2002). This definition includes all the species of the genus *Cercopithecus* as well as *Miopithecus spp.*, *Chlorocebus spp.*, *Allenopithecus nigroviridis* and *Ethryrocebus patas*. The number of species and sub-species recognised varies considerably, with two recent studies recognising 23 species (Grubb *et al.* 2002) and 36 species (Groves 2001) respectively. Regardless of which definition is considered the authority for the term guenon, the *Cercopithecus* genus is the most speciose in the primate order (Fleagle 1999). The diversity seen in habitat, pelage, ecology and social structure has resulted in the guenons being of great interest to evolutionary biologists (Butynski 2002). The first guenon fossils date back to the Pliocene, 3 million years ago (mya), although it is believed that most of the evolutionary radiation of the genus has occurred in the last one million years (Leakey 1988). Considering the relatively recent radiation of the genus it is likely that it is still undergoing speciation (Gautier-Hion *et al.* 1988). The Cercopithecine monkeys have a complex and unclear phylogeny, with different phylogenies obtained for different characters (Appendix i): karyotypes (Ledbetter 1981; Dutrillaux *et al.* 1988), blood proteins (Ruvolo 1988), dental, cranial and postcranial morphology (Strasser & Delson 1987), skull morphology (Groves 2000), mitochondrial DNA (mtDNA) (Disotell and Raaum 2002) and Y-chromosome sequence (Tosi *et al.* 2002). These taxonomic ambiguities could be attributed to their relatively recent speciation. Little is known about the lesser spot-nosed guenon (*Cercopithecus petaurista*; Schreber 1774) with a paucity of studies in existence, largely focusing on behaviour (McGraw 2000) and inter-specific interactions (Buzzard 2006a, 2006b, 2010). Data such as facial markings (Kingdon 1980), chromosomes (Dutrillaux *et al.*, 1988), vocalisations (Gautier 1988) and proteins (Ruvolo 1988) suggest that *C. petaurista* is grouped into the superspecies

Cephus, which includes the currently described *Cercopithecus cephus*, *C. ascanius*, *C. erythrotis* and *C. erythrogaster* species. *C. petaurista* ranges from Guinea Bissau in the North-East to Togo in the South-West, although there has been a recent report of a single male lesser spot-nosed guenon observed in Fongoli study site in South-East Senegal (Pruetz *et al.* 2010), which could suggest a Northern range distribution of the species. Lesser spot-nosed guenons are quadrupedal primates that live

predominantly in the top layer of the forest canopy. Their diet is mainly frugivorous with over 77% consisting of fruit (McGraw 2000). The species is currently divided into two subspecies depending on region, the Western lesser spot-nosed guenon (*Cercopithecus petaurista buettikoferi*; Jentink, 1886) and

Figure 1: Range of *C. petaurista* with sub species range the Eastern lesser Spot-nosed guenon (<http://www.iucnredlist.org/apps/redlist/details/4225/0>) (*Cercopithecus*

petaurista petaurista; Schreber, 1774). The two sub-species are divided by the Cavally River in Liberia which acts as a geographic barrier (Figure 1) (Kingdon 2001; Oates *et al.* 2008). This study is concerned with the Western subspecies, *C. p. buettikoferi*, as samples were collected from the Canhabaque Island in the Bijagos Archipelago off the coast of Guinea Bissau to the west of the Cavally River. There has been no genetic analysis carried out on *C. p. buettikoferi* to date and thus the genetic diversity of the subspecies and relative diversity compared to other closely related species is still unknown. Here we described the HVR1 genetic variation of the Western lesser spot-nosed guenons of Canhabaque Island, we assessed their genetic structure explored their demographic history.

Material and Methods

Study Site

Canhabaque Island (11°15'2"N, 15°43'3"W) lies on the Bijago Archipelago off the coast of Guinea-Bissau, West Africa (Figure 2) and is part of the João Vieira-Poilão Marine National Park.

Figure 2: Map of Guinea-Bissau (<http://www.ibap-gbissau.org/Conexoes/apzcg.html>)

The Bijago Archipelago represents an area of significant ecological importance, not only for birds and marine fauna but also for terrestrial fauna. The area is currently a Biosphere Reserve with an application for UNESCO World Heritage Hotspot ongoing (UNESCO 2006). Canhabaque Island represents one of the areas where *C. petaurista* populations still persist. The average temperature is 26°C and average humidity 70%, with the rainy season occurring from May to October and the dry season from

November to April. The island comprises three main habitat types: mangroves, tropical lowland forest and grassland and only three mammal species are present on the island: *C. petaurista*, duikers (*Cephalophus spp.*) and the Gambian rat (*Cricetomys gambianus*) (R. Sá, personal communication, 15th November 2010).

Figure 3: Map of study sites on Canhabaque Island.

Non-invasive faecal sampling was undertaken during the dry season (February-June 2010) at four main study sites, Menegué, Bane, Inorei and Inhoda covering the entire island (Figure 3). A total of 96 samples were collected, each sample received a unique identification code along with a record of the place of origin, which will allow the geographic structuring of genetic variation on the island to be determined. Faecal samples were stored in one of four ways: silica, ethanol, DMSO or two steps (Roeder *et al.* 2004). Tissue samples were also collected from individuals

killed by local villagers for bushmeat consumption and were stored either in ethanol or in DMSO. A record of sample preservation method was taken so that amplification success rates for the four different methods could be determined. Amplification success rates of preserved samples will be compared between faecal and tissue samples to see if there are any statistically significant differences.

DNA samples were transported from Guinea-Bissau to the United Kingdom in agreement with the Convention on International Trade in Endangered Species (CITES) and for pathogen control regulations (DEFRA).

DNA Extraction

Tissue samples were stored either in ethanol or 20% Dimethyl sulfoxide (DMSO) salt solution. All tissue samples were extracted under sterile conditions using a Qiagen DNeasy Blood and Tissue Kit following an optimised version of the protocol. Two extraction negatives (reagents only) were included in each extraction set to allow monitoring for contamination (Handt *et al.* 1994).

Faecal samples were stored by either DMSO, silica, ethanol or two step method. Extractions were performed under sterile conditions using a Qiaamp mini stool kit following an optimised version of the

HVR1 Primers

Primers specific to Cercopithecidae were created in the D-loop region of the mtDNA by aligning whole mitochondrial and D-loop sequences from old world monkeys (OWM) and great apes (using Bioedit software (version 7.0.5.3, Hall 1999). Primers were then designed in the regions where the sequence was conserved for all Cercopithecidae, but had one or two substitutions in the human and/or chimpanzee sequences making them more specific to OWM mtDNA and reducing the chance of human cross amplification. The resulting primer pair was LNIAHVR1 (5'-CTC-AAA-TGA-ACT-TGC-CTT-3') and HNIAHVR1 (5'-TGA-GGT-AAG-AAC-CAG-ATG-3'). Bases which are substituted in great apes are underlined. The primers were tested using Oligo Analyser (<http://eu.idtdna.com/analyzer/applications/oligoanalyzer>) for basic parameters such as GC content, hairpin, self-dimer and hetero-dimer. Primer optimisation indicated an optimal annealing temperature of 59°C and yielded a fragment size around 656bp in *C. petaurista*. This is an approximate value as no *C. petaurista* sequences were available when the primers were designed.

HVR1 PCR Protocol

PCR was performed on a total volume of 15 µl and contained: 7 µl multiplex (x2), 2 µl of primer mix (2 µm), 0.1 µl BSA (10mg/ml), topped up with 3.9 µl ultrapure water and 2 µl template DNA. Cycling conditions were 95°C for 15 minutes, then 45 cycles

of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 1 minute with a final extension step of 72°C for 10 minutes. Samples that did not produce a band at the 59°C annealing temperature underwent a second PCR cycle at a lower annealing temperature of 54°C as during optimisation this was the annealing temperature at which no samples dropped out. Samples which failed at this reduced annealing temperature were considered as failed extractions. Two PCR blanks (reagents and water) were included in each set of PCR reactions to detect any possible contamination. In order to test the amplification, 2 µl of PCR product was mixed with 3 µl of 1x loading dye and then PCR fragments were then separated on 2% agarose gels (120 V, 40 minutes) using gel electrophoresis and visualised using 2 µl ethidium bromide staining (5mg/ml) per 100ml of agarose gel under a UV transilluminator.

DNA sequencing

The Exo-Sap (Endonuclease I – Shrimp Alkaline Phosphate) method (New England BioLabs) was used to purify all PCR products before sequencing. This protocol removes excess primer and nucleotides from the PCR product, thus purifying the sample. A final volume of 6 µl, containing 2.5 µl Sap buffer (10x), 1 µl Sap (5000 U/ml), 0.5 µl exo (20,000 U/ml) and 2 µl H₂O, was added to 13 µl of PCR product. Cycle conditions were 37°C for 30 minutes, 80°C for 15 minutes and 12°C for 5 minutes. 8 µl of purified sample was then added to 5 µl of either forward or reverse NIAHVR1 primer (5pmol/ µl) for sequencing using Macrogen Europe's EZ-sequencing service.

Data Analysis

Statistical Analysis

Minitab 15.1.20.0 (Minitab Inc 2007) was used to perform chi-squared tests on the amplification success rates of different sample preservation methods. This test indicates whether observed data deviates significantly from expected results. In this

case the expected amplification success rates were calculated as equal proportions of the amplification success for each sample type. Amplification success was defined as presence of a band i.e. DNA amplification, after PCR.

Sequence Alignment and Editing

Forward and reverse DNA fragments were edited by eye and aligned into contig sequences using Sequencher 4.9 (Gene Codes Corp., Ann Arbor, Michigan, USA). The contigs were then re-checked by eye for any discrepancies between the two fragments before conversion into consensus sequences. All consensus sequences were confirmed using BLAST – Basic Local Alignment Search Tool in NCBI to confirm they were *C. petaurista*. The sequences were then exported to Bioedit 7.0.5.3 (Hall 1999) and aligned using CLUSTAL W algorithm. All polymorphic sites and insertions were then re-confirmed through the chromatographs in Sequencher. Polymorphic sites were determined and all insertions, transversion and transistion polymorphisms were re-checked using the chromatograms. Sequences were then corrected so that only true polymorphic sites were present. All sequences were cut to the length of the shortest sequence and exported as a FASTA file for genetic diversity analysis.

Genetic Diversity

DnaSP, version 5 (Librado and Rozas 2009) was used to analyse HVR1 mtDNA sequence polymorphisms. Divergence between populations was calculated for the sequences grouped into location as well as polymorphism and divergence analysis. Mismatch distributions and neutrality tests were conducted including Tajima's D (Tajima 1989), Fu and Li D* and F* (Fu and Li 1993). Haplotype data files were generated.

Analysis of Molecular Variance (AMOVA)

Arlequin 3.11 (Excoffier *et al.* 2005) was used to conduct Analysis of Molecular Variance (AMOVA) to indicate any population substructure in the dataset. Samples were split into groups based on study site for this analysis. AMOVA provides a description of the diversity both within and between populations through presentation of percentage variance described by each component. This is undertaken through analysis of variance of gene frequencies which accounts for the mutational distance between haplotypes (Excoffier *et al.* 2005). The Fixation Index (F_{ST}) provided indicates the degree of differentiation between the population as a whole and sub-populations, ranging from 0 (no differentiation) to 1 (complete differentiation).

Median Joining Network

Network 4.6.0.0 (Fluxus Technology Ltd.) was used to create median joining networks (MJN) of the dataset. Two networks were drawn, one including all sample information and one containing only samples of known origin. A MJN is the combination of all minimum spanning trees (MST) combined into a single network (MSN) using the modified algorithm proposed by Excoffier and Smouse (1994). Missing intermediate nodes are then added to the network via median vectors using a parsimony criterion (Bandelt *et al.* 1999).

RESULTS

A total of 47 sequences of 520bp were obtained showing 12 haplotypes with 26 polymorphic sites including nine singleton variable sites and 17 parsimony informative sites. These comprised 24 transitions and 2 transversions, indicating a 12:1 transition: transversion ratio. The most common haplotype was H4 with 16 individuals having this sequence. There were also a large number of rare haplotypes with six singletons. Overall, nine of the 12 haplotypes contained three or fewer

individuals and only three haplotypes exhibited a higher frequency. This shows that the dataset is dominated by rare haplotypes with few common haplotypes present.

Demographic History

The mismatch distribution analysis using the constant population size model shows the distribution of pairwise nucleotide site differences for observed (dataset) and expected (stable population) values. The graph presented in Figure 4 shows a unimodal pattern; indicating a population bottleneck. The raggedness statistic $r = 0.1371$ (Harpending *et al.* 1993) also indicates a bottleneck with subsequent demographic expansion, giving an erratic distribution and non-equilibrium population (Weber *et al.* 2004).

Figure 1: Mismatch distribution for *C. petaurisa* mtDNA HVR1 sequences showing the frequencies of pairwise differences. The dashed blue line represents the observed pattern and the solid green line represents the expected pattern under the constant population size model.

Geographic Division of the mtDNA Diversity

Only sequences of known origin, i.e. no 'whole island' sequences, were considered for this genetic diversity analysis. The removal of the whole island dataset meant that 2 haplotypes only occurring in these individuals were also lost.

Table 2: Haplotype diversity by location.

Location	N	h	Hd	θ^2	π
Menegue	20	6	0.742	0.074	0.00627 (± 0.00065)
<i>Bane</i>	<i>1</i>	<i>1</i>	-	-	-
<i>Inhoda</i>	<i>3</i>	<i>2</i>	<i>0.667</i>	<i>0.314</i>	<i>0.01282 (± 0.00604)</i>
<i>Inorei</i>	<i>2</i>	<i>2</i>	<i>1.000</i>	<i>0.500</i>	<i>0.00385 (± 0.00192)</i>
All locations	26	10	0.831	0.053	0.00897 (± 0.00132)

In all locations there are high haplotype diversities (Table 7), although small sample sizes for some areas e.g. Inorei, result in a probable over estimation of haplotype diversity. Results from areas with small sample sizes are presented in italics and must be interpreted with caution as lack of sequences can bias the results. DNA polymorphism analysis could not be carried out for Bane, as only one individual was sequenced from this location. The more reliable data from Menegue and all locations support the pattern of high Hd and low π .

Differentiation of Sub-populations

Table 3: AMOVA results.

Source of Variation	Degrees of freedom	SSD	Variance components	Percentage of variance (%)
Among sub-populations	3	2.168	0.10395 Va	21.77
Within sub-populations	22	8.217	0.37348 Vb	78.23
Total	25	10.385	0.47743	
Fixation index	$F_{ST} : 0.21773$			

The results of AMOVA analysis (Table 8) indicate that there is more variation within sub-populations than between sub-populations, the percentage variance explained being 78.23% and 21.77% respectively. However there is a large skew in the number of individuals from each location, with Menegue having a considerably larger N than any other location. This may have confounded the results of the AMOVA, as Menegue also has the largest number of haplotypes which would have resulted in a high within sub-population variation value for this location. The F_{ST} value of 0.21773 given suggests great genetic differentiation between sub-populations (Wright 1978),

indicating that there is substructure of the populations. The significance tests of this AMOVA have a significant $p\text{-value} = 0.00782 \pm 0.00242$, thus showing the significance of the Fixation Index.

Median Joining Networks

Figure 2: Median Joining Network for the entire dataset of lesser spot-nosed guenons on Canhabaque. Each circle represents and haplotype. Circle sizes are proportional to haplotype frequency. Mutational steps are indicated by the red numbers represent the sequence sites that have undergone mutations. Black dots are median vectors of unsampled or missing haplotypes.

The network (Figure 5) shows a complex pattern with 12 haplotypes and 14 mutational steps separating the two most distant haplotypes. The diagram shows a mixture of a few distinct haplotypes (H8 and H10) and many haplotypes that are only two or three mutation steps removed from one another. The diagram shows high haplotype diversity with many rare haplotypes having been sampled. Some differentiation of haplotypes by location is shown; however, the inclusion of ‘whole island’ samples of unknown origin resulted in reduced clarity in the differentiation of haplotypes by location.

Figure 6: Median joining network diagram for the dataset of know origin samples of lesser spot-nosed guenons from Canhabaque. Each circle represents a haplotype. Circle sizes are proportional to haplotype frequency. Mutational steps are indicated in red numbers and represent site that have undergone mutations. Black dots are median vectors of unsampled or missing haplotypes.

The network diagram containing samples of known origin only (Figure 6) shows more clearly the differentiation of haplotypes between different locations on Canhabaque Island. There is high haplotype diversity (10 haplotypes in 27 samples) and 14 mutational steps between the two most distant haplotypes. However, most of the haplotypes are only two or three mutations removed from one another, suggesting relatively recent divergence, with one outlying haplotype (H8) having 6 mutations to the closest haplotype. Most haplotypes are distinct to a specific area, although low sample size in Bane, Inhoda and Inorei suggests that this result is not definite, as a wide range of haplotypes have not been collected from these areas and missing haplotypes may represent shared haplotypes between different areas.

Figure 3: Map of Canhabaque Island with haplotype frequencies by location.

Figure 7 shows the haplotype frequencies found at each data collection site on the island, haplotypes 10 and 11 are not present in this figure as they only occur in the 'whole island' sample set. The haplotype frequencies infer high differentiation between sub-populations with only one common haplotype - haplotype 3 - shared between Inhoda and Menegue. This figure contradicts the results of the AMOVA test as there appears to be a significant level of variation between the populations. However this may have been masked by the large number of haplotypes found at Menegue due to the larger dataset size.

DISCUSSION

The Western lesser spot-nosed guenons of Canhabaque Island have high haplotype diversity (Hd) and low nucleotide diversity (π) a pattern which suggests a past bottleneck followed by a population expansion (Grant and Bowen 1998). Genetic loss during bottlenecks occurs more rapidly in mtDNA than nuclear DNA due to maternal inheritance and therefore a lower effective population size. During population expansion, mutations will accumulate more rapidly in the mtDNA than in the nuclear DNA due to the faster evolutionary rate of mtDNA (Grant and Bowen 1998). This explains the pattern seen in the data, especially as the sequence used was from the hypervariable region of the mtDNA therefore having an even faster evolutionary rate (Howell *et al* 1996). The results of the three neutrality tests performed on the dataset were negative, suggesting population expansion; however the p -values were all greater than 0.05 (Table 6), showing that the *C. p. buettikoferi* population of Canhabaque Island is not significantly expanding.

The unimodal mismatch distribution suggests a population bottleneck (Slatkin and Hudson 1991; Rogers and Harpending 1992). The raggedness statistic agrees with this indicating a bottleneck with subsequent demographic expansion (Weber *et al.* 2004). The x-axis value $\tau = 2\mu t$ indicates that τ is a function of time and that mutation rate and time (generations) are directly proportional; therefore the distance of the peak along the x-axis is a function of time since the bottleneck event (Rogers 1995). In this case the apex of the peak is at $\tau = 5$, indicating a relatively

recent but not contemporaneous bottleneck event. This interpretation agrees with results from the haplotype and nucleotide diversity analysis and the neutrality tests that the population is in post-bottleneck demographic expansion.

The results of the AMOVA were inconclusive. The results suggested higher variance within sub populations however this may have been due to the large skew in sample size towards Menegue. More samples from Inhoda, Inorei and Bane are needed to investigate these results further and determine whether there truly is more variation within the populations, or whether the results are due to different dataset sizes.

The network diagrams show high haplotype diversity with a large number of rare haplotypes present. It is therefore surprising that more common haplotypes are not present, with five missing haplotypes inferred in the diagram. There are several reasons why these sequences could be missing: they truly are extinct haplotypes, data collection was not extensive enough to collect samples of these haplotypes or some individuals have come from other islands bringing new haplotypes without the corresponding ancestral lineage. Nevertheless, the network diagrams conform with the fixation index (F_{ST}) from the AMOVA analysis in suggesting population structure by location for the samples analysed.

To the best of my knowledge this is the first study on the population genetics of *C. petaurista* with the results providing some insights into the population structure, past demographic events and life history of the species. The haplotype sequences gained from this study will be submitted to Genbank for use in future research. Comparison of haplotype diversity (H_d) and nucleotide diversity (π) with other species from the family Cercopithecidae allows the results of this study to be placed in a broader context by determining whether the values are higher or lower than related species. Table 10 shows the H_d and π from other studies. Comparison of these results with the values obtained for *C. p. buettikoferi* shows that many other closely related species also exhibit high H_d and low π . The H_d for *C. p. buettikoferi* falls in the middle of the range of values found, indicating that many Cercopithecine species have high haplotype diversity. In fact the species with the highest haplotype diversity, *Procolobus rufomitratus*, is known to have gone through a recent large bottleneck, however this may have been too contemporaneous to be expressed in

the genetic diversity yet (Mbora and McPeck 2010). Therefore although *C. p. buettikoferi* has a relatively high Hd this does not mean that the population is not in need of conservation protection.

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CONSERVATION AND RESTORATION

Using Genetics as a Tool in Primate Conservation

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

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 reproduction rate, dispersal needs, and degree of specialization, make primates more susceptible to extinction than many other species (Cowlshaw & Dunbar 2000, Harcourt *et al.* 2002, Gibbons & Harcourt 2009). Moreover, the geographical range of threatened primate species often overlaps with areas of high human density (Harcourt & Parks 2003). Recent studies conclude 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).

Figure 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.

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Non-Invasive Genetic 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 several 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) (Figure 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) (Figure 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).

Figure 2: Behavioral 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.

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Figure 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.

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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 & 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 anthropogenic 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).

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 (Figure 4).

Figure 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.

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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).

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 (<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 (Figure 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.

Figure 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.

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Diseases

Disease is another important aspect for primate conservation. The 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 & 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, using 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 ago) and sooty mangabeys (between 1729 to 1875 years ago), the reservoirs of HIV-1 and HIV-2, respectively. Conversely, human transmitted pathogens to great apes such as bacteria (e.g., *Streptococcus pneumoniae*) or viruses (e.g., human metapneumovirus) are causing fatal respiratory outbreaks (Chi *et al.* 2007, Kaur *et al.* 2008, Köndgen *et al.* 2008, 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 using molecular tools.

Figure 6: Primate parasites.

a) *Entamoeba* sp. and b) *Strongylida* fam. gen. sp. (Hookworms) are gastrointestinal parasites that can cause severe infections and weaken the health of the host.

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Applying Conservation Genetics

Primate census

The 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, 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 provide 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 (e.g., see Paektau 1999), such definitions can be key indicators to preserve genetic distinctiveness (evolutionary heritage, genetic diversity and differentiation). For example, Kanthaswamy *et al.* (2006) suggested that the Bornean and Sumatran orangutans should be considered two distinct MUs based on the analysis of mtDNA and microsatellite loci, and consequently the authors advised against 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 modeling 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 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 orangutans from the Kinabatangan floodplain in Sabah, Malaysia, to study the implications of non-intervention, translocation, corridor establishment, and a 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 translocation or corridor establishment alone would not be sufficient to prevent high levels of inbreeding. Instead, a combination of the two measures would retain the demographic stability of even the most isolated subpopulations and constrain localized inbreeding to a sustainable threshold (Figure 7).

	Carrying capacity	Mean (r)	SD	P(E) after 25 / 100 / 250 yr	Initial / mean final	SD	Retained GD after 25 / 100 / 250 yr	Mean F after 25 / 100 / 250 yr	Mean TE
<i>Without inbreeding depression</i>									
PSU 1	230	-0.001	0.04	0 / 0 / 0	230 / 157.7	27.3	0.994 / 0.981 / 0.954	0 / 0.010 / 0.036	0
PSU 2	351	-0.001	0.04	0 / 0 / 0	209 / 39.2	39.2	0.994 / 0.985 / 0.967	0 / 0.009 / 0.023	0
PSU 3	108	0	0.05	0 / 0 / 0.001	63 / 71.48	17.2	0.980 / 0.949 / 0.889	0.001 / 0.030 / 0.096	0
PSU 4	72	-0.001	0.05	0 / 0 / 0.017	61 / 44.36	14.1	0.977 / 0.933 / 0.837	0.001 / 0.037 / 0.131	192.2
PSU 5	313	-0.001	0.04	0 / 0 / 0	293 / 218.63	36.5	0.995 / 0.986 / 0.966	0 / 0.008 / 0.027	0
PSU 6	62	-0.002	0.05	0 / 0.029 / 0.036	55 / 37.72	12.5	0.976 / 0.923 / 0.808	0.001 / 0.041 / 0.150	187.8
PSU 7	54	0	0.06	0 / 0.019 / 0.122	23 / 30.93	11.6	0.947 / 0.964 / 0.732	0.003 / 0.080 / 0.216	149.5
PSU 8	97	-0.003	0.06	0 / 0.022 / 0.076	22 / 38.41	19.1	0.946 / 0.878 / 0.796	0.003 / 0.079 / 0.174	137.5
PSU 9	49	-0.003	0.06	0 / 0.006 / 0.079	49 / 28.43	10.9	0.971 / 0.900 / 0.760	0.001 / 0.052 / 0.204	185.2
PSU 10	31	-0.003	0.08	0 / 0.069 / 0.349	25 / 17.13	7.48	0.944 / 0.827 / 0.628	0.002 / 0.089 / 0.299	152.8
PSU 11	120	-0.001	0.04	0 / 0 / 0	97 / 79.02	19.3	0.987 / 0.966 / 0.906	0.001 / 0.021 / 0.076	0
<i>Including inbreeding depression</i>									
PSU 1	230	-0.002	0.04	0 / 0 / 0	230 / 144.39	30.3	0.994 / 0.981 / 0.952	0 / 0.009 / 0.036	0
PSU 2	351	0	0.04	0 / 0 / 0	209 / 227.16	39.3	0.994 / 0.985 / 0.966	0 / 0.008 / 0.026	0
PSU 3	108	-0.001	0.05	0 / 0 / 0.016	63 / 32.84	19.6	0.980 / 0.949 / 0.874	0.001 / 0.023 / 0.097	218
PSU 4	72	-0.004	0.05	0 / 0.001 / 0.068	61 / 30.43	14.6	0.979 / 0.933 / 0.808	0.001 / 0.030 / 0.133	198.1
PSU 5	313	-0.001	0.04	0 / 0 / 0	293 / 203.83	38.1	1.000 / 0.986 / 0.965	0 / 0.006 / 0.026	0
PSU 6	62	-0.005	0.06	0 / 0.005 / 0.123	55 / 24.37	12.2	0.976 / 0.923 / 0.780	0.001 / 0.039 / 0.155	197.4
PSU 7	54	-0.004	0.07	0 / 0.032 / 0.388	23 / 18.97	10.3	0.947 / 0.963 / 0.702	0.002 / 0.069 / 0.213	173.8
PSU 8	97	-0.002	0.06	0 / 0.038 / 0.273	22 / 33.37	19.3	0.946 / 0.872 / 0.762	0.002 / 0.065 / 0.161	167.4
PSU 9	49	-0.006	0.07	0 / 0.008 / 0.274	49 / 18.1	10.2	0.971 / 0.900 / 0.726	0.001 / 0.030 / 0.189	194.1
PSU 10	31	-0.008	0.08	0 / 0.091 / 0.706	23 / 10.16	6.37	0.944 / 0.874 / 0.611	0.002 / 0.080 / 0.283	164.2
PSU 11	120	-0.002	0.04	0 / 0 / 0.004	97 / 64.03	20.9	0.987 / 0.966 / 0.897	0.002 / 0.020 / 0.076	203.8

Figure 7: Population viability analysis.

The table shows the result of demographic and genetic parameters of the vortex simulation for 25 to 250 years under a non-intervention scenario. Note the mean extinction time (column TE) for most of the sub-populations (PSU) is less than 250 years. P(E): probability of extinction; Pop: population; GD: genetic diversity (from Bruford *et al.* 2010).

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Summary

The extensive use of molecular techniques as tools has provided new opportunities to better understand the mechanisms underlying the evolution and adaptation of primates (Figure 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.

Figure 8: Survival of endangered species.

Interspecific association between western Red and Black-and-white colobus (Cantanhez National Park, Guinea Bissau). Prospects for the survival of these endangered species will benefit from the use of non-invasive genetic tools.

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All authors have contributed equally to this article.

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- Notes from the Field: A Primateologist's Point of View

DAVID LAWSON / WWF-UK

The Trade and Ethnobiological Use of Chimpanzee Body Parts in Guinea-Bissau: Implications for Conservation

Rui Miguel Moutinho Sá, Maria Ferreira da Silva,
Fernando Miguel Sousa and Tânia Minhós

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 to be extinct in the country due to a total absence of information owing largely to political and civil unrest (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; Brugièrre *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/km² in a total area of 17.225 km², corresponding to 40 individuals (Sousa *et al.*, 2011), while in the neighbouring area east of Gadamael, just outside the CNP area, this value decreases to 0.89 nest builders/km² in a total area of

36.513 km², 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 remains 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; Brugièrre *et al.*, 2009). The species is classified by IUCN as Endangered, and listed in CITES Appendix I, and is also protected in Guinea-Bissau. 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.).

This 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

Seven visits, of approximately four hours each, 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 ethnoprimateological 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 market 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

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1. Leopard *Panthera pardus* (1a complete).
CITES Appendix I/IUCN Near Threatened;
2. Chimpanzee *Pan troglodytes verus*.
CITES Appendix I/IUCN Endangered;
3. Nile Crocodile *Crocodylus niloticus*.
CITES Appendix I/II/IUCN Lower Risk/Least Concern.
4. Guinea Baboon *Papio papio*.
CITES Appendix II/IUCN Near Threatened.
5. Possibly Lion *Panthera leo*.
CITES I/IUCN Vulnerable.
6. Possibly African Civet *Civettictis civetta*.
CITES Appendix III/IUCN Least Concern.
7. Antelope horns (species not identified).

Fig. 1. All animal-derived products for human traditional purposes in Bandim market, Bissau, Guinea-Bissau.

and field notes were taken. Informants were assured 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).

Only pieces of animal skins were seen for sale during the surveys (which could have derived from one or more specimens). As the vendors were reluctant to answer questions related to the animal numbers involved in the trade, it was not possible to estimate the number of skins being offered for sale for each species recorded. This paper therefore focuses on the morphological identification of the species and not to the number of skins traded.

RESULTS AND DISCUSSION

Traded species

During visits to Bandim market, morphologically identified dried Chimpanzee skins were found being sold for traditional medicinal purposes. Additionally, dried skins from Temminck's Red Colobus monkeys *Procolobus badius temminckii*, Guinea Baboons *Papio papio* and Olive Baboons *Papio anubis* were also found. The authors also detected trade in 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, Crested Porcupine *Hystrix cristata* spines, teeth, antelope horns, pangolin *Manis* sp. scales, 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 distribution is reported to be in Mali and the Republic of Guinea (IUCN, 2011). The Olive Baboon skin seen was morphologically quite different to the Guinea baboon skins found at the market. While Guinea Baboon skins present red/brownish coloration, the Olive Baboon skin had a green hue, typical of what has been described for the subspecies (Groves, 2001).

Costs, origin and scale

Interviews with urban traders revealed that the cost of a piece of Chimpanzee skin was relatively high, ranging from XOF1500 (CFA Francs) 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 (apart from the elephant hide seen) originated from the "southern part", and frequently mentioned the regions of Cantanhez and Gabú specifically. Vendors considered the "southern part" every location south of Bissau. The authors were told that the elephant hide had come from Senegal. According to Blanc *et al.*, 2007, at least one, and at most 10 elephants remain in Senegal. 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.* to TRAFFIC, 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. See also Marshall (1998).

Two of the vendors also mentioned that individuals from neighbouring countries such as Senegal, Guinea or Gambia are involved in the trade within 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 menstruating 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, pers. obs., 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, as well as both a lack of interest and in-depth study of such practices. As a result, the authors’ observations are opportunistic. However, the use of animals’ body parts for medicinal purposes could seriously threaten the biodiversity of Guinea-Bissau and, in particular, constitutes an additional and significant threat to Chimpanzee populations already menaced by habitat loss and fragmentation, the pet trade and crop-raiding conflicts. Therefore, this phenomenon deserves to be thoroughly investigated (Cá, 2008).

Although not previously 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, 2010). 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 (Robillard, *in litt.* to TRAFFIC, July 2011).

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 Guinea Baboon *Papio papio* is the only baboon species reported and observed in the country (IUCN, 2011). There are striking differences in morphology between both baboon species (namely coat coloration (Groves, 2001)), which enables a distinction to be made based on their skins. Furthermore, in Colobane and Boucotte markets in 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 magic ceremonies (Fernando Sousa, pers. obs., 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, pers. obs., 2008). The respondents pinpointed Cassamance (on the border between Senegal and Guinea-Bissau) as the putative origin of Chimpanzee skins at Boucotte market, and Guinea-Bissau and the 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 *Direção 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 (Anon., 2011)). However, the lack of resources and lack of awareness of management authorities and politicians is hindering law enforcement 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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Distribution of the Entodiniomorphid Ciliate *Troglocorys cava* Tokiwa, Modrý, Ito, Pomajbíková, Petrželková, & Imai, 2010, (Entodiniomorpha: Blepharocorythidae) in Wild and Captive Chimpanzees

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ABSTRACT. Trophozoites of *Troglocorys cava* were detected in all but one of the wild chimpanzee populations from Rubondo Island (Tanzania), with a prevalence ranging between 20% and 78%. However, the ciliate was absent in all captive groups. Prevalence appeared to increase with the number of sequential samples taken from a particular individual and reached 95.5% in wild individuals sampled at least 4 times.

Key Words. Chimpanzees, Entodiniomorpha, intestinal ciliates, prevalence, *Troglocorys cava*.

THE intestine of apes is colonized by a spectrum of protists that cause no or little harm to their host and can be classified as mutualists or commensals. Among them, ciliates are the most prominent group, as they apparently participate in the hindgut fermentation (e.g. Profousová et al. 2010). Reports about the presence of intestinal ciliates in wild apes were mostly a by-product of parasitological examinations (Ashford, Reid, and Wrangham 2000; Murray et al. 2000). However, the proper classification of ciliates requires specialized techniques other than those used for the routine coproscopic detection of parasites, thus representing a serious drawback in their research (Imai et al. 1991).

Most intestinal ciliates of great apes belong to the order Entodiniomorpha and are classified in the genera *Troglophyella*, *Gorilloglyphus* and *Prototapirella*. In addition, a species that had been referred to as a “small” or “unidentified ciliate” (Ashford et al. 2000; Bakuza and Nkwengulila 2009; Murray et al. 2000) was named *Troglocorys cava* and assigned to the family Blepharocorythidae (Tokiwa et al. 2010); the study aimed to investigate the occurrence of *T. cava* in both wild and captive chimpanzees. Herein, we report on the prevalence, diagnostics, and geographical distribution of *T. cava* in populations of wild chimpanzees and discuss the absence of *T. cava* in captive chimpanzees in European facilities, African sanctuaries, and in an introduced chimpanzee population in Tanzania.

MATERIAL AND METHODS

The set of fecal samples of wild ($n = 511$) and captive chimpanzees ($n = 203$) largely corresponds with those discussed by Pomajbíková et al. (2010). It also includes two additional wild chimpanzee populations and one captive facility: Goulougo Triangle, Republic of Congo (DRC) ($n = 20$); Cantanhez National Park (NP), Guinea Bissau ($n = 102$); and Sweetwaters

Chimpanzee Sanctuary, Kenya ($n = 42$). In addition, the numbers of samples from Ugalla and Rubondo Island NP were extended to 119 and 206, respectively. Samples were preserved by 10% formalin and examined by merthiolate-iodine-formaldehyde sedimentation (MIFC), following Pomajbíková et al. (2010).

We calculated the prevalence of *T. cava* differently for habituated (chimpanzees are used to being around people) and unhabituated (the opposite to previous) populations. For a habituated population, the prevalence was expressed as the percentage of individuals infected with *T. cava*; for unhabituated populations, as the percentage of samples positive for *T. cava*. To determine the effect of sequential sampling on the prevalence of *T. cava*, we calculated the cumulative prevalence for selected animals from Kalinzu Forest Reserve. We chose samples only from the wet season ($n = 195$) and calculated the cumulative prevalence based on number of samples per individual: (i) one sample ($n = 36$ animals); (ii) two sequential samples ($n = 29$); (iii) three sequential samples ($n = 25$); and (iv) four or more sequential samples ($n = 22$).

For scanning electron microscopy (SEM), trophozoites of *T. cava* from a chimpanzee from Kalinzu FR were obtained from a filtrated fecal sediment and were preserved with 10% formalin, following the protocol of Jirků et al. (2009).

RESULTS AND DISCUSSION

The general trophozoite morphology, determined by light microscopy and confirmed by NIC, was uniform throughout all localities and consistent with the original description of *T. cava* (Tokiwa et al. 2010) (Fig. 1).

The geographical distribution of *T. cava* includes localities in Tanzania, Uganda, and Gabon (e.g. Krief et al. 2005; Landsoud-Soukate, Tutin, and Fernandez 1995; Murray et al. 2000). We detected *T. cava* in chimpanzee fecal samples from seven of the eight studied wild populations and extended its known distribution in Uganda and Tanzania, and added localities in Nigeria, DRC, and Guinea Bissau. Its prevalence in wild populations ranged from 5% to 98% (Ashford et al. 2000; File, McGrew, and Tutin 1976; Krief et al. 2005; Landsoud-Soukate et al. 1995; Murray et al. 2000). Our

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Fig. 1–7. Diagnostic features of *Troglodytes cava* revealed by light microscopy (1–4) and scanning electron microscopy (5–7) from a formalin-fixed fecal sample from *Pan troglodytes schweinfurthii*, Kalinzu Forest Reserve, Uganda. 1. Right side of the trophozoite showing frontal lobe (arrowhead) surrounded by adoral cilia. 2. Trophozoite showing vestibulum (asterisk). 3. Left concavity (arrow) in dorso-ventral view. 4. Left side of the trophozoite showing the concavity clearly demarcated by a hexagonal line formed by bases of lips (white arrow). 5. Right side of two trophozoites showing typical surface longitudinal striation and both adoral (bottom) and left cilia (above). Arrowhead indicates position of frontal lobe hidden by adoral cilia. 6. Dorso-ventral view showing asymmetrical shape of the trophozoite. The concavity, invisible in this view, is located on the flat left side. 7. Dorso-lateral view of the trophozoite showing the prominent left concavity possessing small round projections on its surface. Scale bars = 20 µm. Fig. 1–4 and 5–7, respectively, are in the same scale.

results fell within this range: Budongo FR, Uganda – 20%; Cantanhez NP – 25%; Gashaka Gumti NP – 33%; Kyambura George – 40%; Goulougo Triangle – 65%; and Kalinzu FR – 78%. In contrast, we did not find *T. cava* in any captive chimpanzees. *Troglodytes cava* was absent also in the wild population of chimpanzees on Rubondo Island, having been introduced onto the island in the 1960s using founders that had spent some period in captivity (Petrželková et al. 2010). Thus, *T. cava* apparently disappears during the captivity; this is confirmed by its total absence in zoos and sanctuaries. These findings are in contrast to the common occurrence of *Troglodytella abassarti* in captive chimpanzees (Pomajbíková et al. 2010). Since nothing is known about the biology of *T. cava* in hindgut ecosystem, its absence cannot be unambiguously explained.

Generally, the populations of commensal ciliates fluctuate greatly, which was demonstrated both in rumen ciliates and *T. abassarti* (Pomajbíková 2008; Williams and Coleman 1991). As a result of this fluctuation, the numbers of *T. cava* in some fecal samples can be reduced below the threshold level of detection. To address this problem we examined serial fecal samples from the same individual and calculated their cumula-

tive prevalence. Prevalence of *T. cava* reached 55.5% in individuals sampled only once, 79.3% in individuals sampled twice, 92% in individuals sampled 3 times, and 95.5% if four and more samples were collected. These results conform to other studies suggesting that ideally three or four samples per animal are necessary to assess the real prevalence of a particular parasite/symbiont (Huffman et al. 1997; Muehlenbein 2005). Therefore, we predict that *T. cava* is present in all individuals in wild chimpanzee populations, a pattern documented previously for *T. abassarti* (Pomajbíková et al. 2010). Despite the fact that none of previous studies included either morphological descriptions or microphotographs, we assume that also other findings of the “small ciliate” in chimpanzees by previous researchers refer to *T. cava*.

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