The enigmatic Bale monkey: Can new analyses shed light on the phylogeny of the *Chlorocebus* genus?

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Abstract

The Bale monkey (*Chlorocebus djamdjamensis*) is a little known primate endemic to the Ethiopian highlands. It is classified as 'Vulnerable' in the IUCN Red List of Threatened Species, and is under threat from increasing anthropogenic pressure. The lack of information about the Bale monkey combined with its threatened status stresses the need for more knowledge. The *Chlorocebus* genus consists of several morphologically similar species, and the correct identification of these has been a challenge for taxonomists. Furthermore, the classification of the species in the genus has become even more complicated because of the apparent frequent hybridization between the *Chlorocebus* monkeys.The taxonomy of the genus has been debated for over a century, but lack of molecular data on the Bale monkey has made it difficult to determine its placement within *Chlorocebus* as a species or a subspecies. Until now, no molecular phylogenies including the Bale monkey have been produced. In this study, mitochondrial markers have been used to investigate the phylogenetic relationship between five of the six species in the *Chlorocebus* genus, using the 12S, COI and cyt b genes to produce three different topologies. The topologies were mainly congruent, but none of the species in the ingroup formed monophyletic clades. Instead, individuals thought to be different species grouped together, pointing towards possible misidentification of individuals because of cryptic variation. Nuclear markers, which would be necessary to resolve the phylogeny completely if hybridization is involved, did not show sufficient variation, and were therefore left out of further analyses. Because of the lack of nuclear data, it is difficult to conclude whether hybridization has taken place between the individuals in this study, and to infer the taxonomic status of the Bale monkey. However, alternative explanations for the incongruence seen in the phylogeny are put forward and discussed based on additional information on the morphology and geographic origin of the sampled individuals.

Introduction

Although humans have populated the earth for only some 200,000 years, the impact of our species on the planet has been profound. From between 10,000 years ago until the year 1750, the population world-wide increased on average by about 67,000 people a year (Weeks 2008); today the number is seven billion and rising (UNPF 2011). In any species, a growth rate of this magnitude in combination with an extensive population expansion will have severe consequences for the surrounding environment. Such consequences include increases in resource-consumption, space and other services, and will inevitably lead to a conflict with other species over resources and habitat.

The use of land to yield goods and services represents the most substantial human alteration of the planet (Vitousek et al. 1997). With the introduction of modern agriculture 10,000 years ago (Matsuoka et al. 2002), the structure and functioning of entire ecosystems became affected, a process that is constant and ongoing today. There is little doubt that human activities have been a major factor in driving species extinctions and invasions - activities that have altered ecological communities and ecosystems, with severe impacts on ecosystem services and goods, leading to irreversible changes in many documented cases (see Hooper et al. 2005). The most severe threat to mammal (and other) species is habitat loss, but habitat fragmentation (discontinuities in an organism's preferred environment) is also a major factor driving decrease in population numbers worldwide (IUCN 2008). Habitat fragmentation decreases the amount of available habitat; it can prevent animals from moving between habitable patches of land, and decreases gene flow between populations, thereby increasing inbreeding and speeding up genetic drift (Frankham et al. 2004).

A species currently under threat from increasing urbanization and habitat destruction is the Bale monkey (*Chlorocebus djamdjamensis*) (Mekonnen et al. 2010a; Mekonnen et al. 2010b; Mekonnen et al. 2011), a primate endemic to the bamboo zone of the Ethiopian Bale Mountains. The Bale Mountains are home to a range of endemic Ethiopian fauna and flora, and are part of the Eastern Afromontane Biodiversity Hotspot (Myers et al. 2000); widely scattered, but biogeographically similar mountain ranges in Eastern Africa. The largest populations of the Ethiopian wolf (*Canis simensis*) can be found in the Bale Mountains, as well as the olive baboon (*Papio anubis*) and the endangered mountain nyala (*Tragelaphus buxtoni*). In 1970 the Bale Mountains National Park was established to conserve the endemic

animals of the region, but protection is made difficult because of lack of law enforcement (Mekonnen et al. 2010a). Rapid deforestation of the bamboo forests in the region is decreasing the available habitat for the Bale monkey, bringing it into closer proximity with humans. Spurred on by a diminishing food supply, the monkeys take to raiding local crops, resulting in many of the animals being hunted and killed (Mekonnen et al. 2011).

From August 2007 to May 2008, Mekonnen et al. (2010b) conducted a study on the behaviour and ecology of the Bale monkey in the Odobullu Forest in South-West Ethiopia, with particular focus on the diet, ranging ecology and activity patterns of the animals. Before this pioneer study, knowledge about the ecology of the Bale monkey had been limited, and the research gave surprising results. It was found that the monkeys exhibited an extreme degree of folivory compared to other relatives in the *Chlorocebus* and *Cercopithecus*, with a remarkably species-poor and specific diet: The animals were observed feeding on only 11 different plant species during the study period, with young leaves of bamboo (*Arundinaria alpina*) comprising 76.7 % of the total.

While previously classified as 'Data Deficient' in the IUCN Red List (Butynski et al. 2008), the status of the Bale monkey was changed to 'Vulnerable' in 2008, based partly on the results of Mekonnen et al. (2010b). In a new report, however, Mekonnen et al. (2011) suggest that the species status should be elevated to 'Endangered' because of the increasing anthropogenic pressure caused by habitat loss and hunting, in addition to possible hybridization with other *Chlorocebus* monkeys.

The *Chlorocebus* taxonomy

Belonging to the Old World monkeys, subfamily *Cercopithecinae*, the Bale monkey is currently placed in the genus *Chlorocebus* (Groves 2001). Since its discovery by Neumann in 1902 (in Groves 2001), the species' placement in the primate phylogeny, along with the placement of the other monkeys of the genus, has been uncertain; see below (Kingdon 1997; Groves 2001; Grubb et al. 2003). As Groves (2001) stated in his book *Primate Taxonomy*: "*The whole genus is in urgent need of revision*". The challenge lies in the limited amount of information available. Although recent studies conducted on the Bale monkey have uncovered new information (see above), many questions yet remain unresolved with regards to the ecology and phylogeny of this species, and so far no molecular data for the species has been generated.

The *Cercopithecidae*, or Old World monkeys, consists of two subfamilies: the *Colobinae* and the *Cercopithecinae.* The *Cercopithecinae* consists of 12 genera, of which my focus will be *Chlorocebus* and *Cercopithecus.* The genus *Chlorocebus*, commonly called 'Savannah monkeys', was first described by Gray (1870), and has been revised several times. Kingdon (1997) described the Bale monkey as a species belonging to *Cercopithecus*, removing the genus *Chlorocebus* altogether. Groves (2001) resurrected *Chlorocebus* in 2001. Although there are now six species classified in the genus – the Bale monkey, the grivet monkey (*Chlorocebus aethiops*), the vervet monkey (*Chlorocebus pygerythrus*), the Malbrouck (*Chlorocebus cynosurus*), the tantalus monkey (*Chlorocebus tantalus*) and the green monkey (*Chlorocebus sabaeus*) – there is still uncertainty with regards to their relationship and taxonomic status. Representatives of four of the six *Chlorocebus* species are pictured in Fig. 1 (a-d). Finding reliable pictures of the tantalus monkey and the Malbrouck proved difficult, as the species are sometimes mistaken for grivet monkeys, and therefore wrongly labeled. Pictures of these monkeys are therefore not shown.

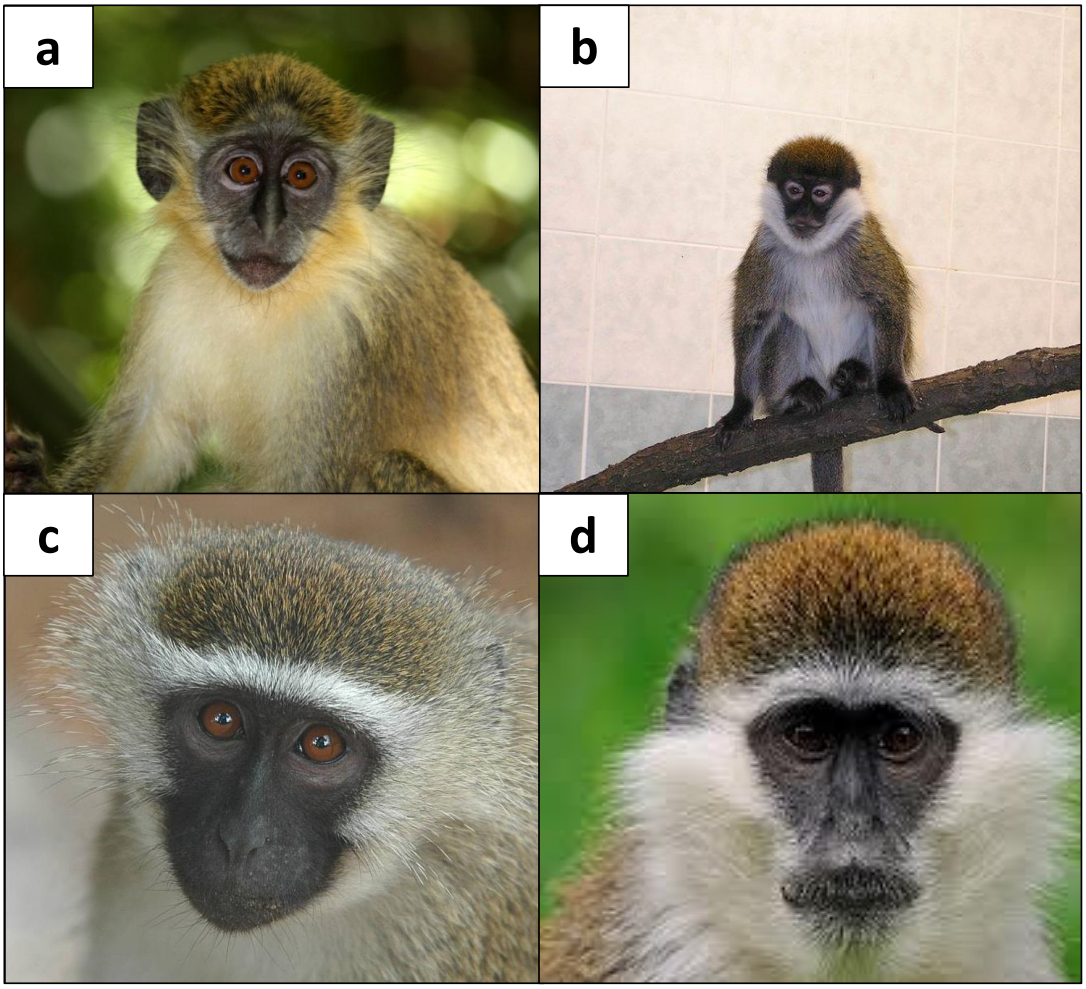

Fig.1: a) Green monkey*, Chlorocebus sabaeus.* Source: Wikimedia commons b) Bale monkey (*Chlorocebus djamdjamensis*). Individual is "Emma", sample from Bojnice Zoo, Slovakia (Picture: Dr. Peter Luptak, Bojnice Zoo. c) Vervet*, Chlorocebus aethiops* (www.onemoregeneration.org 2011) d) Grivet, *Chlorocebus pygerythrus* (http://www.treknature.com/gallery/Africa/Ethiopia/photo226219.htm).

Hybridization

Hybridization in primates occurs mainly between subspecies and species, but has also been documented between genera (Zinner et al. 2011). However, well-documented cases of primate hybridization are rare (Cortes-Ortiz et al. 2007), and in most instances identification of hybrids is based on morphological characteristics alone. One can make the assumption that an individual is a hybrid if it is found in a zone where two sympatric or parapatric (adjacent) taxa have overlapping distribution ranges, and the individual displays a phenotype intermediate between these two (as in Detwiler et al. 2005). Characters intermediate between those of two parental species have been reported for the Hamadryas (*Papio hamadryas*) and olive baboon in Awash, Ethiopia (Nagel 1973). In a visual survey of kinda baboons (*Papio kindae*) and grayfoot chacma baboons (*Papio ursinus griseipes*) of the Kafue River Valley in Zambia, Jolly et al. (2011) observed individuals within groups that had phenotypes intermediate between those of the kinda and grayfoot baboons, and both mitochondrial and Y-chromosome contributions of from both parental species were documented in individuals within these groups.

The Bale monkey is sympatric with the black-and-white colobus monkey (*Colobus guereza*) in some areas, but the species' preference for heights excludes it from the main distribution range of the grivet and vervet monkeys, who prefer lower altitudes. However, as part of their study aimed at increasing knowledge about the Bale monkey distribution in southern Ethiopia, Mekonnen et al. (2011) noted the presence of grivet monkeys at approximately 2,500 m asl, which is within the altitudinal range of the Bale monkey. Although they did not observe any sympatry between the two species, the researchers reported likely hybrids between the two species at three localities in the Oromia Region. The supposed hybrids were reported to have phenotypes intermediate between Bale and grivet monkeys, displaying white tail tips and white brow bands that are larger in grivet monkeys and not present in Bale monkeys. Hybrids were only found in areas without bamboo, perhaps indicating differences in niche selection between parental species and hybrid offspring, or possibly the existence of a hybrid zone, but this needs to be investigated further.

Aims and objectives:

Morphological analyses of the grivet monkey, the vervet monkey and the Bale monkey have been done (Elton et al. 2010), but molecular phylogenies such as in van der Kuyl et al. (1995) have never included the Bale monkey. Shimada (2000), and Shimada et al. (2002) found great haplogroup diversity in RFLP-markers both within and between grivet monkey populations of Central Ethiopia, indicating a large degree of intraspecific variation in mitochondrial DNA. As the taxonomic status of the *Chlorocebus*-monkeys has been debated for a century, this thesis will attempt to clarify the position of the taxa in the genus relative to one another, by constructing a molecular phylogeny which for the first time includes the Bale monkey. Outgroups will be the blue monkey (*Cercopithecus mitis*) and the Hamadryas baboon, both Old World monkeys, belonging to the genera *Cercopithecus* and *Papio,* respectively.

The main questions raised in this thesis are:

I) Do *C. aethiops*, *C. pygerythrus* and *C. djamdjamensis* constitute monophyletic groups within *Chlorocebus*?

II) Can hybridization between *C. djamdjamensis* and *C. aethiops* and\or *C. pygerythrus* be detected?

III) Is *C. djamdjamensis* a separate species within *Chlorocebus*?

An aim of this thesis is to contribute to the conservation of the Bale monkey, by attempting to clarify its taxonomic position within the *Chlorocebus* genus. If the taxonomic status of a population is not determined, this can result in unrecognized species becoming extinct, and non-threatened or hybrid taxa may be prioritized for protection over the endangered species (Frankham et al. 2004). The 'subspecies problem' presents a difficult task for conservation biologists, who need to debate the question of whether conservation efforts should be directed at populations, species or subspecies (Ryder 1986).

Materials and methods

Study species

The Bale monkey

The full distribution range of the Bale monkey is not known, with new populations found only recently in the West Arsi and Guji zones of the Oromia Region as well as the Sidama zone in the SNNP (Southern Nations, Nationalities and Peoples' Regional State) (Mekonnen et al. 2011); Fig.2. It prefers bamboo or forest edge habitats, but has also been observed in degraded bamboo forest and tree dominated forests without bamboo. The total number of Bale monkeys is not known, but is estimated to be between 2,000-3,000 throughout its known distribution range (Mekonnen et al. 2011). Reproduction and population structure has not been studied, but the animals likely form stable social groups. Exact number of individuals in each group has been found to vary from three to 60 individuals (Mekonnen et al. 2011).

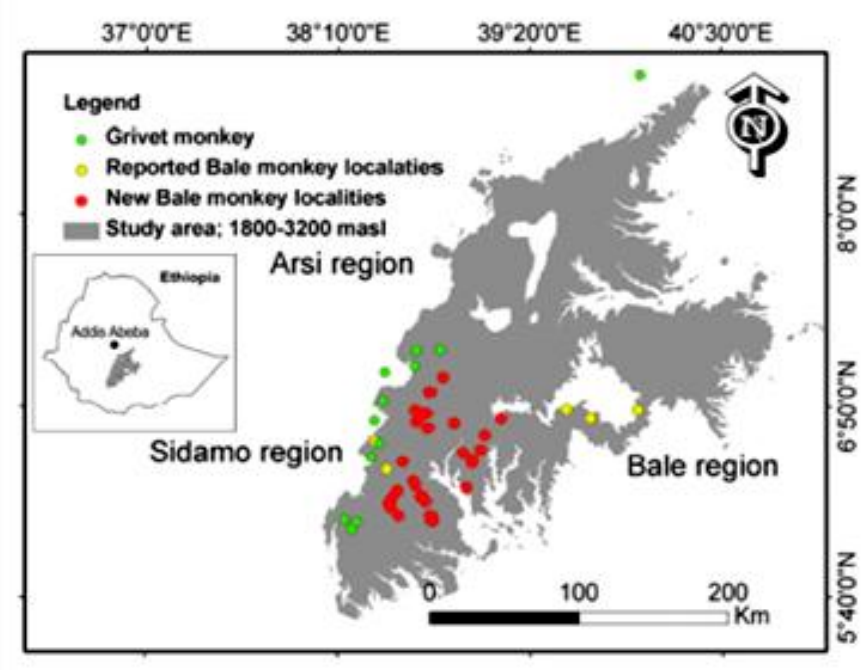

Fig. 2: Map of Bale monkey and grivet monkey locations in southern Ethiopia, adapted from Mekonnen et al. (2011)

The grivet monkey

The range of the grivet monkey extends from Khartoum in the north of Sudan to Mongalla in the south of the country, and it is also found in Djibouti, Ethiopia and Eritrea where it is found south of the River Omo and as far east as the Ethiopian Rift Valley (Butynski et al. 2008); Fig.2. It prefers open grassland and savanna, and feeds mainly on acacia seeds, flowers, foliage and gum. Figs and other fruiting trees can also be included in the diet (Cawthon Lang 2006).

The vervet monkey

Vervet monkeys are among the primate world's most devoted omnivores, but have a strong preference for fruit and flowers (Cawthon Lang 2006). In Africa the vervet monkey is distributed from the Ethiopian Rift Valley, highlands east of the Rift, and southern Somalia in the north, through the eastern lowlands of Ethiopia, Kenya, Tanzania, Uganda, Zambia (east of the Luangwa Valley), Malawi, Mozambique, Zimbabwe, Botswana and South Africa (Butynski et al. 2008); Fig.3. Habitat preferences are mainly like those of the grivet.

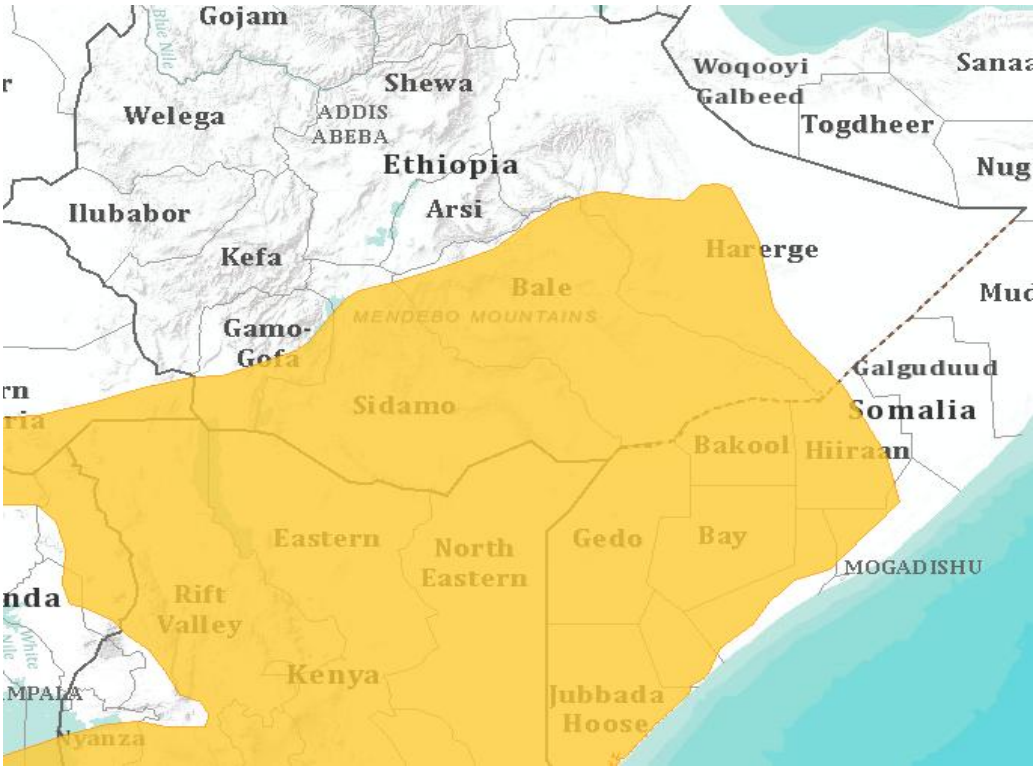

Fig. 3: Vervet monkey distribution in Ethiopia and surrounding countries (from IUCN).

Contacts

In this thesis it has been necessary to gather information by personally communicating with the researchers who have been working in one way or the other on the Bale monkey. These researchers are listed in Appendix 1.

Molecular markers

Finding the optimal molecular marker to distinguish between closely related taxa is a challenge. Both mitochondrial and nuclear markers should be used, to represent both the maternal and paternal lineages; in cases where hybridization between taxa is common, this is particularly important. A good marker should not show too much intraspecific variation, nor should it be over-conservative, which is the case for many protein-coding genes – e.g. those coding for active sites of enzymes; exceptions to this generalization is the major histocompatibility complex (MHC) in mammals, and the self-incompatibility (SI) loci in plants (Frankham et al. 2004). *Microsatellites*, or short tandem repeats (*STRs*), are repeating sequences of DNA, typically 1-5 bases in length. They are frequently used to measure genetic diversity on the population level, but are not commonly used for closely related species. *Restriction fragment length polymorphisms* (*RFLPs*) can be used for investigation of relationships between closely related taxa, as well as for population studies, DNA fingerprinting, gene mapping and studies of hybridization and introgression. The drawbacks of this technique are high expenses, and labor- and technically demanding procedures, requiring large amounts of DNA for each DNA digestion. *Short interspersed nuclear elements* (*SINES*), such as the primate *Alu* elements, have proven useful in phylogenetic studies among suborders, families and genera; one of the reasons for this is the fact that they are nearly homoplasy-free markers, meaning character states observed represent an unique evolutionary event (Li et al. 2009). However, SINEs are sensitive to disruptions because of hybridization. *Y*- and *X*-chromosomal markers are frequently used to resolve phylogenetic relationships, and have been used in primate studies such as inTosi et al. (2006); (2008).

In this study I planned to apply mitochondrial and nuclear sequences to produce a phylogeny. Mitochondrial DNA is used widely in evolutionary studies of mammals because it evolves quickly (Brown et al. 1979; Moritz et al. 1987; Kocher et al. 1989), there is no recombination, and it exists in large numbers in each cell (Robin and Wong 1988). The rates at which animal single copy nuclear (scn) and mitochondrial DNA evolves varies between

different groups of organisms (Moritz et al. 1987), but Brown et al. (1979) estimate that in vertebrates, the rate of mtDNA evolution is 5-10 times faster than vertebrate scnDNA. This rapid mutation rate makes it possible to distinguish closely related species, because a sufficient amount of point mutations have accumulated (Brown et al. 1979; Kocher et al. 1989). The following mitochondrial markers were used in this study:

The 12S rRNA gene

The 12S gene has been used in several phylogenetic studies because of its easy amplification and applicability (Janczewski et al. 1995; Montgelard et al. 1997; Palma and Spotorno 1999; van der Kuyl et al. 2002; Neumann et al. 2006). It has proved suitable for distinguishing primate species, exhibiting a low degree of variation at the population level, but a high resolution at the species and subspecies level (van der Kuyl et al. 2000).

Cytochrome b (cyt b)

Cyt b is an approximately 1140 bp long protein-coding gene found in all mammals (Irwin et al. 1991), and the most widely used gene in phylogenetic studies (Meyer 1994). It has been used to infer phylogenetic relationships in a range of different animal phyla (Irwin et al. 1991; Smith and Patton 1991; Bernardi and Powers 1992; Howell 1993; Parson et al. 2000; Male et al. 2012; Naidu et al. 2012).

The Cytochrome Oxidase (subunit I)-gene (COI)

COI is a protein-coding mitochondrial gene used widely in phylogenetic studies, exhibiting low intraspecific, but high interspecific variation (Folmer et al. 1994; Knowlton and Weigt 1998; Frohlich et al. 1999; Hebert et al. 2003a; Hebert et al. 2003b; Johnson et al. 2012). Over the last decade, there has been increased interest in using this gene for DNA 'barcoding' – a method that employs a genetic marker for accurately determining the species of an organism (Hebert et al. 2003a). Barcoding has proven useful in e.g. wildlife forensics (i.e. poaching and illegal trade of animals and animal products), where species identification can prove difficult (Ogden et al. 2009), but has also proven to be of great use in phylogenetic studies.

The 16S rRNA gene

The 16S gene is more conservative than the above-mentioned markers, but has nevertheless been used to reliably determine relationships between taxa on several taxonomic levels,

ranging from prokaryotes (Bonen and Doolittle 1976; Felske et al. 1998) to mammals (Querouil et al. 2001; Guha et al. 2007; Rueness et al. 2011).

Several nuclear markers designed by Wildman et al. (2009) for New World monkeys (*Platyrrhini*) were used because of their success in resolving the Platyrrhine phylogeny. Markers used are listed in Appendix 2.

Sampling and DNA extraction

Tissue samples of Bale monkey, grivet monkey, vervet monkey and blue monkey were collected from ten locations in Ethiopia (Fig.4). Samples collected were stored on 96 % ethanol. Additionally, zoo samples of two specimens of Bale monkey (taken post mortem) were included, as well as sequences from GenBank (Table 1).

For the DNA isolation, tweezers and a scalpel were used to cut off small fragments of the tissue, then added to marked Eppendorf-tubes containing 20 µl Proteinase K and 980 µl TRIS (tris (hydroxymethyl) aminomethane) buffer. Scalpel blades were used only once for each sample, and tweezers were cleaned after each sample had been processed, using 96 % ethanol and burning. Samples were incubated at 55°C for 1 hour on a thermomixer (Eppendorf, Hamburg, Germany) at 1400 rpm.

For the washing procedure, 800 µl of phenol-chloroform was added to the incubated samples, mixed, and spun down at 4°C for 30 minutes in a Centrifuge 5415R (Eppendorf) at 1400 rpm. 700 µl of the supernatant was transferred to new tubes and mixed with 750 µl phenol-chloroform before it was spun down at 40°C for 30 minutes, at 1400 rpm. The supernatant was transferred to new tubes, mixed with 700 µl 96 % ethanol, and spun down at 4°C for 30 minutes. The supernatant was discarded, taking care not to remove the DNA pellet, and 1 ml 70 % ethanol was added. The samples were spun down for 10 minutes, and the procedure repeated one more time. The samples were dried on a heating block at 55°C to ensure complete evaporation of the ethanol before100 µl MilliQ H_2O was added, and stored at -20°C.

Because of problems during sequencing, the isolated DNA was analyzed using Nanodrop spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, DE, USA). The analyses indicated pollution, possibly from phenol-chloroform residues in the samples, which may inhibit amplification and give poor-quality sequences. New DNA isolations were

therefore performed using the E.Z.N.A. ® Tissue DNA extraction kit (Omega Bio-Tek, Norcross, USA), following the instructions of the manufacturer.

Table 1: List of samples and locations in Africa and Europe (zoo specimens)\ accession numbers (for GenBank individuals). Gene regions sequenced for each individual indicated with **X**. '*Cyt b* partial' represents a short fragment of the gene, '*Cyt b* total' represents the entire gene. Zoo samples: a) Czech Republic: Zoo Jihlava, and b) Slovakia: Bojnice zoo.

Species	**Location (# of specimen)**	**Genbank accession no.**	**12S**	**COI**	**Cyt b partial**	**Cyt b total**
Chlorocebus djamdjamensis Bale monkey	**Odobullu**		**X**	**X**	**X**	**X**
	Czech Republic [a)]		**X**	**X**	**X**	**X**
	Slovakia [b)]		**X**	**X**	**X**	**X**
	Sidamo (#1)		**X**	**X**	**X**	**-**
	Sidamo (#2)		**X**	**X**	**X**	**-**
	Sidamo, Cocosa		**-**	**-**	**X**	**-**
	Sidamo, Arbegona		**-**	**-**	**X**	**-**
Chlorocebus pygerythrus Vervet monkey	**Arbaminc**		**X**	**X**	**X**	**-**
	Robe		**X**	**X**	**X**	**-**
	Tanzania	EF597501.1	**X**	**X**	**X**	**X**
	South Africa	EF597500.1	**X**	**X**	**X**	**X**
Chlorocebus aethiops Grivet monkey	**Jibat (#1)**		**X**	**X**	**X**	**-**
	Jibat (#2)		**X**	**-**	**X**	**-**
	Finote Selam		**X**	**X**	**X**	**-**
	Bahir Dar		**X**	**-**	**X**	**-**
	Awash National Park, Ethiopia	NC007009.1	**X**	**X**	**X**	**X**
Chlorocebus sabaeus Green monkey	Senegal	NC008066.1	**X**	**X**	**X**	**X**
Chlorocebus tantalus Tantalus monkey	CAR (Central African Republic)	NC009748.1	**X**	**X**	**X**	**X**
Cercopithecus mitis Blue monkey	**Jimma**		**X**	**-**	**X**	**X**
Papio hamadryas Hamadryas baboon	N/A	NC001992.1	**X**	**X**	**X**	**X**

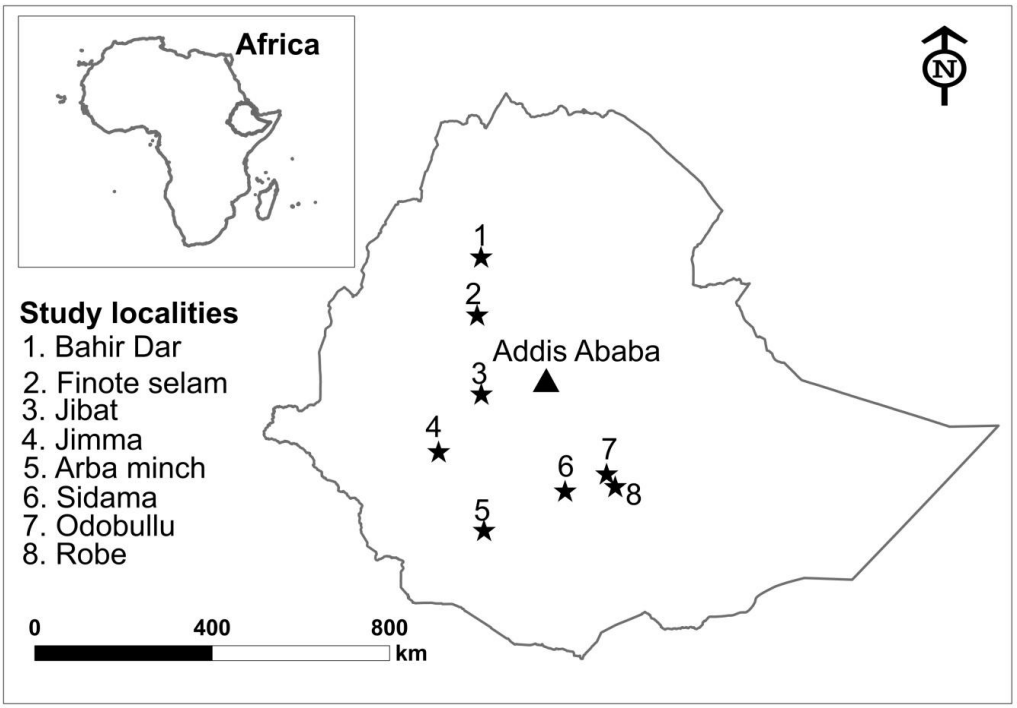

Fig.4: Map of Ethiopia, indicating the eight locations of the samples taken in the field from seven Bale monkeys (locations 6 and 7), two vervet monkeys (locations 5 and 8), four grivet monkeys (locations 1, 2 and 3) and one blue monkey sample (location 4). Locations indicated by stars; numbers correspond to list of study localities.

DNA amplification and sequencing

Altogether, 13 mitochondrial and 10 nuclear primer pairs were tested and applied in this study (see Appendix 2), for amplification of four mitochondrial and 10 nuclear regions.

Hot-start PCRs were performed for all amplifications. The reactions were carried out in a total volume of 25 µl containing 2.5 µl 10x PCR-buffer containing 15 mM $MgCl_2$ (Qiagen, GmbH, Hilden, Germany), 0.8 µl 10 mM primer, 0.5 µl 25 mM $MgCl_2$ (Qiagen), 0.25 µl HotStar Taq Polymerase (Qiagen), 0.20 µl 0.4 % bovine serum albumin (BSA; Promega, Madison, USA), 2.5 µl 2mM dNTP (Fermentas, Burlington, Ontario, Canada), 17 µl MilliQ H_2O. Primers were ordered from www.eu.idtdna.com (Integrated DNA Technologies Inc., Coralville, Iowa, USA) and www.eurofinsdna.com (Eurofins MWG Operon, Ebersberg, Germany). To ensure optimal primer specificity for the study species, I designed primers for amplification of cyt b,COI and 16S, by aligning sequences of the respective genetic markers taken from GenBank *Chlorocebus* individuals, in addition to those sequences I had available from my own samples. I then searched for conserved regions, and chose sequences of 15-20 bases in length. I made reverse primers manually, and used Primer3Plus (Untergasser and Nijveen

2006, 2007) and PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) to find primers with low probabilities of self-priming or formation of needle-pin structures, as well as optimizing the annealing temperature for the primer pairs.

For all regions the following PCR program was used on an Eppendorf Mastercycler ep gradient S: Initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing for 30 sec, and elongation at 72°C for 30 sec, followed by a final extension at 72°C for 4 min. Gradient-PCRs were run to optimize the annealing temperatures of the nuclear primers; see Appendix 2 for annealing temperatures.

PCR products were purified using 2 µl 10 times diluted ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) to 5 µl PCR product, incubated at 37°C for 30 minutes followed by 15 minutes at 80°C. In an attempt to achieve better sequence quality, some of the PCR products were purified using Qiaquick PCR Purification Kit (Qiagen). Prepared amplicons for sequencing at the CEES ABI-laboratory (Department of Biology, University of Oslo) contained 1 µl purified PCR- product, 1 µl 10 µM primer (the same primers as used in the PCR) and 8 µl H_2O. All regions were sequenced in both directions. Sequencing in the ABI lab was done on the ABI PRISM® 3730 Genetic Analyser (Applied Biosystems, Foster City, CA) with BigDye® Terminator v3.1 Cycle Sequencing Kit, and cleaned using Agencourt CleanSEQ® for ABI Big Dye Terminator.

Assembly and alignment of sequences

Editing and assembly of sequences were done in BioEdit Sequence Alignment Editor (Hall 1999) and ContigExpress - a component of Vector NTI Advance 11.5.1 (Invitrogen, Life Technologies, Paisley, UK). Aligning of the cyt b and COI sequences was done manually, and 12S sequences were aligned with ClustalW in BioEdit and in MEGA v. 5.0 (Tamura et al. 2011). The alignments were controlled by referring to the chromatograms. Where ambiguities could not be resolved, the samples were either re-sequenced, or left out of further analyses.

Phylogenetic analyses

Analyses included the 14 samples collected in this study, as well as sequences obtained from GenBank (see Table 1). Cercopithecus mitis and Papio hamadryas were used as outgroups in the 12S and cyt b analyses, but for COI only Hamadryas baboon was used.

Pairwise distances between sequences possibly representing different taxa were calculated in MEGA v.5 (Tamura et al. 2011), based on the entire cyt b gene and using the Tamura-Nei model.

Two methods of phylogenetic analysis were used in this thesis: Neighbor-Joining (NJ) and Bayesian inference (BI). Models for BI analyses were determined by using MrModeltest v.2.3 (Nylander 2004) through the freely available University of Oslo Bioportal server (www.bioportalen.uio.no). For 12S GTR+G was chosen as the best model, while for COI and 'cyt b partial' (a short fragment of the gene; see results) HKY+G was chosen was chosen when using both the Akaike Information Criterion (AIC) and the hierarchical ratio test (hLRT). For 'cyt b total' the best model was HKY+I+G when using hLRT, and GTR+I+G when using the AIC.

BI analyses were performed using MrBayes v.3.2.0, with two independent runs with four chains for each analysis (one cold chain and three heated chains); the Markov Chain Monte Carlo (MCMC) algorithm was set for 1,000,000 or 2,000,000 generations dependent on the dataset. All analyses sampled every 100 generations, discarding the first 25% of these (a burn-in of 2500 or 5000 for 1,000,000 and 2,000,000 generations, respectively).

The NJ analyses were performed in PAUP* 4.0 (Swofford 1998) through the University of Oslo Bioportal server, using HKY as distance measure for COI and *'cyt b partial'* and GTR for 12S. Branch support was estimated with 10,000 bootstrap replicates.

Results

Of the 10 nuclear and four mitochondrial markers used in this study, only three mitochondrial markers gave sequences of sufficient quality to be used in the analyses.

The entire COI gene was successfully amplified, and the final alignment of 1384 bp used nine individuals from this study and six sequences from GenBank (Table 1).

The 12S gene was successfully amplified for 365 bp, using twelve individuals from this study in addition to six from GenBank (Table 1).

I could only successfully amplify 'cyt b total' (~1140 bp) for five of my samples (Table 1). Upon examination of the aligned sequences, which had been amplified for the entire fragment, I located the primer sequence, but discovered nucleotide variation compared to the original sequence. Naidu et al. (2012) speculated that the primers designed by Kocher et al. (1989) may not be specific enough to be able to amplify or sequence the gene for all mammal species. This led me to assume that the failed sequencing was due to a mismatch between primer and template, and new primers were designed, but without amplification success. The cyt b phylogeny were thus based on a 416 bp long fragment of the 5'-end of the gene ('cyt b partial'), with 14 individuals from this study and six individuals from GenBank included in the analyses (Table 1). The 'cyt b total' dataset included sequences of four individuals from this study and six individuals from GenBank.

Table 2: Pairwise distances between four of the taxa in this study for the cyt b gene, using the Tamura-Nei model in MEGA v.5 (Tamura et al. 2011). Numbers above vertical columns correspond to the numbers for each individual (indicated in parentheses).

Sample	1	2	3	4
(1) *C. djamdjamensis* - Odobullu	-			
(2) *C. djamdjamensis* - Slovakia	0.054	-		
(3) *C. aethiops* - NC007009.1	0.057	0.021	-	
(4) *C. pygerythrus* - EF597500.1	0.052	0.041	0.048	-

Degli-Esposti et al. (1993) found that towards the 3'-end the cyt b gene is less conserved, indicating that an exclusion of this part of the gene might have consequences when used in phylogenetic analyses. Preliminary analyses were done on the '*Cyt b* total' dataset, to control for any changes in topology compared to the '*Cyt b* partial' dataset, when the entire gene was analyzed. The analyses did not show any notable incongruence (results

not shown). Furthermore, several authors have based phylogenetic analyses on partial cyt b-sequences with reliable results (Janczewski et al. 1995; Lopez et al. 2000; Ren et al. 2004; Rego et al. 2007). Parson et al. (2000) claimed that a 358 bp fragment of cyt b has the widest taxonomic representation in nucleotide databases. When retrieving primate sequences from GenBank, I found that the majority of cyt b sequence data was of this short fragment.

Although 16S was amplified during PCR, sequences were not of sufficient quality to be used in the analyses, despite repeated attempts of reamplification and resequencing

The nuclear markers amplified showed little or no variation upon aligning the sequences, with variation mainly found between the ingroup taxa and the outgroup, *C. mitis*. The nuclear data has therefore been excluded from further phylogenetic analyses.

Pairwise distances between selected sequences from the *'*cyt b total*'* dataset (Table 2) showed that *C. djamdjamensis* from Odobullu differ between 5.2 and 5.7 % from the other individuals, which is a somewhat larger difference than between any other pair of sequences. The difference between *C. djamdjamensis* from Slovakia and *C. aethiops* from GenBank is 2.1 %, while between the former and *C. pygerythrus* from GenBank the difference is 4.1 %.

The final phylogenetic analyses were performed on three mitochondrial datasets: CO1, 12S, and 'cyt b partial' which all showed variation within the ingroup. However, not all samples were amplified successfully for all three regions, and thus not included in the respective phylogenies (Table 1).

Overall, the three regions analyzed give the same picture, although with varying degrees of resolution, with the COI tree (Fig. 5) providing the best resolved topology. A combined analysis of the three regions was performed, but the topology did not differ much from the COI topology and did not lend more support to the clades (results not shown). The NJ analyses produced overall the same topology as the BI analyses. The results are, thus, not shown but bootstrap branch support is displayed together with the posterior probabilities in the topologies (Fig. 5-7).

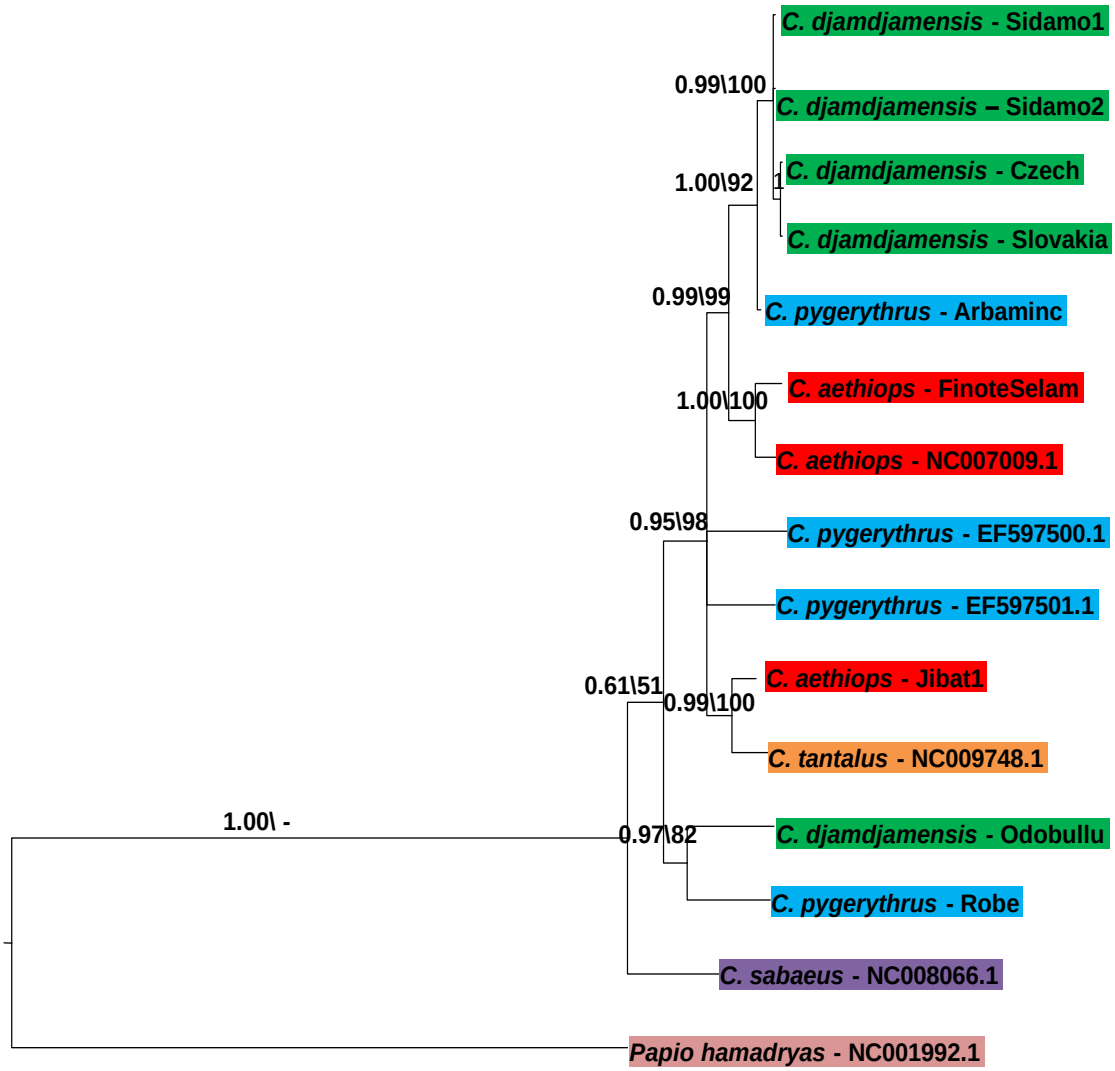

Fig.5: Bayesian Inference (BI) majority rule consensus tree of COI (with model HKY+G) for 14 *Chlorocebus* individuals identified as Bale monkey (*C.* djamdjamensis), grivet monkey (*C. aethiops*), vervet monkey (*C. pygerythrus*), tantalus monkey (*C. tantalus*) and green monkey (*C. sabaeus*). The Hamadryas baboon (*Papio hamadryas*) was used as outgroup. Posterior probabilities \ bootstrap support from NJ-analyses is shown above branches.

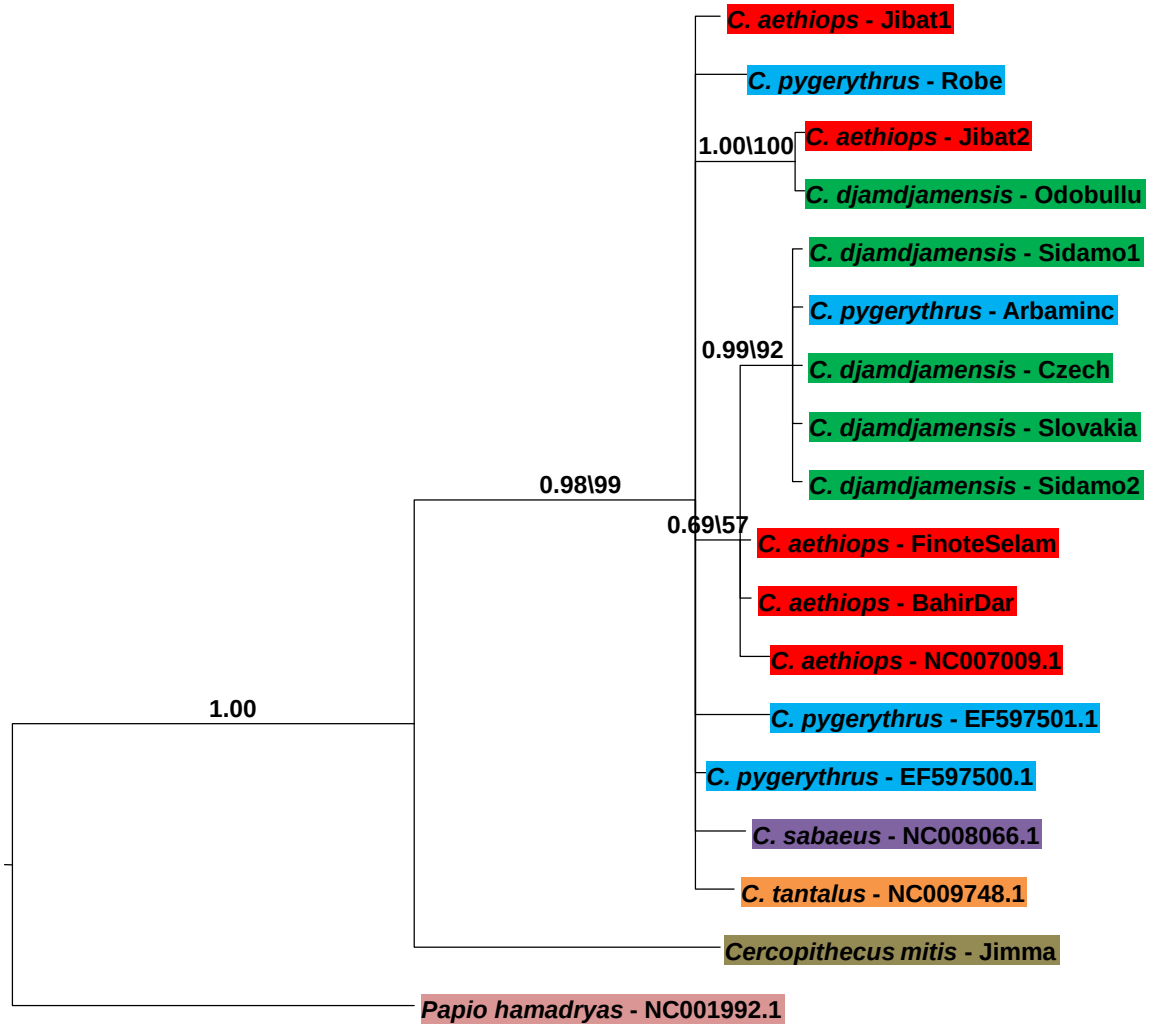

Fig.6: Bayesian Inference (BI) majority rule consensus tree of 12S (with model GTR+G), for 16 *Chlorocebus* individuals identified as Bale monkey (*C.* djamdjamensis), grivet monkey (*C. aethiops*), vervet monkey (*C. pygerythrus*), tantalus monkey (*C. tantalus*) and green monkey (*C. sabaeus*). The Hamadryas baboon (*Papio hamadryas*) and the blue monkey (*Cercopithecus mitis*) were used as outgroups. Posterior probabilities \ bootstrap support from NJ-analyses is shown above branches.

In each of the BI majority rule consensus trees (Figs. 5-7), the ingroup consisting of the *Chlorocebus* individuals in principle forms a polytomy. In the COI tree (Fig.5), the *C. sabaeus* individual is sister to this polytomy, although with very low support (posterior probabilities, PP = 0.61 \ bootstrap support (BS) = 51). All trees show highly supported clades within the polytomy.

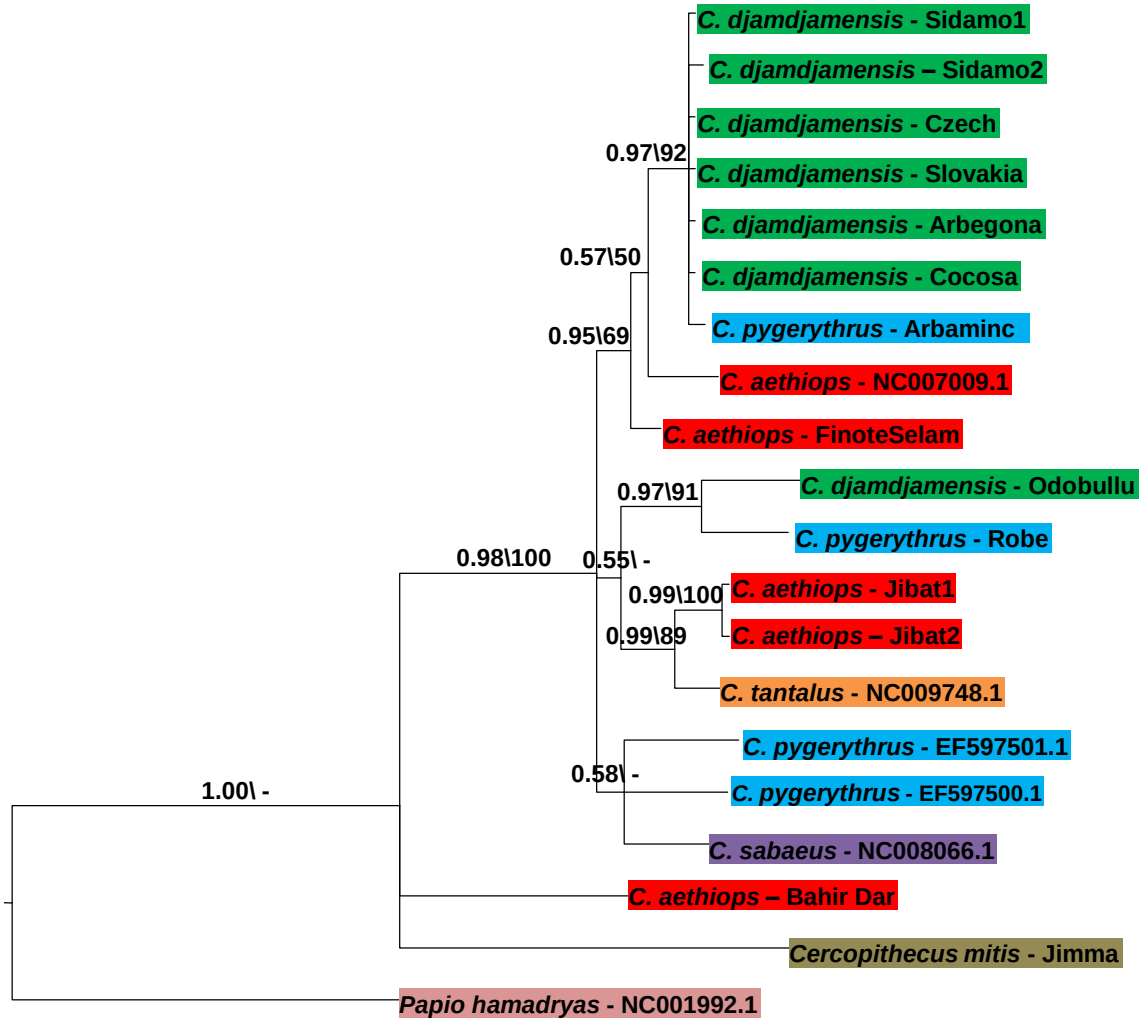

Fig.7: Bayesian Inference (BI) majority rule consensus tree of cyt b (with model HKY+G), for 18 *Chlorocebus* individuals identified as Bale monkey (*C.* djamdjamensis), grivet monkey (*C. aethiops*), vervet monkey (*C. pygerythrus*), tantalus monkey (*C. tantalus*) and green monkey (*C. sabaeus*). The Hamadryas baboon (*Papio hamadryas*) and the blue monkey (*Cercopithecus mitis*) were used as outgroups. Posterior probabilities \ bootstrap support from NJ-analyses is shown above branches.

One such clade, found consistently with high support (PP = 0.97-0.99 \ BS = 92-100) in all trees, includes all the *C. djamdjamensis*-individuals, except for the individual from Odobullu. *Chlorocebus pygerythrus* from Arbaminc consistently groups together with the *C. djamdjamensis*-clade; in CO1 as a sister to the *C. djamdjamensis* individuals, and in 12S and cyt b as part of a polytomy (Fig.6 and 7, respectively). *Chlorocebus pygerythrus* from Robe and *C. djamdjamensis* from Odobullu form a well-supported clade in the COI (PP = 0.97\ BS = 82) and cyt b (PP = 0.97 \ BS = 91) trees. Notably, in the 12S topology, *C. djamdjamensis* from

Odobullu forms a clade (PP = 1 \ BS = 100) with *C. aethiops* individual #2 from Jibat. The *C. aethiops* individuals do not constitute a monophyletic clade in either of the trees. In the COI tree, the C*. aethiops* individual from Finote Selam forms a highly supported clade (PP = 1 \ BS = 100) with the GenBank specimen of *C. aethiops* from Awash National Park, which is sister group to the *C. djamdjamensis* clade (PP = 0.99 \ BS = 99). The close relationship of these two to the *C. djamdjamensis* clade is seen also in the cyt b and 12S trees, although the individuals do not constitute a clade, but are part of a polytomy (in 12S also including the *C. aethiops* individual from Bahir Dar). In the *Cyt b* tree, the individual from Bahir Dar has, however, a quite divergent position, as part of a basal polytomy together with the outgroup taxon *Cercopithecus mitis* and the clade consisting of the remaining in group taxa. It is worth noting that *C. aethiops* from Jibat does not form a clade with the C*. aethiops* individuals from Finote Selam or the GenBank specimen of *C. aethiops* from Awash National Park in any of the trees. In the COI and cyt b trees the *C. aethiops* individuals from Jibat (only individual # 1 was amplified for COI) form a clade (PP = 0.99 \ BS = 100 for COI and PP = 0.99 \ BS = 89 for cyt b) with the GenBank specimen of *C. tantalus* from the Central African Republic (CAR). In the 12S tree, *C. aethiops* # 1 from Jibat is part of the basal polytomy, whereas *C. aethiops* # 2, as mentioned, forms a highly supported clade with *C. djamdjamensis* from Odobullu.

As with *C. aethiops,* the *C. pygerythrus* individuals do not constitute a monophyletic group in any of the topologies. In all trees *C. pygerythrus* from Arbaminc groups with the *C. djamdjamensis* individuals (with the exception of the Odobullu individual), or is sister to these. *Chlorocebus pygerythrus* from Robe forms a clade with *C. djamdjamensis* from Odobullu in the COI and cyt b trees, but in the 12S tree its relationship to the other taxa is unresolved. The *C. pygerythrus* individuals from GenBank are part of a polytomy in the COI and 12S trees, while in the cyt b topology they group together with *C. sabaeus,* although with very low support (PP = 0.58).

Discussion

It is not difficult to understand why taxonomists like Kingdon (1997) would describe all taxa in the *Chlorocebus* genus (treated as *Cercopithecus* by Kingdon) as subspecies of C. *aethiops*. The morphological similarities between the different *Chlorocebus* species are apparent to the observer, and misidentification seems common. In the aforementioned study by Shimada (2000), this author admitted having difficulties in distinguishing grivet monkeys by morphology in the wild, which is not surprising given the likeness especially between the grivet, vervet and Bale monkey. This confusion is confirmed by a simple search on the World Wide Web; attempting to attain pictures of a grivet will yield search results of pictures ranging from tantalus monkeys to Bale monkeys. An information search on any of the *Chlorocebus* monkeys will produce a range of conflicting results, giving both the generic names *Cercopithecus* and *Chlorocebus*, and often describing most of the species as subspecies of *C. aethiops.*

As this study produced phylogenies based only on mtDNA, establishing the Bale monkey's status as a genetically distinct species of *Chlorocebus* cannot be done with certainty. The disadvantage of using mtDNA in phylogenetic studies of closely related species is that it is maternally inherited (Potter et al. 1974), unlike nuclear DNA, which is inherited both from mother and father. Thus, analyses of mtDNA will mask hybridization events, because one will only be able to identify the maternal mtDNA lineage. Inferences on taxonomic relationships can be made based on the available data and constructed phylogenies, but the inclusion of nuclear markers is vital in assigning the correct taxonomic status to a taxon, particularly for species where hybridization is common.

Hybridization within the *Chlorocebus* genus has been described by Mekonnen et al. (2011) and Zinner et al. (2011), and is thought to be frequent between Bale monkeys and grivet monkeys (Mekonnen et al. 2011). Hybridization between grivet monkeys and vervet monkeys is also known (Groves 2001). Possible misidentification of individuals caused by morphological similarities, together with the suggested frequent hybridization events between the *Chlorocebus* monkeys has made the interpretation of the phylogenetic trees in this thesis a challenge.

Do C. aethiops, C. pygerythrus and C. djamdjamensis constitute monophyletic groups within Chlorocebus?

Going into this thesis I had three initial questions. The quick and easy answer to this first question is a negative. Neither *C. pygerythrus* nor *C. aethiops* form monophyletic clades within the phylogenies produced in this thesis and overall do not cluster consistently with their own conspecifics or the other species in the topologies, although with some exceptions. Similar results have been found by van der Kuyl et al. (1995) based on the 12S gene, and also in unpublished results using cyt b (Anagaw Atickem, pers. comm.). van der Kuyl et al. (2000) also demonstrated using the 12S gene that *Chlorocebus* (referred to as *Cercopithecus* in the paper) individuals in captivity were possibly misidentified on the basis of morphology. This implies that misidentification may also be a possibility in this study.

The grouping of the *C. aethiops* individuals from Jibat with the *C. tantalus* specimen from the Central African Rebublic (C.A.R) in the COI and cyt b trees is surprising, given that the known distribution ranges of the two species do not overlap. However, two of the countries bordering on Ethiopia and theC.A.R - Sudan and South Sudan- are areas severely affected by political instability. It is therefore possible that the range of the tantalus monkey actually extends into Ethiopia, and that the Jibat individuals are tantalus monkeys, or hybrids between the two.

The relationship of *C. sabaeus* to the other *Chlorocebus* is not clear; the individual included here forms a low-supported clade with the *C. pygerythrus* individuals from GenBank in the cyt b topology. It is possible that the *C. sabaeus* individual may actually be a vervet, as the origin of the sample is Senegal - a part of the distribution range of the vervet. However, the clade is not well-supported, and it is difficult to make any firm conclusions based on it.

Most of the *C. djamdjamensis* individuals form a clade with high support in all topologies together with the vervet individual from Arbaminc. The only exception is the inconsistent placement of the Odobullu individual. However, as I will discuss, there may be possible explanations for this result.

Can hybridization between C. djamdjamensis and C. aethiops and\or C. pygerythrus be detected?

The grouping in the COI and cyt b trees of the Bale monkey from Odobullu with the vervet monkey from Robe is a conundrum. The Odobullu individual was a juvenile found dead inside the Odobullu forest, most likely killed by a leopard. Odobullu is a secluded, densely forested area where Bale monkeys occur in high densities (Mekonnen et al. 2010a). The only other primate species found in this area is the black-and-white colobus and it therefore seems unlikely that the Odobullu individual is anything but a Bale monkey. However, some alternative explanations can be put forward. The first possibility is that both the Robe and the Odobullu individuals were vervets. Although vervet monkeys have not been observed in the Odobullu forest, they are found throughout southern Ethiopia and to the north of the Odobullu forest. One solution then is that the leopard killed the Odobullu monkey outside the forest, near Robe perhaps, and transported it to the location where it was found, for instance to feed cubs. This is of course speculation, but if it were the case, it would lead to the erroneous identification of the Odobullu individual as a Bale monkey. It is not likely that this was a vervet monkey that had ventured into the area on its own, as juvenile Cercopithecine monkeys tend to stay with their family group until they mature (in vervets around five years of age for males) (Macdonald 2004).

The second possibility is that the Robe individual was indeed a vervet, but that the individual found in Odobullu forest was a vervet x Bale monkey hybrid. The third possibility is that both the Robe and Odobullu individuals were Bale monkeys or Bale monkey x vervet hybrids. The vervet from Robe was found, killed by farmers while raiding crops, and was identified on the spot as a vervet monkey by skilled field-assistants (Anagaw Atickem, pers. comm.). However, if there has been hybridization, direct or through back-crossing, morphological characters may not be reliable, and the Robe individual could have been carrying Bale monkey mtDNA but not displaying the phenotype of a Bale monkey. What is puzzling is that neither phylogeny shows any consistent grouping of these individuals with any of the other *Chlorocebus* species. In the cyt b topology the Robe + Odobullu clade comes out as a sister group to the grivet Jibat + tantalus monkey clade, although with low support. The 12S topology only contributes to the confusion, as the Odobullu individual forms a clade with grivet # 2 from Jibat. However, in the case of grivet # 2 the sample proved difficult to

amplify, and when amplified of lower sequence quality than the other samples. Contamination is therefore a possibility that must be taken into consideration. In the 12S tree the vervet from Robe is part of a polytomy, with its relationship to the other vervets and the grivet # 2 from Jibat remaining unresolved, thus providing no further resolution.

The Bale monkey clade (not including the Odobullu individual) groups together with high support in all topologies. However, the clade also includes the vervet from Arbaminc, and four alternative explanations can be put forward for this grouping: 1) The samples have been mixed in the field or in the laboratory; 2) the Arbaminc individual is a Bale monkey x vervet hybrid; 3) all the Bale monkeys are vervets or vervet monkey x Bale monkey hybrids; or 4) alternatively that all the supposed Bale monkeys in this clade are grivet x Bale monkey hybrids, and the vervet from Arbaminc a grivet x vervet hybrid.

The possibility of the samples being mixed is highly unlikely. Samples of all individuals were taken directly in the field and added to correctly marked tubes. DNA was isolated twice, amplified and sequenced several times, and preliminary topologies based on the first samples isolated produced the exact same results (based on cyt b) as the final topologies, which were based on sequences from the second isolation. As to the other alternative explanations, it is possible that the specimens from Sidamo and the two zoo specimens are all hybrids, based on morphological characters and the possible hybridization occurring in the Sidamo area; and particularly if hybridization is common. Pictures of Emma, the Bale monkey from Bojnice Zoo in Slovakia (Fig.1), reveals a thin, white brow band thought to be absent in 'pure' Bale monkeys and only present in hybrids and introgressed individuals (Mekonnen et al. 2011). The feature was also observed in putative Bale monkey hybrids by Mekonnen et al. (2011); see Appendix 3. However, Dr. Jan Robovsky of Zoo Jihlava in the Czech Republic, does not agree with the 'white brow band-hypothesis', and refers to a holotype of the Bale monkey in the Berlin Museum für Naturkunde, also possessing this trait (Jan Robovsky, pers. comm.). Hybrids are also thought to display an intermediate coat colour and a tail of intermediate length (Mekonnen et al. 2011), but it is unclear if all the specimens displayed these traits, and if these traits may be results of individual phenotypic variation.

Based on the genetic proximity of the grivets from Finote Selam and GenBank to the Bale monkey + Arbaminc vervet - clade, one possibility is that this latter clade consists of grivet x Bale monkey hybrids. This would then imply, as mentioned above, that the vervet

from Arbaminc is not a vervet, but a vervet x grivet hybrid. If the vervet from Arbaminc is the result of a grivet female hybridizing with a vervet male, it could display a vervet phenotype, but its mtDNA would be that of a grivet. Assuming then, that the vervet from Robe (grouping together with the Odobullu individual) is not in fact a 'pure' vervet but a Bale monkey x vervet hybrid, this chain of reasoning opens up for the possibility that the Bale monkey + Arbaminc vervet clade does not contain any 'pure' Bale monkey individuals, and that the Bale monkey from Odobullu is the only 'pure' Bale monkey of the individuals in this study. However, left with only phylogenies based on mtDNA, it is not possible to discriminate between these alternative explanations, and nuclear data is strongly needed to make firm statements about the importance of hybridization for the observed results.

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Is C. djamdjamensis a separate species within Chlorocebus?

This is perhaps the most difficult question to answer, based on the phylogenies available. The fact that the majority of the *C. djamdjamensis* individuals (excluding the Odobullu individual form a well-supported clade in all topologies points to the conclusion that *C. djamdjamensis* is in fact a separate species. However, to draw this conclusion, based on my topologies, I would have to make two assumptions linked to the argumentation above. One assumption would be that the entire *C. djamdjamensis* clade (including the vervet from Arbaminc) is in fact Bale monkeys, and not vervet or grivet hybrids. The other would be that the Bale monkey from Odobullu is not a Bale monkey, but a hybrid between a Bale monkey and a vervet or grivet. Based on my previous argumentation, I consider it, however, a possibility that the Bale monkey from Odobullu is the only 'pure' Bale monkey individual in this study, and that the Bale monkey + Arbaminc vervet clade does not contain Bale monkeys, but grivets or grivet x Bale hybrids.

If there is indeed ongoing hybridization between Bale monkeys and other *Chlorocebus* monkeys, and this hybridization produces fertile, viable offspring, applying the *biological species definition* would not assign species status to the taxon. Focusing on genetic rather than reproductive isolation, Baker and Bradley (2006) argue for a different view, defining a genetic species as "*a group of genetically compatible interbreeding natural populations that is genetically isolated from other such groups*". They furthermore put forward a quantitative measure to define genetic species (of mammals), based on the cyt b gene, and propose that

<5 % genetic divergence is sufficient to assign species status to a taxon. The distance calculated for the 999 bp fragment of the cyt b gene in this study shows a 5.2-5.7 % difference between the Odobullu individual and the three other sequences included (vervet, grivet and Bale monkey from Slovakia; Table 2), and only a 2.1 % difference between the Bale monkey from Bojnice Zoo, Slovakia, and the grivet from GenBank This is consistent with the topologies, and may lend support to the hypothesis that the Bale monkey clade contains grivet x Bale monkey hybrids rather than 'pure' Bale monkeys. If the Bale individual from Odobullu is indeed the only pure Bale monkey in this study, then the assignment of species rank would be justified, based on the criteria put forward by Baker and Bradley (2006). However, this conclusion is based on very few individuals, and further studies on the *Chlorocebus,* based on a larger sample size than in this thesis and using complete gene sequences for all species in the genus is needed.

It may be possible to argue for the Bale monkey's status as a species on the basis of its narrow ecological niche. The specialization on bamboo, which is a trait the Bale monkey does not share with any members of its genus, may require specialization of the gut because of the high cyanide content in the plant (Glander et al. 1989; Mekonnen et al. 2010b). If there are indeed differences in the structure of the intestinal system of the taxa, this may point towards a division of the taxa. As previously mentioned, Mekonnen et al. (2010b) reported that <80 % of the Bale monkey's diet consisted of young leaves of bamboo. Glander et al. (1989) reported this same preference in the gentle bamboo lemur (*Hapalemur griseus*) of Madagascar, but they found that young leaves of the bamboo species in their study (*Cephalostachyum perrieri*) contained no cyanide. The specialization seems therefore to be behavioral rather than related to gut morphology. Furthermore, preliminary investigations of the stomach morphology of the Bale monkey and vervets do not indicate any such differences, but a more exhaustive study is needed to draw any conclusions (Jan Robovsky, pers. comm.).

Conservation

Ceballos and Ehrlich (2009) suggest, based on their evaluation of discoveries of new mammal species, that many organisms in all groups may likely have gone extinct without being noticed, which implies an underestimation of species extinction. It is estimated that over the last millennia about 10 % of the world's species of birds have been eliminated by humans, and on a local scale, extinctions have reached more than 90 % in some cases (Dirzo and Raven 2003). A review of extinction rates for many groups of organisms, including vertebrates, presents the gloomy fact that over the last 300 years levels of extinction have been least several hundred times greater than the expected rate based on the geological record (Dirzo and Raven 2003).

A report by the IUCN Red List (IUCN 2008) showed that of the 5,487 mammal species assessed, nearly one-quarter of species (22.2 %) are globally threatened or extinct, with almost half of all extinct species being rodents (Order Rodentia). The dugongs and manatees of the order Sirenia are all classified as threatened, with one species already extinct in modern times. Of the odd-toed ungulates in the order Perissodactyla 81 % are threatened or extinct. Although the IUCN Red List has not updated its species status, in November 2011 it was officially declared that one such ungulate, the western black rhino (*Diceros bicornis longipes*), had become extinct in the wild (IUCN 2011). In the amphibian order, nearly one-third (32 %) of the world's species are known to be threatened or extinct, 43 % are known to be non-threatened, and 25 % have insufficient data to determine their threat status. At least 42 % of all amphibian species are declining in population numbers, and as many as 159 species may already be extinct. The order with the highest number of species with a higher than average level of extinction threat is the primates (Order Primates); of the 414 known species of primates, 49 % are considered threatened (IUCN 2008).

As a vulnerable species with a narrow niche, the Bale monkey is particularly exposed to the threats of human population expansion, and the resulting degradation and destruction of its habitat. Harvesting of bamboo forests in its distribution range diminishes the natural food supply and likely increases incidents of crop raiding, leading to conflicts with humans that in many cases result in killing of the animals (Mekonnen et al. 2010b; Mekonnen et al. 2011). The loss of genetic diversity is a concern for small populations, but hybridization with more common species - which may be the case for the Bale monkey - is

also a serious threat, and may cause a species to be 'hybridized out of existence' (Frankham et al. 2004).

Conservation actions require adequate knowledge about the genetics, ecology, distribution and behaviour of endangered taxa, and molecular techniques can aid in this task by monitoring the genetic status of species and populations. One aim of this thesis was to contribute to the knowledge on the Bale monkey, by producing the first molecular phylogeny of the *Chlorocebus* with the Bale monkey included. To resolve the taxonomic debate over the correct assignment of species or subspecies status for the members of the genus, however, is beyond the scope of this thesis. The surprising incongruence between the morphological identification of the sampled individuals in the field and the genetic data observed may indicate the presence of cryptic variation. Correct taxonomic classification of the species becomes more complicated with the possibility of hybridization. To resolve the uncertainties of the *Chlorocebus* phylogeny additional research is required, using a larger sample size and including nuclear data.

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Appendix 1

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Jean-Marc Lernould	CEPA - Conservation des Espèces et des Populations Animales, France

Appendix 2

Mitochondrial primers

Primer name	Sequence (5'- 3')	Annealing Temperature	Reference
16s-ulviF	TCGTAAACCCTATTGTCGAT	55°C	Eli Rueness
16s-ulviR	CCAATCAGTGAAATTGACCT	55°C	Eli Rueness
Cytb1_F (L14724)	CGAAGCTTGATATGAAAAACCATCGTTG	55°C	(Irwin et al. 1991)
Cytb1_R (H15149)	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	55°C	(Kocher et al. 1989)
Cytb2_F (L15162)	GCAAGCTTCTACCATGAGGACAAATATC	50°C	(Irwin et al. 1991)
Cytb2_R (H15915)	GGAATTCATCTCTCCGGTTTACAAGAC	50°C	(Irwin et al. 1991)
COI_H (HCO2198)	TAAACTTCAGGGTGACCAAAAAATCA	40°C	(Folmer et al. 1994)
COI_L (LCO1490)	GGTCAACAAATCATAAAGATATTGG	40°C	(Folmer et al. 1994)
12s (L1091)	TGACTGCAGAGGGTGACGGGCGGTGTGT	55°C	(Kocher et al. 1989)
12s (H1478)	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	55°C	(Kocher et al. 1989)
16S_A718F	CGCAAGTGCCCCTTCTCGCA	63°C	This study

16S_A1246R	TGCTATTTTGTGCTTGGGTGGTGTT	59,5°C	This study
16S_B1247R	TTGCTATTTTGTGCTTGGGTGGTGT	59,5°C	This study
16S_B314F	GCCACCGCGGCCATACGATT	63°C	This study
Cytb_Ane1_F	CCCACTCTCACACGATTCTTCA	56°C	This study
Cytb_Ane2_F	ATCCCATATATCGGAACTAAT	47,5°C	This study
CO1 a=mtD12	CCAACAGGAATTAAAATTTTTAGATGATTAGC	54°C	http://crandalllab.byu.edu/PrimerDatabase.aspx
CO1 b	TCCAATGCACTAATCTGCCATATTA	54°C	http://crandalllab.byu.edu/PrimerDatabase.aspx
Cytb2_C784R	GTGGGTCGGCTGGGGTGTAG	62,6°C	This study
Cytb2_D1038R	GGGCTGGCTTACTGGCTGGC	63,8°C	This study
CO1_A986R	CCATGAAGTGTAGCAAGTCAG	53°C	This study
CO1_A62F	TATTCGGTGCATGAGCTGG	55°C	This study
CO1_B1498R	GGGGACAGCCATTTAATCATTC	54°C	This study
CO1_B520F	CCCGCCATATCCCAGTATC	54,5°C	This study
CO1_C1498R	GGGGACAGCCATTTAATCATTC	54°C	This study
CO1_C1001F	GATCTGCCGCAATACTTTGAG	53,5°C	This study

Nuclear primers from Wildman et al. (2009)

Primer name	Sequence	Annealing Temperature
1p31.1_F	GTAAAGTATCCTTATTGCAGCATG	40
1p31.1_R	CCAACTTAATACCTATTTTGTCTG	
1q31.3_F	TGAAGGTCTAGATTTCATTTTAAGTTCAC	52,7
1q31.3_R	CAGTGTTATCTCAAACCCCTGACA	
2p22.3_F	AGTGTTCCTTTTGGAAATGACCAGAAGT	53
2p22.3_R	CTTCCACCTCTCTAGCTTTCAGRCTC	
3q22.2_F	AATGTGATCAGAGTTGAAATGAAATCAT	55,4
3q22.2_R	AGCAATAGAAATGGAATTAATCATGACTC	
5p15.33_F	TCTGGTTTTCAGCTGCTCATGCCAG	61
5p15.33_R	TTCGATAAACCTAGGACCYGTCCAGG	
6p22.3_F	GCTGATTTCACCTGTTTCACAGTG	59
6p22.3_R	TTTGTCTCTTTTACACTGCCACAATC	
8q23.1_F	ATAGAAACATGTTATRGAAACTAGTCACA	50

8q23.1_R	AGGACAAAGACAAARGGATAGTATG	
10p12.33_F	ATTCTATTCCCTGTGATGAWAGCAGA	53
10p12.33_R	TCTTTTCACTCAACATATGCCTGGA	
10q23.1_F	CACTATTTCTAGAAAGAGCTGGCCCAC	62
10q23.1_R	TATCCTAATTCTGCATCTCTGAACATGC	
Xq22.1_F	GCCCTTYGAGTACTTCCCATATGC	60,5
Xq22.1_R	GACCCAAGTCATAACCAATGG	

Appendix 3

Photographs of *Chlorocebus* monkeys

"Emma", the individual from Bojnice Zoo, Slovakia:

(Photo: Peter Luptak, Bojnice Zoo)

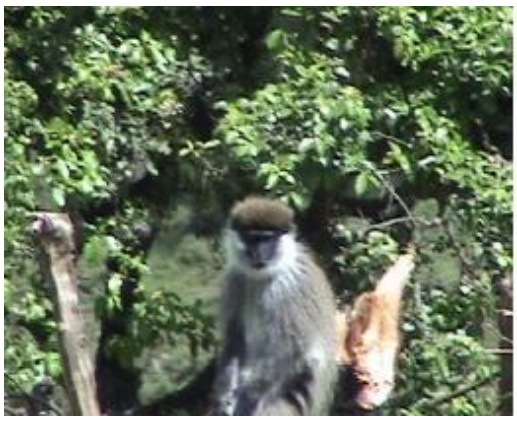
(Photo: Peter Luptak, Bojnice Zoo)

Vervet monkey:

Vervet monkey from Robe population (Photo: Addisu Mekonnen)

Putative Bale monkey hybrids:

Adult male putative hybrid of Bale monkey x grivet monkey at Wotiye, Ana Sora Woreda (Photo: Addisu Mekonnen, January 2010)



Sub-adult male putative hybrid Bale x grivet, Ekuma mountain, Kokossa (Photo: Addisu Mekonnen, February 2010)

Grivet monkeys:

Grivet monkey from Bahir Dar population (Photo: Addisu Mekonnen)

Grivet monkey from Awash national Park (Photo: Addisu Mekonnen, February 2010)

Captive Bale monkeys from Sidamo:

Captive young Bale monkey, Bodie Mt, Bensa (Photo: Addisu Mekonnen, May 2010)

Captive Bale monkey, Hagere Selam town (Photo: Addisu Mekonnen, February 2010)