NONINVASIVE ANALYSIS OF POPULATION GENETIC STRUCTURE OF MOUNTAIN GORILLAS (*Gorilla beringei beringei*) OF BWINDI NATIONAL PARK IN UGANDA

BY

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Declaration of independence

I Julliet Nafula Ogubi, B.Sc. (Hons), solely declare to have conceived and written this dissertation, entitled "Population genetic structure of mountain gorillas (*Gorilla beringei beringei*) in Bwindi National Park, Uganda", without any inadmissible help and /or material that has not been explicitly indicated. All sources of information that were used have been clearly indicated. This dissertation has not been submitted elsewhere, neither inside nor outside Uganda.

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Dedication

This piece of work is dedicated to my dear loving parents, Dorothy and Joseph Ogubi.

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Abstract

Several studies on mountain gorillas have focused on behavior and the recent census. Very little is known about the genetic structure that exists in this endangered species despite the numerous intervention measures being put to try and save the rapidly reducing numbers as a result of poaching, habitat destruction and population pressure on the same resources. In this study, data analyzed from 98 feacal samples collected from Bwindi Impenetrable and Mgahinga National Park at 13 highly polymorphic tetra-nucleotide loci were used to infer the population structure of mountain gorillas. Genetic diversity was measured in terms of number of alleles, observed and expected heterozygosity. Population structure was defined by comparing the genetic diversity within and between different groups and the number of migrants between groups and population clusters. Two clusters were computed based on the altitudinal home ranges of the gorillas with the Mgahinga group clustering with high altitude groups in Bwindi. Despite the two hierarchical genetic clusters, six significantly differentiated genetic groups were computed evidenced by the F_{ST} results, AMOVA and the unrooted genetic tree. Samples collected from Mgahinga were genetically different from those collected in Bwindi. Likewise there was much differentiation among the low altitude gorillas compared to the high altitude gorilla groups. Six private alleles were observed in four different populations (Shonji, Kyaguliro, Habinyanja and the Mgahinga population) at four loci in some cases with high frequencies of up to 0.58. Inspite of a migration rate of 1.24 migrants per generation, the relatively low levels of heterozygosity coupled with the high levels of genetic differentiation and high frequencies of private alleles implicate genetic drift as the major driving force shaping the genetic structure of these gorilla groups. The future survival of mountain gorillas is threatened by habitat fragmentation due to human activities and the rapid spread of zoonotic diseases. Therefore conservation efforts should by geared towards mitigating this trend.

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CHAPTER ONE

INTRODUCTION

1.1 Background to the study

The use of non-invasively collected samples for genetic studies from wild populations has been made possible by the technical advances in molecular genetics (Taberlet *et al.*, 1996; Constable *et al.,* 2001; Morin *et al.,* 2001; Nsubuga *et al*., 2004, 2008; Okello *et al*., 2005 and Thalmann *et al*., 2007). Application of genetic tools to conservation has provided critical insights into history of endangered species, the amount of genetic variation present in both individuals and populations and descriptions of how these variations are geographically distributed (Avise, 2000; Vigilant and Bradely, 2004). There is a general perception that genetic variation present in wild populations is directly related to the population size and therefore species that have exhibited recent constrictions have reduced variation (Frankham, 1999; Vigilant & Bradley, 2004). However, low variation could be present in a population despite the lack of evidence to show decline. This could be attributed to the number of individuals and the variations present originally (Amos & Balmford, 2001).

Endangered, sensitive species under the verge of extinction yet undergoing intensive behavioural study are often disrupted by the presence of humans. They often end up undergoing a lot of stress which in turn has negative effects on their feeding and reproductive behavior. Non invasive sampling and genotyping becomes a preferred alternatives technique in studying these species and also in sample collection as it does not involve interfering with the normal activities of the study species.

Earlier on, a more conventional approach of genetic sampling was used whereby animals had to be captured or killed. This was done by trapping them, a procedure that was stressful and destructive at the same time. Currently, destructive sampling is neither preferred nor allowed in the cases where endangered species are involved thus, the development and encouragement of alternative methods for studying wild species. DNA from non-invasively collected samples such as urine (Hayakawa & Takenaka, 1999), ejaculates (Domingo-Roura *et al.*, 2004), faeces (Goossens *et al.*, 2000), shed hairs (Lorenzini *et al.*, 2004) and saliva have demonstrated reliability in analysis of various population and behavioural aspects in wild animal populations.

Many animal populations today live in patchy discontinuous habitats as a result of alteration of the environment by humans. When small populations become fragmented and migration between subpopulations decreases or stops, consequent increases in inbreeding and loss of genetic diversity can have serious negative effects on the long-term viability of fragmented populations and by extension, the species as a whole (Frankham, 1996; Frankham *et al*., 2002). Determining which subpopulations are in migratory contact with each other can highlight important dispersal corridors as well as identify isolated areas, thereby suggesting priority populations for conservation. Genetic studies can also identify migrants between habitat fragments and individuals of mixed ancestry thus suggesting persistent reproductive contact (Bergl & Vigilant, 2007).

Since the beginning of research on wild mountain gorillas in the Virungas some 40 years ago, the number of nests and the size of the dung left in nests has been used to estimate the number, group composition, ages and sexes of gorillas through observation (Schaller, 1963). With observation, information acquired can be used to infer the direction of movement, the number of groups and the age/sex composition of social groups at site. Mountain gorillas have relatively small range sizes of

the protected areas (Virungas: 450 km^2; Bwindi: 331 km^2) and average home range depends on availability of resources; this makes it easy for their nests and trails to be accessed by researchers. Gorillas live in social groups with members constructing individual nests each night. These nests are cohesively distributed at the group's nesting site and each individual typically defecates in or next to the nest before leaving the site in the morning **(**Lindsley & Sorin, 2001). Field research based on non-invasive fecal collection combined with robust molecular genetics techniques has provided powerful new tools for gathering valuable biological information with minimal disturbance to the study species (Guschanski *et al.*, 2009). Molecular techniques have been used extensively in the monitoring and determination of genetic variability at intra-specific and population levels in a number of species. The combination of observational data from wild animals and the use of molecular markers to infer genetic relationships among individuals has provided insights into mating systems, reproductive strategies, dispersal patterns, genetic relatedness, and the influence of kinship on social behavior (Griffin *et al.*, 2002; Di Foire, 2003; Smith *et al*., 2003; Nsubuga *et al*., 2008). In particular, analysis of genetic structure of a population provides a better understanding of the evolutionary consequences of varied social organization in animal populations (Lukas *et al.*, 2005). Population structure, level of gene flow and differences in genetic diversity among populations are very important factors that should be considered when developing management plans for all species. Genetic sampling and analysis methods complementing field observations for investigating the population structure and estimating levels of differential migration of sexes of natural populations can provide a better understanding of the causative evolutionary pressures, the ecological and genetic consequences (Guschanski *et al.*, 2009). The survival and proper management of a species greatly depends on the amount of genetic variation within and between the different populations of the species in question. Therefore, a central

problem in conservation of biodiversity is the identification of discrete populations, management units and evolutionarily significant units for targeted management (Crandall *et al.,* 2000).

In this study, the genetic population structure of 94 habituated mountain gorillas from Bwindi Impenetrable National Park was analyzed together with four samples obtained from Mgahinga Gorilla Nation Park. Results from this study will provide an insight into the number of distinct genetic clusters to which these habituated gorillas belong and how much within and between-group genetic diversity and differentiation exists. These findings are expected to provide more understanding of how fit the population is and help improve directed conservation efforts.

1.2 Objectives of the study

The overall aim of this study is to analyze the population genetic structure of the different social groups of the Mountain Gorillas in Bwindi Impenetrable National Park and Mgahinga Gorilla National Park using molecular tools.

1.2.1 Specific objectives.

1. To determine the extent of genetic variation within and between the seven habituated groups of mountain Gorillas in Bwindi Impenetrable and one habituated gorilla group from Mgahinga National Park.
2. To determine the number of distinct genetic clusters and the extent of genetic differentiation among the eight habituated groups of mountain gorillas in Bwindi Impenetrable National Park and Mgahinga Gorilla National Park.

1.3 Problem statement/ Justification

Though much information is known concerning mountain gorilla behaviour from direct observation (Robbins *et al*., 2001), comparatively, few efforts have focused on studies at molecular level. Unlike with other large mammals, no reported work has been done to characterize the population structure of Mountain gorillas in Uganda. Since some of the mountain gorilla groups are in constant contact with each other and are also known to migrate and emigrate at sexual maturity (Doran & McNeilage, 1998; Stokes *et al.*, 2003; Watts 2003; Nsubuga *et al*., 2008), there is a likelihood of homogenization of gene flow between the different gorilla family groups. Mountain Gorillas are economically important endangered species currently existing in small isolated but threatened pockets of habitats. This in turn threatens the genetic diversity of this species. Effective conservation and management of the mountain gorillas, in the face of increasing pressure from human population increase, poaching and habitat destruction requires accurate genetic information regarding the distribution of genetic variation between the different gorilla groups. Such information can be obtained through noninvasive genotyping of individuals at a set of reliable polymorphic genetic markers in order to measure the genetic diversity in populations.

Out of the approximately 700 remaining mountain gorillas worldwide, about 302 live in Bwindi Impenetrable National Park (Guschanski *et al.*, 2009). Seven groups in this National Park are habituated but they range among several unhabituated and lone groups/individuals. The Habinyanja, Mubale and Rushegura groups are found in Buhoma tourist site and have been habituated for tourism purposes. The Nkuringo group is found in Nkuringo while Bitukura and the Shonji groups found in Ruhija and Rushaga areas respectively are also habituated for tourism. The Kyaguliro group found in Ruhija is habituated for research purposes (McNeilage *et al*., 2006). The

choice of these seven groups of mountain gorillas was influenced by the fact that they are constantly being monitored and their home ranges are known, making it easy to collect samples from them.

CHAPTER TWO

LITERATURE REVIEW

2.1 Geographical distribution, social organization and dispersal of Mountain gorillas

The critically endangered mountain Gorilla (*Gorilla beringei beringei*), is one of the two subspecies of the eastern gorillas occurring in two extant populations, one in the Virunga Volcanoes on the borders of Rwanda, Democratic Republic of Congo and Uganda, and the other in Bwindi Impenetrable National Park, Uganda. Approximately 71% of the Virunga gorillas live in habituated groups and can be directly counted. The total population was estimated to contain 380 gorillas in 2003 (Gray *et al.,* 2005). A much smaller proportion (approximately 35%) of habituated gorillas in Bwindi can be directly observed and counted.

Gorillas have a relatively limited range in equatorial Africa as compared to other primates like the chimpanzees (Yamagiwa, 1999). There has been a striking discontinuity between the numerous western gorillas (*Gorilla gorilla*) and the perhaps 12,000 remaining eastern gorillas (*Gorilla beringei*) (Doran & McNeilage, 1998). Mountain and eastern lowland gorillas are classified by IUCN—International Union for Nature Conservation. The World Conservation Union as endangered, while western lowland gorillas are considered threatened (Lee *et al.* 1988).

There is strong evidence from museum specimens to showing decades ago, gorillas lived in regions where they no longer exist today (Hofreiter *et al.,* 2003). Thalmann *et al,* (2007) argues that eastern and western gorillas once co-existed but were separated during the formation of the Great Rift Valley. The divergence of this species including other primates like bonobos and chimpanzees is attributed to the formation of the Great Rift Valley approximately 20 million years ago. Genetic

evidence depicts a single ancestry suggesting a possible split between western and eastern gorillas followed by a reduced male mediated gene flow from eastern to western gorillas (Thalmann *et al.*, 2007). Unlike in humans great apes including gorillas are known to have a high nucleotide diversity and therefore their effective population sizes in centuries to come is rated to be higher than in humans; 10, 400 for humans and 25, 200 for gorillas (Yu *et al.*, 2004).

Mountain gorillas are highly social and live in relatively stable, cohesive groups held together by long-term bonds between adult females and males. Relationships among females are relatively weak (Stewart & Harcourt, 1987). The groups are non-territorial, with the silverback defending the group other than the territory. The dominant silverback determines the activities and the direction of movement of his group.

The dominant silverback is believed to monopolize mating and therefore perceived to sire all the siblings born during his tenure. In multimale groups, lower-ranking males are nonetheless successful in gaining paternities through opportunistic matings or through absenting themselves with a female from the community (‘‘consortships’’) or by ‘queuing’ for dominance status, (Nsubuga *et al.*, 2008). During group fission, studies have shown that dominant males tend to remain with their off springs and the subordinate ones form new groups. This is consistent with the proposal that the outcome of group fission in primates is not only influenced by maternal relationships among individuals, but also by patrilineal relationships (Nsubuga *et al*., 2008).

While primates may live in social groups to reduce the risk of predation, this decision comes with increased competition for mates and food. As the group sizes increase, home ranges of particular groups are also forced to expand to cater for the additional members. If this is not done the average food intake per individual will lead to reduced reproductive success (Nkurunungi, 2004).

Dispersal decisions made by animals living in groups play a very important role in determining their individual survival, reproductive success, population dynamics, genetic differentiation and in turn the social system of their species (Pusey & Packer, 1987, Robbins & Robbins 2005). Emigration can be voluntary or forceful where the reigning dominant individual can evict his potential competitors which are normally the sexually mature males (Robbins & Robbins, 2005). Voluntary emigration can be in search of mates (Perrin & Mazalov, 2000), inbreeding avoidance (Pusey & Packer 1987), and better resources such as food, water and shelter (Robbins & Robbins, 2005). However this is a risky decision associated with greater aggression (Steenbeck *et al.*, 2000), increased infant mortality for species prone to predation (Alberts & Altmann, 1995) and the high cost of obtaining resources in a new unfamiliar environment (Dobson *et al.,* 1998).

Unlike in most humans populations where the males are more philopatric and the females disperse, both females and about 45% males leave their natal groups at sexual maturity (Stoinski *et al.*, 2009). Males leave at the age of 11 years and often the separation process is slow; they spend more and more time on the edge of the group until they finally leave altogether (Lindsley & Sorin, 2001). They may travel alone or with all-male groups for 2-5years before they can attract females to join them and form a new group though about 43% of the dispersing males leave with females (Stoinski *et al.,* 2009). Females typically emigrate at about 8years old, either by moving directly into an established group or joining a solitary male. Females often transfer to a new group several times before they settle down with a particular silverback male (Watts, 1990).

Although both males and females disperse from their natal homes at sexual maturity (Doran & McNeilage, 1998; Stokes *et al.,* 2003; Watts, 2003), field observation suggest that males migrate further than females (Yamagiwa, 1987). Little is known about the lone male dispersal patterns

though males are said to occasionally transfer their genes over longer distances which could explain the alleles that are identical at certain nuclear loci in both western and eastern gorillas (Thalmann *et al.*, 2007).

2.2 Noninvasive sampling and its relevance in genetic studies of endangered species

Noninvasive sampling involves collection of sample for analysis without distracting the normal daily activities of the target species. Samples collected include faeces, urine, saliva from food remains, and shed hair and feathers. While studying protected and endangered species including primates, collection of invasive samples such as blood and biopsies as a source of DNA is limited if not prohibited. However with the advance in technology, non-invasively collected samples such as faeces, hair and saliva from food remains can now be used as a reliable source of DNA.

Most comprehensive genetic studies of primates have relied upon faeces because they are easy to collect, always abundant and in addition, they yield DNA extracts that are sufficient enough for analysis (Taberlet *et al*., 1999; Morin *et al.*, 2001; Vigilant, 2002; Nsubuga *et al*., 2004). Shipment and exportation of faecal material from the field to the laboratory does not require the CITES permit thus, making it less strict compared to blood or biopsies.

Generally, analysis of faeces has been used to identify the species and food habits of animals. The information obtained from faeces is important to elucidate behavioral and ecological features of target animals. However, that alone cannot help identify the species among sympatric animals with similar body sizes and food habits (Tatara and Doi 1994, Kurose 2005). In such instances, faecal DNA analyses can provide species identification, variability at species and individual level, sex, answers to kinship questions and many more. Feacal DNA can be used to evaluate the extent of genetic variation among populations and to discuss kinship within a population (Gerloff *et al.*,

1995), distinguish between individuals (Paxinos *et al.*, 1997), and identify species (Murakami, 2002). In order to examine the ecological and social features of animal populations, sex of the individual members under investigation should be identified and can be done by amplification of the amelogenin gene (Ennis & Gallagher, 1994; Yamauchi *et al.*, 2000; Nsubuga *et al.*, 2008; Guschanski *et al.*, 2009). Animals sharing the same feeding ground can have different preferences in terms of selecting foods to eat. Apart from physical observation, analysis of feacal DNA can help deduce these preferences. The Advantages involved in noninvasive sampling are sufficient enough to predict increasing use in the coming years. Despite the many advantages, this method also provides serious drawbacks in terms of reliability, if applied without extreme care.

2.2.1 Technical challenges and limitations of non invasive sampling

Several studies have revealed many technical challenges involved in analysing DNA extracted from non-invasively collected samples (Gerloff *et al.*, 1995; Taberlet *et al.*, 1997; Gossens *et al.*, 1998; Taberlet *et al.*, 1999; Smith *et al.*, 2000; Morin *et al.*, 2001; Bradley & Vigilant, 2002; Fernando *et al.*, 2003; Maudet *et al.*, 2004; Okello *et al.*, 2005). In the light of the rapidly growing interest in noninvasive sampling, it is imperative that researchers fully understand the challenges and difficulties involved. Some of these challenges include the risk of contamination during collection or extraction, lack of sufficient knowledge on sample preservation, genotyping errors, and failure of amplification among others. Once these are properly addressed, the future of non invasive genetic sampling is thus quite promising.

Employing either physical dehydration, freezing and/or chemicals like ethanol, RNAlater preservation buffer or desiccating silica beads, helps introduce harsh conditions for the enzymatic activity of nucleases (Nsubuga *et al.*, 2004). The two way storage method of faecal samples

described by Nsubuga *et al.,* (2004) have so far been reliable and successfully employed in many studies (Nsubuga *et al.,* 2004, 2008; Bergl *et al.,* 2008; Guschanski *et al.,* 2008; Guschanski *et al.,* 2009). Recent extraction kits such as the QIAmp Kit, Qiagen have been developed based on the silica method and they have proved quite effective in removing PCR inhibitors (Pompanon *et al.,* 2005).

DNA expected after extraction from faeces, feathers or hairs is only a few picograms at times less than one picogram. In such instances, the PCR technique, an enzymatic process capable of producing exponential amounts of target DNA sequences from just a few copies becomes very necessary. A genotyping error occurs when the observed genotype of an individual does not correspond to the true genotype. Although genotyping errors occur in all but the smallest data sets that are generated in genetic studies, they have almost exclusively been recognized in linkage analyses, in forensic analysis and in non invasive genotyping (Pompanon *et al.,* 2005). With noninvasive samples the total DNA available for genotyping is normally in picogram range and therefore if the PCR allows detection of only one target molecule, leading to allelic dropout thus producing false homozygotes. Allelic drop out, which is the amplification of only one of the two alleles present at a heterozygous locus (Foucalt *et al.,* 1996; Gagneux *et al*., 1997; Taberlet *et al.,* 1997; Goossens *et al.,* 1998), and the occurrence of 'false alleles' (artefact generated by PCR), and human error which accounts for up to 93% of genotyping errors (Hoffman and Amos, 2005).

Genotyping errors that are not accounted for lead to false conclusions especially in studies involving individual identification, population assignment, kinship and census studies because they rely on the individual genotypes obtained (Taberlet *et al.,* 1997; Ellegren, 2004; Hoffman and Amos, 2005). The effect of genotyping error remains largely unknown in this field, though several

recent studies try to account for these errors in analysis (Marshall *et al.*, 1998; Bonin *et al.*, *2004;* Dakin and Avise, 2004; Okello *et al.,* 2005). Genotyping errors can be minimized by employing the multiple tube approach (Navidi *et al.*, 1992) and results from Hardy-Weinberg tests can hint on whether there are genotyping errors in the data set (Xu *et al.,* 2002; Hosking *et al.,* 2004). Highly reproducible errors such as null alleles cannot be detected by replicating the genotyping assays and so require Hardy-Weinberg tests while on the contrary, stochastic allelic dropouts might not be detected by Hardy-Weinberg tests, but by replicating the genotyping assays (Pompanon *et al.*, 2005).

2.2.2 Choice of molecular markers

The choice of genetic marker is extremely important because it has consequences for the subsequent analyses. An ideal genetic marker should exhibit high heterozygosity, provide good amplification and should be easy to score. High heterozygosity is ideal because it will require few loci to reach a desired probability of identity (PI) and thus the costs involved in running several extra loci is reduced.

2.2.3 Microsatellites and their use in population genetic and behavioural studies

These are tandem repeats of 1-6 nucleotides also known as Simple Sequence Repeats (SSR) found scattered in the nuclear genome of most taxa, however dinucleotides, trinucleotides and tetranucleotides are most commonly used (Selkoe and Toonen, 2006). Microsatellites have emerged as the most popular and versatile type of molecular marker for ecological studies. Microsatellite loci vary in repeat length from 5 to 40. Microsatellites used as genetic markers are embedded in the noncoding DNA either in the intergenic sequence or in the introns and are generally assumed to

evolve neutrally. They are surrounded by flanking regions which do not participate in coding process during protein synthesis.

Microsatellites are single locus co-dominant markers and a number of loci can be combined in the genotyping process thus saving on time and the costs involved in the process. They have been documented to be generally shorter compared to sequenced loci but the recent of Nextgen sequencers can now produces much shorter fragments. They can therefore amplify via PCR even from very low concentrations or degraded DNA (Taberlet *et al.,* 1999). During degradation, DNA breaks in shorter strands and chances of finding microsatellites intact are high even after high levels of degradation which commonly occurs with non-invasively collected samples.

Most of these molecular markers are species specific or can be used with very closely related species and therefore there are lower chances of cross contamination by non target species that could be present in the extract compared to other techniques that involve the use of universal primers (Selkoe and Toonen, 2006). Unlike other markers with low mutation rates, microsatellites mutate fast resulting into a very high allelic diversity in the population. This makes them very informative in the analysis of small fragmented and recently bottlenecked populations as compared to allozymes where only loci with highest mutation rates will be informative (Hedrick, 1999 and Selkoe & Toonen, 2006). The selection of a marker depends on the question to be addressed, however in the case of paternity analysis, identity analysis, clonal structure, population structure or migration that employ allele frequency estimates (Wilson & Rannala, 2003), microsatellites are the best choice since only a few loci can provide a lot of information on a number of questions and unique genotypes 'identification tag' (Queller *et al.,* 1993).

Despite the numerous advantages, the use of microsatellites has its own limitations among which are; unclear mutation mechanisms, the need to isolate species specific markers, hidden allelic diversity (homoplasy) and amplification problems. The mode of mutation is not very clearly understood, though it is thought that microsatellites evolve by step-wise mutation, his overshadows point mutations that do not result into fragment length change.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study sites and study groups

Bwindi Impenetrable National Park (BINP) is a montane forest (331 km^2) located in Southwestern Uganda (08530–18080 N; 298350–298500 E) with an altitude between 1,1600m on the western side and 2,607m on the eastern part above sea level (Butynski & Kalina, 1993). This study focused on five habituated groups, two groups under habituation and a single group from the Virungas. The Kyaguliro group has been closely monitored since August, 1998, and identities of all gorillas are well known. Currently, this group contains one adult male with silvery hair on the back (silverback), six females, four mature males that have not developed the silver hair (blackbacks), three juveniles and three infants. The Habinyanja group which ranges in the Buhoma and Nyamishamba areas of the park has been habituated for tourism since 1998 and originally contained thirty members. Fission of the group occurred in February, 2002, producing the Habinyanja group with two silverbacks, seven blackbacks, five adult females and six juveniles\infants and Rushegura which ranges in Buhoma most of the time, with one silverback, five adult females, two subadults, two juveniles and three infants. The Mubale group ranging in Buhoma consists of one Silverback, one blackback, four Adult Females and two infants while the Nkuringo group is now left with two Silverbacks following the death of the oldest silverback (Nkuringo), five Blackbacks, four adult females, three Juveniles and five Infants. The Bitukura group a new group under habituation, consists of four silverbacks, four adult females, three juveniles and two infants. Shonji is a new group being monitored for possible habituation and hence less information is known about it.

Mgahinga Gorilla National Park is located in the southwestern corner of Uganda. The Park covers the northern slopes of the three northernmost Virunga Volcanoes: Mt. Muhavura (4,127 m), Mt. Gahinga (3,474 m), and Mt. Sabinyo (3,645 m). The Park is bordered to the south by the Republic of Rwanda and to the west by the Democratic Republic of Congo. Mgahinga Gorilla National Park is 33.7 km^2, comprising of only 8% of the Virunga Conservation Area and consists of the partly forested slopes of three extinct volcanoes.

3.2 Fecal Sampling and storage

A total of 131 faecal samples were non-invasively collected from mountain gorillas of Bwindi Impenetrable and 6 from Mgahinga National Parks between March and July 2008. Ninety eight faecal samples were successfully extracted and genotyped at 13 microsatellite loci including 4 from Mgahinga Gorilla National Park. Samples were collected from the night nests of the groups and as such the individual identities were not known but were rather assigned based on the dung size (Schaller, 1963) and later genotyping results.

Sampling was done in the morning before noon since high ambient temperature at such a time of collection is known to catalyze the rate of DNA degradation (Nsubuga *et al.,* 2004). The outermost part of the faecal bolus, suspected to contain more epithelial cells discarded from the gut lining, was targeted for collection. Such types of samples yield sufficient DNA and possess low levels of PCR inhibitors (Fernando *et al.,* 2003; Okello *et al.,* 2005). Where possible, multiple samples were collected from the same individuals on different days. During sample collection, all the materials and equipment were sterilized and care was taken to eliminate cross contamination.

Approximately three grams of faecal samples from each individual were collected and put in 20ml vials containing absolute ethanol as previously described by Nsubuga *et al*., (2004). After 24 – 36

hours, the samples were transferred and stored in 50ml vials containing approximately 30g of desiccating silica gel beads (Sigma S7625). The samples were kept at ambient temperature in the field but were kept at 4°C in the laboratory at Uganda Virus Research Center before being shipped to the genetics lab at San Diego Zoo Institute for Conservation Research (California, USA) for further analysis.

3.3 DNA isolation

Genomic DNA was extracted from 0.1g of dried faecal material using the QIAamp® DNA Stool Kit (Qiagen) following the manufacturer's instructions and a few modifications (Nsubuga *et al*., 2004; 2008). This process was done in a room exclusively dedicated to DNA extraction to avoid any possible contamination. Ten to 20 samples were extracted at once along with two negative controls. DNA was recovered in a final volume of 200µl of elution buffer provided with the extraction kit. The amount of DNA in each extract was quantified using a NanoDrop® ND-1000 Spectrophotometer machine and finally stored at 4°C.

3.4 Genetic analyses

All gorilla DNA samples were genotyped via polymerase chain reaction (PCR) at up to 13 tetra-nucleotide repeat microsatellite loci (Bradley *et al*., 2000; Table 1) originally characterized in humans but some of which had previously been used extensively for analysis of gorillas (Clifford, 1999; Bradley *et al*., 2000, 2004, 2005; Zhang *et al*., 2001; Bergl *et al*., 2008; Nsubuga *et al*., 2008). Sex was determined via analysis of a segment of the amelogenin locus on the X and Y chromosomes (Bradley *et al*., 2001). PCR amplification was performed in a total volume of 15 µL containing (2 – 10 ng) DNA template 1.5 µL 10x PCR buffer, 1.5 µL $MgCl_2$ (25mM stock), 0.3 µL each of Forward and Reverse primers (10 µ M), 0.6 µL dNTPs (10mM), 0.15 µL of Taq Gold (5 U/

μL) (Perkin-Elmer Applied Biosystems); 0.6 μL bovine serum albumin (BSA: 10 mg/ μL) and 8.05 μL ultra-pure H_2O. Samples were PCR-amplified using a 2720 Thermal Cycler (Applied Biosystems) and a Master Cycler gradient (Eppendorf) using the following cycling parameters: initial denaturation 3 min at 95 °C; 35 cycles of 40 s at 95 °C, 40 s at 54 °C to 60 °C (see Table 1), 30 s at 72 °C, and a final extension step of 30 min at 72 °C. It is believed that diluting the template also dilutes the PCR inhibitors present in high amounts in non-invasively collected samples, therefore, the template was diluted in a ratio of 1:2 to help dilute the possible PCR inhibitors.

The PCR products were visualized under UV light after electrophoresis on 2% agarose gels stained with Ethidium bromide. The 5' end of each forward primer was fluorescently labeled, and the PCR products were separated using capillary electrophoresis on an ABI 3100 genetic analyzer (Applied biosystems). Alleles were scored relative to internal size standard (500 ROX) using GeneMapper 3.0 software (Perkin-Elmer Applied Biosystems).

To minimize genotyping errors such as allelic dropout (Taberlet et al. 1996), heterozygotes were confirmed after two independent replications and homozygotes after at least four independent replications.

A post-PCR multiplexing system was used to genotype the loci based on allelic fragment sizes and the fluorescent labels used as follows; Loci [D5s1457(NED), D4s1627(FAM)]; Loci [D3s2459(FAM), D7s817F2(HEX), D10s1432(NED)]; Loci [D5s1457(NED), D7s2204(HEX)]; Loci [D2s1326(FAM), D5s1457(NED), D7s817F2(HEX), D5s1470(HEX), D16s2624(FAM)] and [D1s550].

3.5 Genetic marker assessment and diversity analyses.

The level of variation of each of the genetic markers used in genotyping was assessed using the program CERVUS ver3.0.3 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007) to estimate the allele frequency, the expected heterozygosity, *HE*, the exclusionary power of the loci, and the probability of null alleles. Deviations from Hardy-Weinberg equilibrium, *HWE*, for each locus were examined using Fisher exact tests (Guo & Thompson, 1992), and genotypic linkage disequilibrium, *LD*, among pairs of loci was assessed using the program GENEPOP version 4.0.9 (Rousset, 2008). Because multiple testing enhances type *I* error, critical levels of significance were adjusted in accordance with the sequential Bonferroni correction for multiple comparisons (Rice, 1989). In addition, mean individual heterozygosity, *HI*, was calculated as the mean of the number of loci at which each gorilla was heterozygous, divided by the total number of loci at which each gorilla was scored (Slate *et al.*, 2000).

Nei's (1973) expected heterozygosity, observed number of alleles per locus, *NA*, and effective number of alleles, *AE*, and gene flow calculated from the number of migrants per generation using the formula $F_{ST} = 0.25(1-F_{ST})/F_{ST}$ were calculated using program POPGENE ver 3.2 (Yeh, 1999). Heterozygosity values for the Bwindi gorilla population was compared with heterozygosities reported in other wild primate populations such as western gorillas (Bradley, 2004); rhesus macaques, (Charpentier *et al.*, 2008); howler monkeys (Milton *et al.*, 2009) and baboons (Nguyen *et al.*, 2009). To correct for differences in sample size, the allelic richness was computed, *AR*, as implemented in FSTAT 2.9.3.2 (Goudet, 2001). The population structure was also defined by comparing the allele frequency distributions with the explicit assumption that significant differences in allele frequency distributions is indicative of reproductively isolated populations. F_{ST}

pairwise comparison as a measure of genetic differentiation was computed in POPGENE 3.2 (Yeh, 1999) and a population tree showing the genetic distances determined.

To be certain that the markers utilized here could accurately distinguish between any two individuals, population allele frequencies based on wild-born individuals sampled (n = 98) were used to calculate the 'probability of identity' (P_{ID}), defined as the probability that any two individuals picked at random from the population would share the same multilocus genotype by chance, and the probability of identity between siblings ($P_{ID\text{-sibs}}$) (Paetkau & Strobeck, 1994; Mills *et al.,* 2000; Waits *et al*., 2001). Furthermore, to uncover accidental multiple sampling of the same individual, CERVUS version 3.0.3 was used to perform an identity analysis by conducting pairwise comparisons of all multilocus genotypes. One to three mismatches were allowed in the output options in order to spot potential mismatches due to genotyping error or allelic dropout. Samples that yielded the same genotypes across all screened loci were deemed to represent multiple sampling of the same individual.

Assignment of individuals to the groups where they were sampled was done based on the multilocus genotype implemented in ARLEQUIN version 3.1 (Schneider *et al*., 2000). Lastly Analysis of molecular variance (AMOVA) was performed on Bwindi groups based on the altitudes they range in. Nkuringo, Habinyanja, Rushegura and Mubale that range at the lower altitude were grouped together while Bitukura, Kyaguliro and Shonji that range at a higher altitude formed another group. The Mgahinga group was not included due to insufficient data. F_{IT}, F_{CT}, F_{SC} and F_{IS} defined as the differentiation within the entire sampled population, between the different altitudes, differentiation within each altitude and the inbreeding index in the whole population respectively were calculated using the program ARLEQUIN version 3.1 (Schneider *et al.*, 2000)

3.6 Population Structure Analysis

The existence of population genetic structure between gorillas from different sectors of the Bwindi Impenetrable National Park (BINP) were assessed using a Bayesian model-based clustering method as implemented in program STRUCTURE 2.1 (Pritchard *et al*., 2000; Falush *et al*., 2003). The model assumes that the multilocus genotypic data utilized is generated from markers that are in *HWE* and linkage equilibrium within populations. In this model, it is assumed that there are *K* distinct populations or genetic clusters (where *K* may be unknown) in a set of sampled multilocus genotypes, each with a distinctive set of allele frequencies at each locus. Tests were conducted using a range of different numbers of population clusters (*K*) to guide an empirical estimate of the number of identifiable populations. For each value of *K* (1 to 10), to ensure consistence of results, 10 independent replicates were run with a burn-in period of 500,000 steps followed by 10^6 iterations.

To estimate the most likely value of *K* that best fits this data, the method of Evanno *et al.,* (2005) was followed. All samples were pooled and were assumed to belong to an unknown number of genetically unique clusters (*K*). After establishing the number of genetic clusters (*K*), STRUCTURE was then used to probabilistically evaluate the individual membership proportional (Q) (based on the individual genotype in the sample) to each of the two inferred clusters, with an admixture model allowing joint assignment to the two clusters. Individuals with a threshold of Q = 0.8 were assigned to one cluster while those with Q < 0.8 were assumed to be admixed (assigned jointly to more than one cluster). Genotypes from adult males and females (n = 77) were analyzed separately because adult male-female dyads within group are generally unrelated. If genotypes of juveniles and infants (related individuals) are included in the data, one risks getting a significant amount of deviation

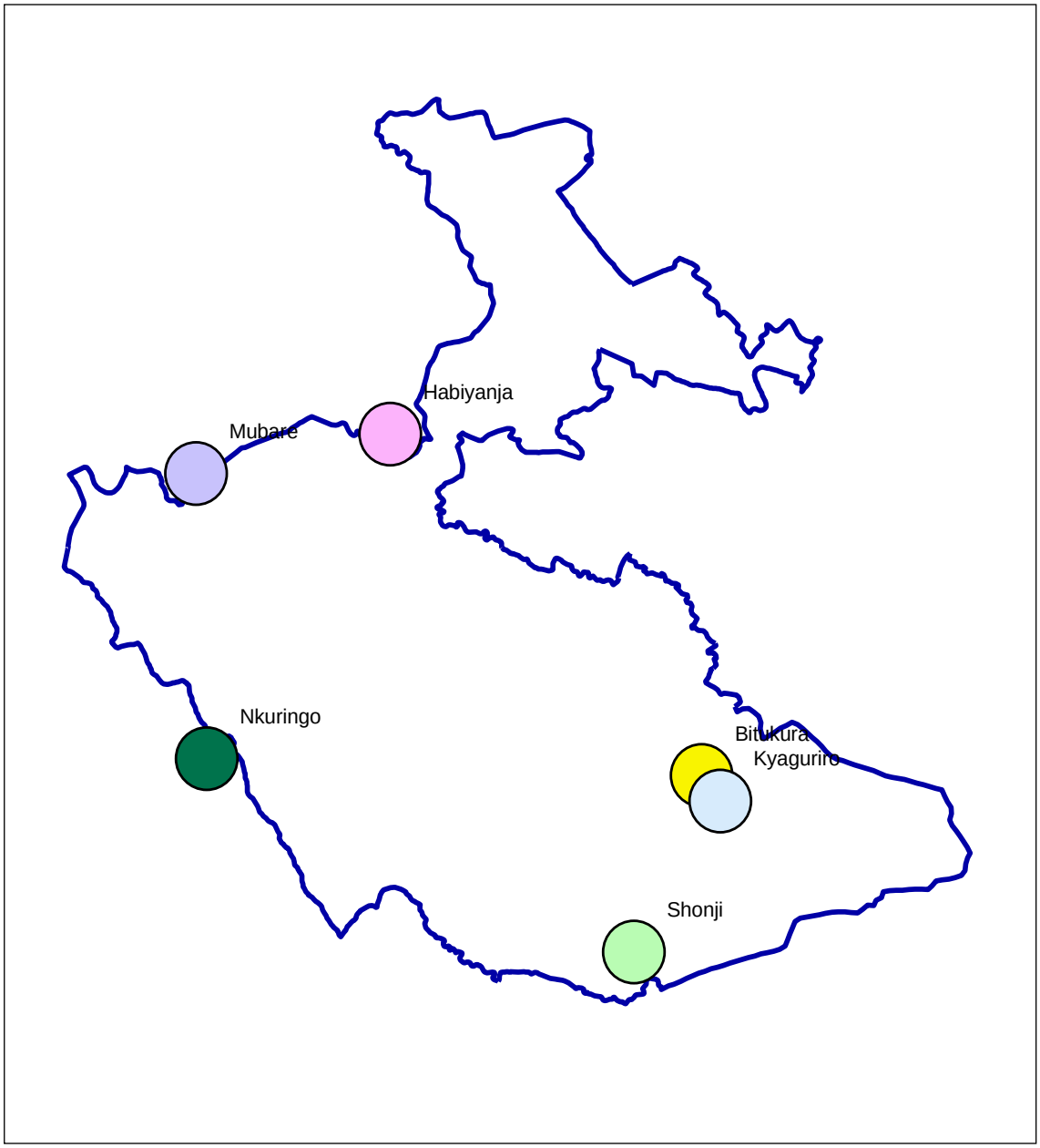
from Hardy-Weinberg Equilibrium at these loci. However the results from the analysis of adults were compared to those of the whole population including juveniles and infants. When using STRUCTURE, data from closely related individuals were eliminated to avoid obtaining figures with significant deviations from the Hardy-Weinberg Equilibrium, depression of heterozygosity and also to prevent the risk of over estimating *K*. (Lukas *et al.*, 2005; Bourgain *et al.*, 2004; Guschanski *et al.*, 2008). In gorilla social systems, adult males and females are less related as a result of dispersal at sexual maturity (Nsubuga *et al.*, 2008) while juveniles and infants are kin and inherit half of their alleles from each parent. Since these alleles are already represented in the data set through their parents their inclusion is not necessary.

Table 1; Summary of the set of 13 tetra nucleotide loci used in this study.

Locus	Primer sequence	Fluorescent label	Allele size range
vWF	F: CCCTAGTGGATGATAAGAATAATC R: GGACAGATGATAAATACATAGGATGGATGG	HEX	135–167
D1s550	F: CCTGTTGCCACCTACAAAAG R: TAAGTTAGTTCAAATTCATCAGTGC	FAM	170–202
D2s1326	F: AGACAGTCAAGAATAACTGCCC R: AGGGAATTCTCTGAGCTAATAC	FAM	242–286
D3s2459		FAM	196-248
D4s1627	F: AGCATTAGCATTTGTCCTGG R: GACTAACCTGACTCCCCCTC	FAM	230–258
D5s1457	F: CTGGTTTGGGCTGTTATGG R: AGGGACTTAGAAAGATAGCAGG	NED	111-135
D5s1470	F: CATGCACAGTGTGTTTACTGG R: TAGGATTTTACTATATTCCCCAGG	HEX	173-189
D5s1505		FAM	203-219
D7s817F2	F: TAAATCTCTTTATGGCTGACTG R: GGGTTCTGCAGAGAAACAGA	HEX	130-150
D7s2204	F: TCATGACAAAACAGAAATTAAGTG R: GTTCACTGTAGAGTTCCTTTATGC	HEX	239–259
D8s1106	F: TTGTTTACCCCTGCATCACT R: TTCTCAGAATTGCTCATAGTGC	FAM	123–151
D10s1432	F: CAGTGGACACTAAACACAATCC R: TAGATTATCTAAATGGTGGATTTCC	NED	156–180
D16s2624	F: TGAGGCAATTTGTTACAGAGC R: TAATGTACCTGGTACCAAAAACA	FAM	124–144

F: Forward primer, R: Reverse primer.

Figure 1: Map of Bwindi Impenetrable National Park showing the sampling sites for six groups of mountain gorillas



CHAPTER FOUR

RESULTS

4.1 Genetic diversity

A total of 98 samples were genetically sexed and genotyped at 13 microsatellite loci with 81 of these being adults and 17 juveniles and infant. Faecal samples collected from Bwindi Impenetrable National Park totaled to 94 and 4 samples were from Mgahinga Gorilla National Park. Out of the 94 samples representing 28% of the total Bwindi population, 77 were adults and 17 were juveniles and infants combined. The P_{ID}, the chance of finding two randomly sampled individuals with identical genotypes, was very low at 4.00×10^{-12}, as well as the, $P_{ID\text{-}sibs}$, the more conservative P_{ID} among siblings, at 2.26×10^{-5}. This indicates that it is highly improbable that any pair, including sibling pairs exhibited the same genotype profile across all 13 loci. Therefore, all the 98 individuals were considered in further analysis. Out of the total sample of 98 individuals, 90.6% of the gorillas genotyped from Bwindi, had complete genotypes for all the 13 loci while the rest were successfully genotyped for 6 – 12 loci. The number of alleles per locus in each population ranged from four to eight (mean = 6 ± 1.27). The expected and observed heterozygosities (H_E and H_o) ranged from 0.50 to 0.82 and 0.34 to 0.82 respectively. No locus showed significant deviation from Hardy-Weinberg proportions after a Bonferroni correction with analysis of 94 gorillas of Bwindi. Likewise, none of the pairs of loci exhibited significant linkage disequilibrium after a Bonferroni correction for multiple comparisons (all $p \geq 0.05$; $\alpha = 0.05$). Hence, the entire set of 13 loci was retained for further analyses.

When the data from the whole sampled population including the four samples from Mgahinga National Park was analyzed, 89.8% had complete genotypes, and the number of alleles per locus ranging from four to nine (mean = 6 ± 1.47). The expected and observed heterozygosities (H_E and H_o) ranged from 0.51 to 0.83 and 0.52 to 0.78 respectively. The mean individual heterozygosity, H, for all sampled populations of Bwindi and Mgahinga was 0.62. Two loci showed significant deviation from Hardy-Weinberg proportions after the Bonferroni correction (Table 2).

4.2 Population structure

A total of six private alleles were observed in four different groups; at locus D1s550, alleles 186 and 194 are private to Shonji. At locus D5s1470, alleles 177 and 197 are private to Kyaguliro. In addition, alleles 258 were private to Habinyanja, and 216 to Mgahinga at loci D4s1627 and D3s2459 respectively (Table 3). The distribution of the private alleles among the different groups was not random; only one of the groups sampled at low altitude (Habiyanja) possessed a single private allele with a low frequency (0.05) at locus D4s1627 while three out of four of the groups sampled at high altitude (including the Mgahinga group) possessed private alleles sometimes at very high frequencies of up to 0.58. No private alleles were recorded in 9 out of the 13 loci analyzed (Table 4).

A hierarchical analysis of molecular variance (AMOVA) revealed no significant differentiation in the whole population (F_{IT} = 0.069; P = 0.089). However, significant differentiation was observed between groups sampled at low and high altitudes (F_{CT} = 0.041; P = 0.023). Likewise, there was significant genetic differentiation between groups sampled at each altitude (F_{SC} = 0.059; P < 0.001). No significant excess homozygosity was observed in the whole sample (F_{IS} = -0.030; P = 0.818).

Pairwise group comparisons revealed strong genetic structuring between groups in Bwindi impenetrable forest with 17 out of 21 possible comparisons showing significant genetic differences ($P < 0.05$) (Table 5). The presence of a strong genetic structure was further corroborated by results from the assignment tests whereby 95.9% of all the individuals' multilocus genotypes were correctly assigned to the groups where they were physically sampled from while 4.1% were assigned to other neighbouring groups (Table 6). About 6.67% of individuals sampled in Nkuringo were assigned to Kyaguliro, 9.09% sampled in Habinyanja were assigned to Bitukura and 12.5% sampled in Bitukura were assigned to Kyaguliro group.

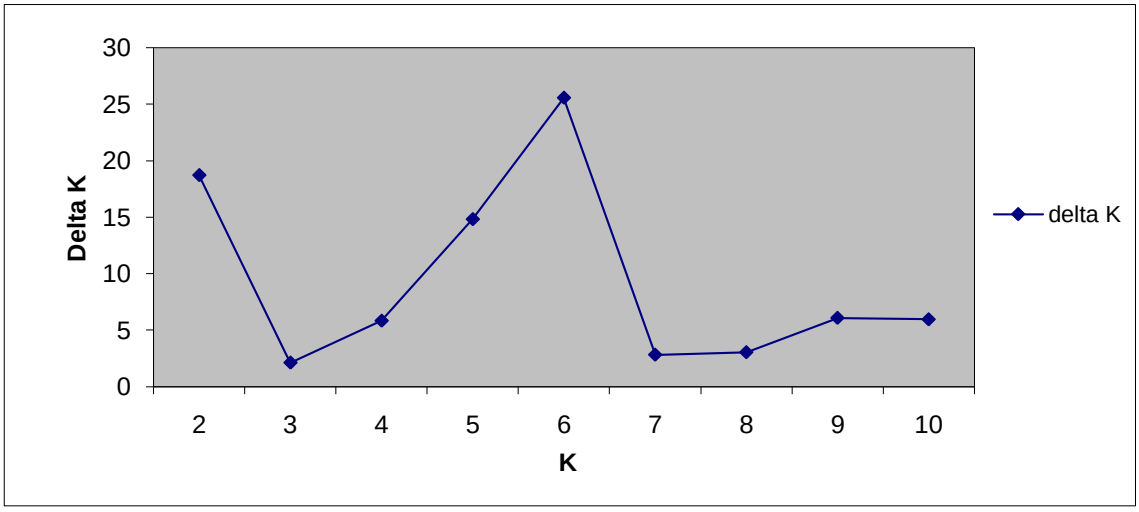
The extent of gene flow for the total sample quantified as the average number of migrants between groups per generation (*Nm*) and estimated as $Nm = 0.25(1 - Fst)/Fst$, was high at locus D4s1627 with a value of 2.66 and lowest at locus D7s2204 with a value of 0.49. The mean number of migrants per generation for the combined data set of Bwindi and Mgahinga was 1.24 (Table 7).

4.3 Structure Results

Assessment of the posterior probability values for *K* ['Log probability of data', *L (K)*] suggested a strong subdivision at $K = 6$ than at $K = 2$ (Fig. 2). There is normally an increased variance in L (*K*) at higher values of *K*, as observed elsewhere by Evanno *et al.* (2005) therefore, subdivision at the values $K = 2$ was selected for analysis in this study. Straightforward identification of *K* was hampered by this variance. Hence, despite estimation of delta *K* from structure output producing a two modal values at both $K = 2$ and $K = 6$ (Fig. 2), $K = 2$ was considered optimal on basis of the following: whenever the STRUCTURE program produces clustering results with similar results at different values of *K*, the smallest value is typically the most accurate (Pritchard *et al*., 2000; Bergl & Vigilant, 2007; Pritchard & Wen, 2004). Despite $K = 2$ being considered in the selection of

clusters, the strong subdivision at K = 6 suggested 6 genetically differentiated individual groups. Thus hierarchical genetic structuring. This finding corroborates the observed significant pairwise F_{ST} values between groups (Table 6)

Fig. 2. Graph showing Rate of change in the likelihood function of *K for mountain gorillas of Bwindi Impenetrabel and Mgahinga National Parks.**

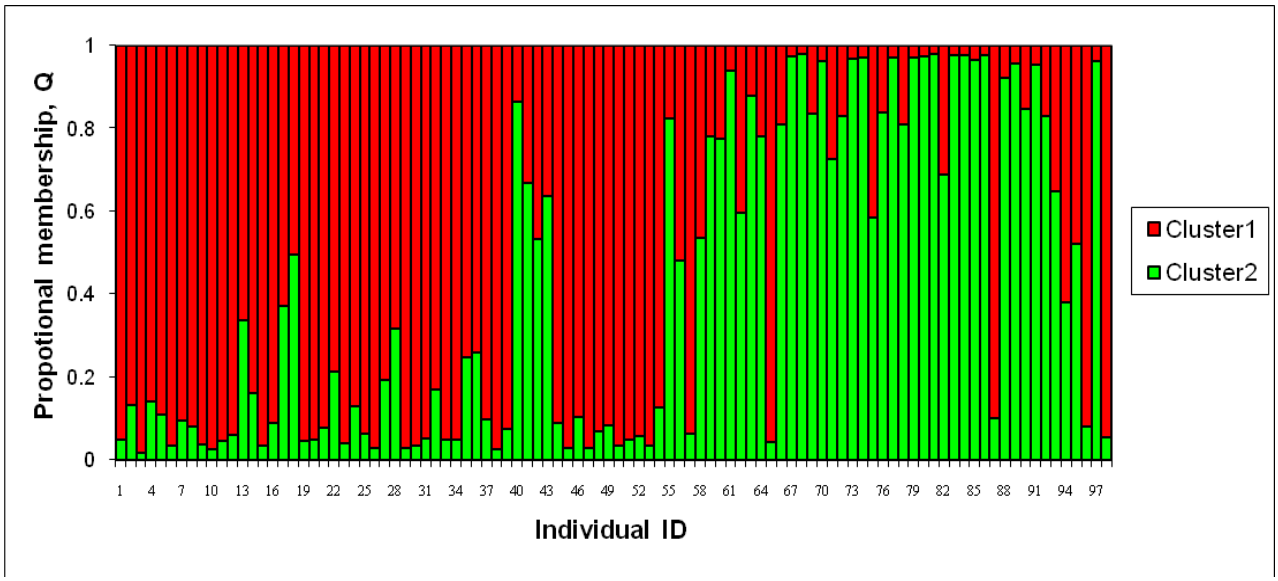


* K is the number of genetic clusters detected by STRUCTURE based on allele frequencies.

STRUCTURE analysis inferred two and six genetic clusters in Bwindi adult gorillas only and the entire sampled population including adult gorillas, juveniles and infants together with the Mgahinga group. (n = 81 and n = 98 respectively; Fig. 3). Nkuringo, Habinyanja, Mubare and Rushegura gorilla groups assigned together in cluster 1, while Bitukura, Shonji and Kyagurilo groups assigned to cluster 2. The Individuals with membership (Q) values between 0 and < 0.2 or > 0.8 and 1.0 were classified as assigned to one cluster, while those between 0.2 and 0.8 had admixed ancestry in both clusters (Figs. 3).

The Mgahinga group assigned to cluster 2, although three of four gorillas from Mgahinga are admixed between the two clusters (Fig. 3). The proportion membership Q assigned 47% of adult sampled gorillas to have origin in cluster one ($Q > 0.2$). One individual from cluster 1 with $Q = 0.81$ is an immigrant from cluster 2. The rest are of admixed origin between the two clusters ($Q > 0.2 < 0.8$). On the other hand, 59% of gorillas sampled in cluster 2 are correctly placed in their locality of origin and 49% of admixed origin. No individual in this group exclusively belonged to cluster 1.Analysis of 98 individuals including juveniles and infants showed that 86% of gorillas sampled belong in cluster 1 ($Q < 0.2$) and 14% are of admixed origin (Fig. 3). Nonetheless, 56% have their origin in cluster 2 ($Q > 0.8$), 10% are migrants from cluster 1 ($Q > 0.2 < 0.8$) and 34% are of admixed origin ($Q < 0.2$)

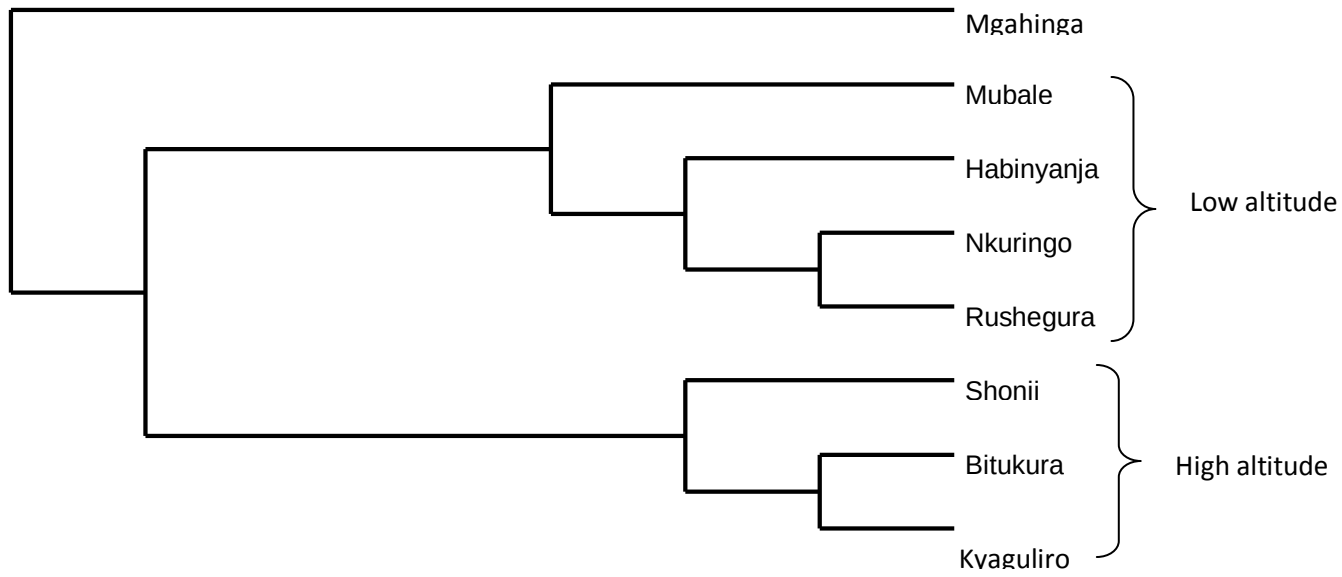
Fig. 3: Proportional membership coefficient (Q) for mountain gorillas in Bwindi Impenetrable and Mgahinga National Parks.



Proportional membership coefficient (Q) in each of the two clusters inferred using STRUCTURE without prior population (group) information for 94 gorillas (irrespective of age) in Bwindi National Park, and four gorillas from Mgahinga Gorilla National Park. A proportion of each bar is red and green coded to represent the assignment probability for each individual to the two population clusters. X-axis represents group ID: individual ID 1 – 16 are from Nkuringo group; 17 - 28 are from Habinyanja; 29 – 39 from Mubare; 40 – 43 from Mgahinga; 44 - 54 from Rushegura; 55 – 64 from Bitukura; 65 – 86 from Shonji; and 87 – 98 from Kyagurilo group.

Further results from the genetic tree show the subdivisions in the eight sampled populations of mountain gorillas. Using Nei's (1972) Original measures of genetic identity and genetic distance, groups Bitukura and Kyaguliro are similar and closely related to Shonji whereas Nkuringo and Rushegura groups were more similar and closely related Habiyanja, than they were to the rest of the groups (Table 7, Figure 4). As expected the gorilla group from Mgahinga was the outlier (Fig. 4 and Table 7).

Fig 4. Phylogenetic tree showing relatedness in different gorillas groups from Bwindi Impenetrable and Mgahinga National Park.



The dendrogram is based on Nei's (1972) Genetic distance method (UPGMA) modified from NEIGHBOR procedure of PHYLIP Version 3.5.

Table 2: Genetic variability in 13 microsatellite loci of 98 mountain gorillas of Bwindi Impenetrable and Mgahinga National Parks

Locus	Temp °C	*NA*	*N*	H_O	H_E	*AE*	*AR*	*PIC*	P_{HW}	F_{null}
vWF	60	8	96	0.67	0.77	4.22	7.99	0.74	0.91	0.06
D1s550	60	8	92	0.71	0.69	3.16	7.33	0.63	0.73	-0.02
D2s1326	60	6	87	0.67	0.73	3.64	5.98	0.68	0.08	0.04
D3s2459	55	9	88	0.71	0.83	5.80	7.99	0.81	0.92	0.08
D4s1627	60	6	76	0.57	0.73	3.60	6.00	0.68	0.01*	0.11
D5s1457	55	7	96	0.73	0.79	4.76	6.80	0.76	0.13	0.04
D5s1505	58	5	95	0.53	0.70	3.25	5.00	0.64	0.06	0.12
D5s1470	60	5	92	0.52	0.55	2.20	3.97	0.45	0.62	0.02
D7s817F2	60	6	95	0.78	0.79	4.76	6.00	0.76	0.80	0.01
D7s2204	55	6	77	0.57	0.65	2.81	6.00	0.62	0.42	0.06
D8s1106	55	4	93	0.36	0.51	2.03	4.00	0.47	0.03*	0.17
D10s1432	55	6	97	0.68	0.75	3.97	5.99	0.71	0.50	0.05
D16s2624	55	5	98	0.57	0.57	2.31	4.96	0.50	0.66	-0.01
Mean		**6**		**0.59**	**0.57**	**3.57**	**6.00**	**0.65**		
SD		**1.47**		**0.12**	**0.11**	**1.18**	**1.23**			

Temp °C- annealing temperature; *NA*-number of alleles; *N*-number of genotyped individuals; *AE*- effective number of alleles; *AR*- is allelic richness; *Ho*- observed heterozygosity; H_E- expected heterozygosity; *PIC*-polymorphic information content; P_{HW} -corrected *P*-value associated with Hardy-Weinberg equilibrium test
* statistically significant after Bonferroni correction at p value = 0.05. SD- standard deviation.

Table 3: Allele frequency at 13microsatellite loci in 8 groups of mountain gorillas

Locus vWF	**Nkuringo NK**	**Habinyanja HB**	**Mubale MU**	**Mgahinga MG**	**Rushegura RU**	**Bitukura BT**	**Shonji SH**	**Kyaguliro KY**
135			0.05	0.17				0.08
139	0.37	0.82	0.36	0.17	0.45	0.35	0.32	0.38
143	0.03	0.09	0.14	0.66	0.09	0.35	0.16	0.08
147						0.05	0.09	
151	0.03	0.09				0.25	0.04	0.13
155	0.41		0.40		0.09		0.07	0.20
159	0.16				0.37		0.07	
163			0.05				0.25	0.13
Locus D1s550								
178					0.05		0.16	0.08
182	0.37	0.30	0.64		0.45	0.37	0.55	0.34
186							0.02	
190	0.40	0.30	0.27	0.50	0.32	0.50	0.20	0.54
194							0.02	
198	0.03			0.50				
202	0.20	0.40	0.09		0.18	0.13	0.05	0.04

Locus D2s1326								
242	0.18	0.14	0.18		0.05	0.22	0.32	0.13
254		0.08		0.75				
258	0.39	0.58	0.59		0.30	0.44	0.18	0.50
262	0.14	0.08					0.15	0.04
270		0.08		0.25		0.06	0.15	
274	0.29	0.04	0.23		0.65	0.28	0.20	0.33
Locus D3s2459								
196	0.10	0.14	0.18			0.28	0.03	0.08
200	0.30	0.14		0.75	0.22	0.17	0.03	0.22
204	0.07		0.14		0.06	0.11	0.28	0.33
216				0.25				
232	0.03	0.18	0.14		0.11	0.11	0.08	0.25
236	0.20	0.09	0.23			0.11	0.30	
240			0.04		0.06			
244	0.23	0.05	0.27		0.55	0.22	0.28	0.04
248	0.07							0.08

Locus D4s1627								
234	0.13	0.05	0.19	0.50			0.09	0.08
238	0.31	0.27	0.44	0.50	0.39	0.44	0.46	0.54
242	0.03	0.27	0.06		0.28	0.17	0.27	0.13
246	0.40	0.36	0.19		0.22	0.39	0.18	0.25
250	0.13		0.12		0.11			
258		0.05						
Locus D5s1457								
111	0.06	0.21	0.04		0.04	0.30	0.38	0.17
115	0.69	0.42	0.14		0.64	0.25	0.12	0.29
119	0.06	0.04	0.18	1.00	0.18	0.05	0.07	0.08
123	0.03	0.21	0.41				0.17	0.25
127	0.16	0.12	0.23		0.14	0.20	0.24	0.17
131						0.20	0.02	0.04
Locus D5s1505								
203			0.09		0.30	0.11	0.07	0.13
207	0.75	0.13	0.73	0.63	0.50	0.22	0.14	0.24
211	0.22	0.54			0.20	0.50	0.34	0.50
215	0.03	0.29	0.18			0.17	0.32	0.13
219		0.04		0.37			0.13	

Locus D5s1470								
177								0.08
181	0.31	0.29	0.68	0.83	0.44	0.28	0.53	0.26
185	0.09					0.11		0.08
189	0.59	0.71	0.32	0.17	0.56	0.61	0.47	
197								0.58
Locus D7s817F2								
130	0.28	0.08	0.14		0.18	0.22		0.08
134	0.09	0.21	0.36	0.67	0.41	0.06	0.26	0.34
138	0.25	0.17		0.33	0.14	0.22	0.36	0.46
142	0.03	0.12	0.41		0.04	0.28	0.07	0.04
146	0.25	0.42	0.09		0.23	0.22	0.29	0.08
150	0.09						0.02	
Locus D7s2204								
239		0.05	0.09			0.11		0.09
243	0.09		0.14		0.20	0.06	0.08	
247						0.22	0.11	0.18
251	0.68	0.95	0.59		0.70	0.28	0.54	0.18
255	0.09		0.18		0.10	0.22	0.11	0.23
259	0.14					0.11	0.16	0.32

Locus D8s1106								
139			0.09				0.21	
143	0.03	0.14	0.05	0.17	0.05	0.15	0.05	0.13
147	0.75	0.68	0.77	0.83	0.67	0.80	0.36	0.83
151	0.22	0.18	0.09		0.28	0.05	0.38	0.04
Locus D10s1432								
160	0.06	0.08			0.05			0.17
164	0.03					0.05		0.04
168	0.31	0.54	0.77		0.55	0.30	0.14	0.13
172	0.60	0.21	0.09	0.50	0.30	0.25	0.14	0.33
176		0.13		0.25		0.30	0.47	0.29
180		0.04	0.14	0.25	0.10	0.10	0.25	0.04
Locus D16s2624								
124			0.05			0.05	0.11	0.04
128	0.31	0.54	0.36	0.25	0.36	0.25	0.20	0.25
132				0.38			0.03	0.04
136	0.56	0.46	0.59	0.25	0.64	0.70	0.59	0.63
140	0.13			0.12			0.07	0.04

Table 4: Population pairwise F_{ST} values

	NK	HB	MU	RU	BT	SH
HB	0.092*					
MU	0.137*	0.074*				
RU	0.031^{ns}	0.032*	0.093*			
BT	0.080*	0.055*	0.121*	0.053*		
SH	0.144*	0.073*	0.125*	0.092*	0.023 ^{ns}	
KY	0.070*	0.073*	0.115*	0.056*	0.019 ^{ns}	0.041 ^{ns}

* Significant at P = 0.05; ns not significant at P = 0.05

Table 5: Percentage assignment of individuals to groups wher they belong based on their multilocus genotypes

	NK	HB	MU	RU	BT	SH	KY
NK	**93.33**						**6.67**
HB		**90.91**			**9.09**		
MU			**100.00**				
RU				**100.00**			
BT					**87.50**		**12.50**
SH						**100.00**	
KY							**100.00**

Table 6: F-Statistics and Gene Flow for all Loci in all the sampled populations of mountain gorillas of Bwindi Impenetrable and Mgahinga Gorilla National Parks

Locus	Sample size	Fis	Fst	Nm*
vWF	96	0.01	0.17	1.22
D1s550	92	-0.13	0.18	1.16
D2s1326	87	-0.06	0.20	0.99
D3s2459	89	0.10	0.15	1.40
D4s1627	77	0.24	0.09	2.66
D5s1457	96	-0.12	0.25	0.76
D5s1505	95	0.05	0.17	1.20
D5s1470	93	0.01	0.15	1.42
D7s817F2	95	-0.03	0.12	1.82
D7s2204	77	-0.01	0.34	0.49
D8s1106	98	0.21	0.10	2.31
D10s1432	97	-0.17	0.17	1.24
D16s2624	98	-0.12	0.08	2.93
Mean	**91**	**-0.030**	**0.17**	**1.24**

Table 7. Nei's Original Measures of Genetic Identity and Genetic distance for mountain gorilla groups of Bwindi Impenetrable and Mgahinga Gorilla National Parks

Group ID	NK	HB	MU	MG	RU	BT	SH	KY
NK	****	0.8424	0.8046	0.4198	0.8593	0.8102	0.7018	0.8113
HB	0.1714	****	0.8478	0.3649	0.8652	0.8063	0.7934	0.7586
MU	0.2174	0.1652	****	0.4292	0.7973	0.7609	0.7385	0.7555
MG	0.8680	1.0082	0.8459	****	0.3724	0.3962	0.3973	0.4373
RU	0.1516	0.1448	0.2265	0.9877	****	0.7963	0.7318	0.7807
BT	0.2105	0.2153	0.2733	0.9259	0.2278	****	0.7971	0.8846
SH	0.3542	0.2314	0.3031	0.9230	0.3122	0.2268	****	0.7740
KY	0.2091	0.2763	0.2804	0.8271	0.2476	0.1226	0.2562	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal). NK-Nkuringo, HB-Habiyanja, MU Mubale, MG Mgahinga, RU Rushegura, BT Bitukura, SH Shonji and KY Kyaguliro.

CHAPTER FIVE

DISCUSSION

There was a high allelic diversity and a high genetic diversity in the sampled mountain gorillas. Gorilla groups ranging at the same altitude were found to be more genetically similar to each other compared to those sampled at different altitudes. This is clearly evidenced by the partitioning of the different groups into sister clades concordant with the two altitudes where sampling was conducted (Fig. 5). The Mgahinga gorillas sampled approximately 25 km away from Bwindi gorillas (Thompson *et al.,* 2009) were much more genetically differentiated from the Bwindi groups. The observed heterozygosity in this study (0.59) is comparatively lower than what has been observed previously among other non-human primates (when compared to Western lowland gorillas 0.83, Bradley et al., 2004; Baboons, 0.81, Nguyen *et al.,* 2009; Rhesus macaques, 0.73, Charpentier et al., 2008). Although two hierarchical genetic clusters have been identified among the seven groups sampled within Bwindi, extensive sampling of the whole Bwindi population at 16 microsatellite loci, revealed three genetic clusters and higher levels of heterozygosity (0.68) in the previous study of Guschanki *et al.* (2009).

The significant genetic differentiation between groups as evidenced by the significant F_{ST} values (Table 6) coupled with the relatively low levels of heterozygosity and high frequency of private alleles are indicative of genetic drift operating as a major driving force in shaping the population genetic structure of the different gorilla groups of Bwindi and Mgahinga. There was limited gene flow in the entire sampled population though it was more pronounced in the groups sampled at a higher altitude with three out of four groups possessing private alleles and in some cases with high frequencies.

The genetic structuring of the Bwindi and Mgahinga groups could have been accentuated by habitat fragmentation due to human settlement coupled with the social organization of gorilla groups which favors mating within groups but not between groups and sometimes with only the dominant male within the group. Bwindi is divided into the northern and the southern sections and until now no gorilla groups have been observed to occupy the middle section know as the 'neck'. This section was severely logged and damaged during the political insecurity and gorillas seem to have avoided it since. This could also be seen from gorilla locations during the census (Guschanki, *et al.,* 2009). This evidence is shown by having southern gorillas clustering together and the northern ones too (Figure 3). The results clearly indicate that if nothing is done this may lead to these two clusters becoming more genetically distinct with time. To therefore harmonize gene flow conservation efforts should be geared towards rehabilitating this section of the park to encourage gorillas to occupy it and thus facilitate gene flow.

Although low heterozygosity was observed, the existent strong genetic differentiation evidenced by the significant 17 out of the 21 possible pairwise group comparisons in Bwindi gorillas suggested limited geneflow between groups and this was further supported by the results from the assignment tests where 95.9% of the sampled individuals were correctly assigned to the groups where they were sampled. Despite a migration rate of 1.24 migrants per generation, the forces of genetic drift seem to act strongly on the study population resulting in the intergroup genetic differentiation. Interpopulation differentiation can be as a result of genetic drift, mating systems or founder effects. In addition Wahlund effect could also result in underestimation of the genetic variability within a population. However, in the case of gorillas, family groups were treated as distinct sampling units and therefore the Wahlund effect had no effect on the extent of genetic differentiation. This kind of mating system in mountain gorillas does not favour random choice of mates by females both within

and between the groups. Gorillas, like in other social species, males prefer philopatry as compared to females and will thus queue for dominance after the death of the reigning dominant male and still hinder the random mating resulting into females mating with close relatives within the group hence low heterozygosity levels as observed in this study. This type of social behavior however, is beneficial as it enhances co-operation between male within groups to the extent that males within groups have been observed to cooperate in preventing males from other groups abducting females during group encounters (Sigg *et al.* 1982; Hammond *et al.,* 2006). The cohesive social structure of gorillas coupled with philopatry favours limited gene flow between groups, increased inbreeding within groups thus favouring accumulation of private alleles as observed in this study. Evidence of inbreeding was seen at some loci especially those with private alleles occurring with high frequencies.

Population genetics theory predicts that reductions in population size and limited migration decrease local genetic variation, triggering negative genetic processes such as inbreeding depression and loss of adaptive potential (Frankham et al., 2002). Geographically distant populations tend to show greater levels of differentiations due to lower levels of gene flow (Slatkin, 1993). This is evidenced by the results (fig. 5) showing the group from Mgahinga as an outlier and very different from the Bwindi groups. On the other hand, little or no genetic differentiation in geographically isolated populations is an indication of a recent separation (Alcaide et al, 2009).

The observed inbreeding levels (F_{IS}) coupled with genetic isolation of different groups caused by habitat fragmentation due to increased human population pressure poses a threat to the future survival of this range restricted species and as such appropriate conservation measures need to be reinforced to mitigate this trend.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion and future directions

This study has provided insights into the enormous applications of noninvasive sampling in defining the population structure of critically endangered and elusive animals. All the loci were in *HWE* with analysis of Bwindi gorillas only and two loci deviated with analysis of the entire sampled population including the Mgahinga gorillas. Results of this study imply that while there is considerable genetic diversity in the species as a whole, this diversity is distributed over the various populations, each having comparatively little intra genetic variation.

Although individual populations exhibited positive fixation indices at some loci, the overall fixation index for the total sample across all loci was negative, suggesting that this habituated population is after all not as highly inbred as it may have been speculated. Though an average of 1.24 migrants per generation was calculated, the forces of genetic drift still act strongly on this population. Mgahinga gorillas seemed to cluster together with the gorillas sampled at higher altitude; although the unrooted genetic tree showed they are outliers.

The results from this study were biased to only habituated groups in Bwindi and Mgahinga National Parks but extensive sampling of all gorillas population both habituated and non-habituated is needed in order to make dependable decisions which will direct the responsible authorities into coming up with reliable management plans based on these scientific findings.

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