Morphological Variation of Two Howler Monkey Species and Their Genetically-Confirmed Hybrids

by

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To Mom and Dad

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PREFACE

I chose to develop my dissertation into three stand-alone papers which are written for individual publication. In the introduction chapter, I explain how the three papers together address the main goal of my dissertation, and in the conclusion, I discuss the implications of my findings and future directions. While I refer to all the work in the first person, the work would not have been possible without the collaboration of several other researchers. Those individuals are acknowledged or will be acknowledged not only in this publication but also in the respective journals where each paper will be submitted.

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ABSTRACT

Hybridization challenges traditional species definitions, the most common being that a species comprises reproductively isolated individuals (Mayr, 1963). Although hybridization has been reported for several primate species, this dissertation is the first to investigate morphological variation in a Neotropical primate hybrid system. Two related howler monkey species, *A. palliata* and *A. pigra*, are known to hybridize in an area within Tabasco, Mexico. Using mitochondrial DNA, the SRY gene, and microsatellites, I identify hybrid individuals of different generations of crossbreeding and backcrossing to answer questions about hybrid morphology. What do hybrids look like when compared to purebred individuals? Is there a sex bias in the expression of hybrid morphology? I begin by comparing and contrasting the morphology of the two parent species and reporting how differences between them are shaped by differences in the extent of sexual selection. This will not only provide a basis for understanding the morphological variation present in hybrid individuals but also lay the groundwork for future research on the selective forces that hybrids are subject to. Therefore, the dissertation is comprised of three parts: 1) A review of the contributions of and the approaches used in the study of primate hybridization, 2) the impact of intra-sexual selection on sexual dimorphism and testes size in *A. palliata* and *A. pigra*, and 3) the morphology of hybrid versus purebred howler monkeys. My work sheds light on the range of variability in

morphological expression when genetically distinctive populations crossbreed. It will also serve as a model for evaluating the issue of hybridization in the primate fossil record.

CHAPTER 1: INTRODUCTION

The study of extant primates has provided many insights for understanding human evolution. The Neotropical primate system considered in this dissertation is a particularly valuable one. Howler monkeys of the genus *Alouatta* are aptly named due to their possession of an enlarged hyoid bone that aids them in the production of loud calls. These monkeys are interesting because of the large degree of inter- and intra-specific variation in social organization (Crockett and Eisenberg, 1987). While it is one of the most studied of all the New World monkeys, a detailed analysis of their systematics based on molecular data was only completed recently (Cortés-Ortiz et al., 2003), and there is much to learn about the intricacies of the genus with respect to how different species came to occupy their current geographic distributions, the evolutionary factors that played a role in their speciation, and why differences exist in their demography, behavior, and morphology. This dissertation provides and addresses the data needed to answer some of these questions. In particular, it deals with the morphological variation in two specific howler monkey species, *Alouatta pigra* and *Alouatta palliata*, as well as their hybrids from Tabasco, Mexico.

The subspecies of *A. palliata* that I studied for this dissertation is *A. p. mexicana*, but there are other documented possible subspecies, including *A. p. aequatorialis*, *A. p. palliata*, *A. p. coibensis*, and *A. p. trabeata*. The range of *A. palliata* covers southern Veracruz, Oaxaca, and Tabasco in Mexico, possibly parts of

Guatemala, and extends south through Honduras, Nicaragua, Costa Rica, Panama, Colombia, Ecuador, reaching the northwestern tip of Peru in the Tumbes region. *A. pigra* occupies a smaller geographical range, which covers the Yucatan peninsula in Mexico, Belize, and parts of Guatemala (Cortés-Ortiz et al., 2007).

Cortés-Ortiz et al. (2007) had confirmed hybridization between *A. palliata mexicana* and *A. pigra* in their area of contact in Tabasco, Mexico. Understanding hybridization of this system is of importance to evolutionary biology and anthropology because of the role that hybridization may play in speciation and how it affects the genetic diversity of the interacting taxa (Mallet, 2007). More specifically, it has implications for understanding the isolating mechanisms that can maintain species identity and the forces that these species have to face to occupy their current distributions. In addition, the study of hybridization in a primate may shed light on the question of whether hybridization could have occurred in our own recent evolution. Hybridization could have caused different degrees of reticulation within the hominin phylogeny (Holliday, 2003). Other primate hybridization systems have been proposed as good models for interpreting evidence of hybridization in the human fossil record (Jolly, 2001). However, knowledge of the extent of variation in the genotype and phenotype of hybridizing taxa and their hybrid offspring is needed to better understand what factors affect the development of reproductive isolation mechanisms.

In chapter two, I discuss some of the studies that contributed to our current understanding of primate hybridization and the approaches they utilized to identify hybrid individuals, study hybrid zones, and infer the extent of ancient hybridization responsible for shaping current patterns of primate species

variation. I point out the strengths and weaknesses of the methods employed in such studies and offer suggestions for future directions, including the need for the type of data generated from this dissertation's research.

In chapter three I examine morphological data from *A. palliata* and *A. pigra* and interpret how it correlates with the two species social systems. One of the goals of physical anthropology and primatology is to understand how primate social systems influence the evolution of sexually selected traits. Howler monkeys provide a good model for studying sexual selection due to differences in social systems between related species. I use a resampling approach to analyze differences in sexual dimorphism of body and canine size. In addition, I compare testes size as a way of gauging the intensity of sperm competition in both species. The discussion on sexual dimorphism and its relationship to male and female competition has a long history (Plavcan, 2001). This study provides a good example of the complexity of this relationship, in addition to informing those who infer behavior from the morphology of hominin fossil specimens. Moreover, this study generated the data necessary for establishing the extent of morphological variation in each species to be compared with hybrid morphological variation.

In chapter four, I compare the data produced in chapter three with morphological data obtained from hybrid individuals. Instances of hybridization have been reported in primate species, where hybrids are identified based on morphology. However, multigenerational hybrids may not always be detected using such methods. I investigate the morphology of howler monkey hybrids detected by genotyping uni- and bi-parentally inherited markers (mtDNA, SRY genes, and microsatellites). *A. pigra* and *A. palliata* diverged approximately 3 mya

(Cortés-Ortiz et al., 2003). The two species can be distinguished based on their overall appearance, but they are similar enough such that hybrids can be difficult to identify. Moreover, evidence exists for directionality of hybridization, where hybridization may only occur between *A. palliata* males and *A. pigra* females, potentially biasing the morphology of the hybrids (Cortés-Ortiz et al., 2007). Hybrid males are only found in *A. pigra* groups (where they resemble *A. pigra* in outward appearance) whereas hybrid females are found in groups of both species and resemble the species of the group in which they reside. I compare genetically confirmed hybrids to pure individuals for several morphometric traits for each sex and discuss the implications of the findings for using morphology as a diagnostic tool and the longevity of the signature of hybridization.

Overall, data on morphology of *A. pigra*, *A. palliata*, and their hybrids, combined with our understanding of the parental species social systems and extent of evolutionary divergence, provides an informative picture to infer possible fitness associated to different genotypes and phenotypes within and between species and their hybrids.

CHAPTER TWO: History of the study of primate hybridization

Introduction

Recently, primate hybridization has been garnering increased attention. One reason is that hybridization in primates is now a fairly well-documented phenomenon (Arnold and Myer, 2006; Cortés-Ortiz et al., 2007). Primate hybrids were recognized as early as the late nineteenth century (Chiarelli, 1973). As of 2007, approximately 34 primate species were known to naturally hybridize (Cortés-Ortiz et al., 2007). Primates have been shown to exhibit interspecific as well as intergeneric hybridization (Jolly, 2001), suggesting variation in the extent of reproductive isolation across this order. Primate hybridization can inform studies of primate evolution about the role that hybridization has played in shaping the diversity within this lineage (Arnold, 2004). Therefore, primate hybridization is of general interest for the purposes of understanding primate diversity, especially with implications for conservation (Detwiler et al., 2005) and determining the origins of particular genes and adaptations (Evans et al., 2006; Hawks and Cochran, 2006) in primates. This chapter reviews some of the main research that has contributed to the development of this field and points out the strengths and weaknesses of particular approaches to the study of primate hybridization.

Hybridization as it is used here is defined as the interbreeding between individuals from genetically distinct groups (Harrison, 1990). It has also been

defined as the interbreeding of individuals from parental taxa that are distinguishable by one or more heritable traits (Evans, 2001). Because species' definitions continue to be debated (Holliday, 2003; Coyne and Orr, 2004), the term hybridization here will be used regardless of the taxonomic distinctions of the parental taxa. Several terms that may arise in this discussion that are often associated with hybridization include backcrossing, crossbreeding, gene flow, admixture, introgression, and reticulation. When a hybrid individual mates with a purebred individual, the result of the mating is said to be a backcrossed individual. Hybridization and crossbreeding are often used interchangeable, but crossbreeding sometimes refers to the reproduction between two distinguishable groups within a species. Gene flow refers to the transfer of genes from one population to another while Admixture (or intermixture) specifically refers to the production of new genetic variants through recombination (Allendorf et al., 2001). Another term used in hybridization studies is introgression, or introgressive hybridization, which was originally introduced by Anderson and Hubricht (1938). It refers to movement of novel alleles from one species into another through the repeated backcrossing of hybrids with one of their parental taxa. Introduced genes may face possible epistatic interactions subject to selection in novel environments (Hawks and Cochran, 2006), so genes that are beneficial often persist for several generations. Finally, when hybridization takes place between lineages after their initial divergence, it leads to web-like phylogenetic relationships and is referred to as reticulation (Holliday, 2003).

Different approaches have been taken to recognize and study hybridization in primates. In this chapter, I structure my discussion based on the type of approaches utilized in these studies. The most direct methods for

identifying hybrid individuals are those where interspecific mating was observed in captivity. Therefore, I review findings from studies based on captive primate populations. However, identifying hybrid individuals in the wild is of importance to those interested in studying the dynamics of natural hybrid zones and the implications of hybridization to the evolution of taxa. I initially explore studies where wild hybrids are identified using morphological characteristics. With increasing availability of molecular markers, more studies are uncovering instances of primate hybridization, so I dedicate a section to review the types of molecular data that can be used for this purpose. Finally, many studies suggest that hybridization can sometimes lead to discordant phylogenies created from different data sets. I address examples of primate studies where hybridization was inferred from the presence of such discordances. Taken together, these methods have provided a way to ascertain the role of hybridization in primate evolution. I conclude by offering suggestions for the kind of research that is necessary to move forward.

Hybridization studies based on captive populations

Some of the earliest discoveries of instances of primate hybridization took place in captivity, including zoos, sanctuaries, colonies, and research centers (Chiarelli, 1961; Buettner-Janusch, 1966). Identifying hybrids in captive settings is often straightforward, specifically when a hybrid offspring is produced from the observed or inferred mating of individuals from two recognized subspecies, species, or genera. Further, backcrossing of F1 (first generation hybrids) can also be documented, allowing for research on more long-term effects of hybridization. In many cases, interspecific hybrids were first identified in captivity before they

were studied in their natural environments such as some species of macaques (Bernstein, 1966) or have been confirmed when natural hybridization has been suggested such as in howler monkeys (De Sousa Jesus et al., 2010).

Hybrid research in captivity is useful for identifying mechanisms of reproductive isolation. If populations of two parental species are in contact but do not hybridize in the wild, then hybridization and the production of viable offspring in captivity could provide evidence for prezygotic isolating mechanisms. That is, the parental species, under natural conditions, either do not recognize potential mates from the other species, or are incapable of copulating and achieving successful fertilization (Coyne and Orr, 2004). As Bernstein noted (1966), "Separation mechanisms dependent upon geography are inoperative under the usual conditions of captive confinement, and many behavioral mechanisms may be overcome under artificial conditions." Even in the absence of isolating geographic barriers, some animals may form polyspecific associations in nature but only actually hybridize in captivity (Godfrey and Marks, 1991).

A disadvantage of using captive populations is that it is not possible to obtain an accurate idea of hybrid fitness. The production of viable and fertile offspring through crossbreeding in captive conditions would suggest a minimum or null effect of endogenous selection on hybrid individuals, but hybrid fitness can also be affected by exogenous selection (Burke and Arnold, 2001). Researchers may be able to rule out a hybrid fitness disadvantage due to inherent incompatibilities affecting its development but would not be able to test whether the hybrid offspring would be adapted to the environment of the parental taxa. The availability of ample nutritional resources, lack of predators,

and medical treatments in captive settings may bias any inferences one may make on the likelihood of hybrids to survive in natural settings.

Nevertheless, captive hybrid primate studies have provided useful evidence for the potential of species, and even different genera, to crossbreed. It is often the case that crossbreeding between genera only occurs when it is induced in captive settings. Well known intergeneric hybrid primates include rheboons (mating between baboons and rhesus macaques, Kuel and Harris, 1995; Markarjann et al., 1974) at the Southwest Foundation. The rheboon hybrid was sterile and so it is assumed that if any were to occur in the wild, they would be unlikely to reproduce (Jolly, 2001). There are also larcons, which are produced from mating between Hylobates lar and concolor-group gibbons (Hirai et al., 2007), although it is not known whether these individuals are fertile. Yet another example is siabon, hybrid offspring of gibbons and siamangs (Myers and Shafer, 1979; Wolkin and Myers, 1980). Finally, there are reports of crosses between different guenons and papionins (Lernould, 1988) as well as geladas and hamadryas or anubis baboons (Markarjan et al., 1974). These examples indicate that hybridization can occur despite very long divergence times, sometimes up to an estimated 14 mya.

Captive hybrid primates can be good candidates for some studies that address the longevity of hybridization signals after several generations of interbreeding and backcrossing with the parental taxa. Some captive primate colonies are monitored such that relatedness among individuals is known through observations of reproductive events or the use of genetic data (Godfrey and Marks, 1991). Such data of known pedigrees provide information as to whether hybrid individuals are first generation or backcrossed with the parental

species and therefore allow for analysis of hybrid phenotype for hybrids of different genetic backgrounds. Specifically, many researchers have studied the morphology of hybrids produced in captivity. Earlier, Smith and Scott (1989) measured crown-rump length and weight in Chinese-Indian hybrid rhesus macaques and found heterosis (defined as dimensions larger than midparental average values) in hybrids compared to non-hybrids. Hybrids of different saddle-back tamarins also exhibited heterosis for both cranial (Cheverud et al., 1993) and post-cranial (Kohn et al., 2001) morphological traits as well as an increase in infant survival (Jaquish, 1994). Kohn et al. (2001) interpreted these results as evidence against Templeton's idea that coadaptation in gene complexes is disrupted during outbreeding. Ackermann et al. (2006) also found heterosis, though less pronounced, for hybrid (olive x yellow baboons and backcrosses) cranial traits, in addition to increased variation and the presence of novel phenotypes for nonmetric traits. The authors concluded that qualitative morphological signatures, rather than heterosis in metric traits, are better for detecting hybridization between taxa that are not well differentiated.

In sum, hybridization studies based on captive primates, where pedigrees are sometimes known, have provided a foundation for understanding the extent that hybridization can occur in primates as well as some of the phenotypic outcomes of hybridization. In addition, captive studies can reveal whether isolating mechanisms between species are primarily pre- postzygotic. Apart from the limitations of such studies for predicting hybrid fitness, studies in captive settings should consider whether particular genetic signatures or morphological anomalies have resulted from hybridization or whether they are an artifact of

founder's effect, a phenomenon that can occur in zoo populations which were established from small populations (Ackermann et al., 2006).

Studies of wild primate hybrids detected through morphological features

While captive primates provide ample evidence for the potential of different primate genera and species to crossbreed and produce viable offspring, field studies instead contribute to our understanding of the mechanisms involved in natural hybridization and the structuring of hybrid zones in the wild (Mayr, 1942; Woodruff, 1973; Barton and Hewitt, 1985; Hewitt, 1988; Arnold and Hodges, 1995). Many studies of primate hybridization the wild have relied on morphological observations combined with statistical methods to identify hybrid individuals in hybrid zones and are discussed in the following sections. However, there are several concerns over using such data, including the availability of morphological markers than can be clearly defined in the hybrids, the inability to distinguish between hybrids of different generations, and the deviations from expected morphological values due to genetic interactions.

Many primary reports of instances of hybridization describe such morphological observations for purported hybrid zones. This was the case for many primate taxa, including baboons (Samuel and Altman, 1986), macaques (Groves, 1980; Ciani et al., 1989; Watanabe and Matsumura, 1991; Bynum et al., 1997), langurs (Choudhury, 2008), guenons (Struhsaker et al., 1988), lemurs (Sterling and Ramarason, 1996), squirrel monkeys (Jones et al., 1973; Thorington, 1985), marmosets (reviewed in Marroig et al., 2004) and howler monkeys (Aguiar et al., 2008; Agostini et al., 2008; Bicca-Marques et al., 2008).

For many of these studies, quantitative methods were employed to attempt to identify hybrid groups based on morphology and include multivariate statistical procedures such as discriminant function analysis (DFA) and principal component analysis (PCA). Researchers may employ these multivariable methods on morphometric traits to determine whether differences in overall morphology exist between groups. DFA can reveal which morphological variables distinguish populations, and have been performed for some primate hybridization studies (Supriatna, 1991; Schillaci et al., 2005; Gligor et al., 2009). These studies were able to distinguish hybrids from the parental species using morphometric data. When dealing with many variables, a common method is data reduction, such as factor analysis or PCA, where the goal is to reduce all considered variables into a number of uncorrelated components (Sokal and Rohlf, 1995). Differences in the component scores between groups can then be used in statistical tests. Whereas univariate methods such as ANOVA or ANCOVA often show how variables compare in magnitude between groups, PCA variable loadings may reveal not only size but also shape differences (Hayes et al., 1990). Data reduction techniques have been utilized in primate hybrid morphology studies (Cheverud et al., 1993; Schillaci et al., 2001; Hamada et al., 2006; Ackermann and Bishop, 2010; Delmore et al., 2011). In these studies, several morphometric variables were highly correlated, and the first principal component explained the most variation and helped to discriminate among groups when subjected to statistical testing. Care should be taken not to violate the numerous assumptions underlying most multivariable methods, such as the assumption of normality, which is usually difficult when analyzing data from natural populations of primates with small sample sizes.

Rather than using metric traits, some primate hybrid studies rely on external nonmetric features. In such cases, hybrids can be more easily identified when the two parental species differ in readily observable morphological characteristics as the intermediacy or mosaic phenotypes can be apparent, particularly where the parental species are in contact, such as in baboon hybrid zones (Jolly, 2001) and/or when polyspecific social groups exist (Southwick and Southwick, 1983). Such hybrids are described as having intermediate features, which often mean they display features distinctive to both pure forms and/or features that are the average between them, for traits such as pelage coloration and other external features (Jolly et al., 1997; Hamada et al., 2006). Anubis x hamadryas baboon hybrids have been identified along the border of the two species distributions in the Awash Valley, where hybrid groups apparently expressed a range of phenotypes that included characteristics from both parental species (Nagel, 1973). Yet, as Tung et al. (2008) pointed out, morphological variables for identifying hybrids are often pre-defined by the researchers and are subject to observer bias. The same study also brought up the fact that phenotypic differences only reflect variation at a few loci and may not be accurately representative of the degree of hybridization, a phenomenon that can affect that whole genome.

Only a few studies have attempted to correlate traits scored from external features of individuals in natural hybrid zones with genetic evidence of their ancestry. These studies have shown a correlation between morphology and genetics, but the morphological markers used included face color, mane color, cheek tuft color, hair length, tail shape, anal patch shape, etc. (Nagel, 1973; Bergman and Beehner, 2004) and were found to be highly correlated with genetic

ancestry of the hybrids (except in Tung et al., 2008). It is possible that such features are not subject to dominance or epistatic effects. These traits are used to construct what has been called the morphological hybridity index (MHI) or phenotypic hybrid index (PHI), which is calculated based on knowing the character traits for the parental species (Nagel, 1973; Sugawara, 1988; Froehlich and Supriatna, 1996; Bynum et al., 1997; Alberts and Altmann, 2001; Bynum, 2002; Tung et al., 2008; Charpentier et al., 2008; Delmore et al., 2011). This index is based on traits that sort independently (Nagel, 1973). In baboons, it was later modified for a greater resolution of intermediate phenotypes using additional intermediate character states (Bergman and Beehner, 2003). While this morphological index has more or less coincided with a genetic hybridity index, it may not be reliable for other primate hybrid systems with a general lack of F1 hybrids, when there is difficulty in detecting clear patterns in hybrid morphology, or when pelage coloration is not sufficient for discriminating between taxa (Steinberg et al., 2009). Even in cases where the correlation of genetic and morphological data for the hybrid index has been attempted, the correlation was weakened because of cryptic hybridization (Tung et al., 2008). Phillips-Conroy et al. (1991) noted: "In the investigation of hybrid zones, the primatologist has the advantage of highly visible subjects, whose individual identity and long-term history can often be determined." However, this has only been the case for a few primate studies, including the baboon hybrid zones that have been under observation for a few decades. Molecular data is often necessary to confirm hybridization and the MHI only serves to generalize overall patterns of hybrid morphology (Phillips-Conroy et al., 1991; Alberts and Altmann, 2001).

Some morphological methods for identifying hybrids are validated based on captive individuals with known pedigrees, such as for Chinese and Indian rhesus macaques (Hamada et al., 2006). However, studies that use morphological evidence to suggest hybridization may in fact underestimate the degree of hybridization for some taxa. This has led some to coin the term "cryptic hybridization," a phenomenon that occurs where hybridization does not produce morphologically discernible traits (Rees et al., 2003). This can happen if the genes that have been introduced through hybridization are not linked to morphological phenotypes (Ackermann, 2010). Molecular data has confirmed cases of hybridization in non-primate animals that would have remained undiscovered using morphological evidence alone (Rees et al., 2003; Gaubert et al., 2005). Therefore, when possible, molecular data should be incorporated in primate studies as well.

Using morphology to identify hybrids can also be problematic because of variation in the interactions between the two parental genomes. Primate hybrids of known pedigrees have been measured for departures from the expected average of the parental values (such as in Smith and Scott, 1989; Cheverud et al., 1993; Schillaci et al., 2005; Ackermann et al., 2006). Intermediate, or average, features are expected only when additive traits account for the differences between taxa that diverged recently or regularly exchange migrants (Falconer and Mackay, 1997). However, dominance and epistatic allele interactions can lead to significant variation in expression of morphological traits in hybrids and a departure from the expected values under an additive allele model, particularly for hybridizing taxa with long divergence times (see Ackermann, 2010 for an in-depth discussion). As shown in captive studies, the extent of variation could

include heterosis (hybrid vigor), dysgenesis, or transgressive hybridization, where some hybrid individuals may possess values outside the range of the parental taxa (Ackermann, 2010). Many primate hybrid morphological studies have revealed this range of phenotypic expression for cranial and postcranial traits (Smith and Scott, 1989; Cheverud et al., 1993; Kohn et al., 2001; Ackermann et al., 2006; Kelaita et al., 2009). While first generation hybrids are known to express much variability in morphology (Ackermann et al., 2006), the inclusion of multigenerational hybrids leads to an even more variable sample (Schueler and Rising, 1976). Knowing the generation of the hybrid could reduce some of that variability and give a better idea of the phenotypic expectations for certain hybrid crosses; however, this type of information for natural populations is often unavailable as it requires long-term research of the hybridizing populations.

One concern for morphological studies of hybridization, and a reason for the need to combine morphological data with other kinds of data, is that morphology often reflects variation at one or a few loci, whereas genetic data are often acquired from loci from across the genome (Tung et al., 2010). In general, the high degree of morphological variability observed for many primate hybrids either metrically or non-metrically (Phillips-Conroy and Jolly, 1986; Froehlich and Supriatna, 1996; Bynum et al., 1997; Bynum, 2002; Peres et al., 1996; Ackermann et al., 2006; Aguiar et al., 2008) shows that morphological traits should be used with caution as a diagnostic tool, and that there is much to learn about the factors that influence their expression.

Finally, while it is clear that morphological features are not as reliable for detecting hybrid individuals and their degrees of hybridization, it is still important to understand the morphology of hybrids of known genetic ancestry.

One reason is that understanding the fitness of particular phenotypes is crucial for knowing the contributions of hybridization to the genetic diversity of species. Another reason is that morphological analysis is usually the only way to explore fossil specimens, with the exception of fossils where ancient DNA can be extracted. Possible examples of hybridization in human evolution based on morphological data have been reviewed recently (Holliday, 2003; Arnold and Meyer, 2006; Schwartz and Tattersall, 2010; Ackermann, 2010) and recent molecular work has supported some of these cases. Recently, a study based on 4 billion nucleotides from three Neanderthals suggested that gene flow occurred between Neanderthals and ancestors of non-Africans before they occupied Eurasia (Green et al., 2010).

While for some primate taxa studies morphological evidence has been utilized to identify some hybrid individuals in the wild, the interbreeding of other primate taxa would be missed with the exclusive use of this type of data. Evidence cautions against the single use of such diagnostic tools and instead suggests the incorporation of molecular markers. Nevertheless, further studies are needed to understand different aspects of hybrid morphology, including the variation observed in distinct hybrid systems as well as the longevity of hybrid morphological signatures, especially due to the implications for hybrid fitness as well as recognizing hybrids in the fossil record.

Genetic studies for detecting hybridization in wild primates

Hybrids can be identified with molecular and cytogenetic methods because hybridization results in the exchange of genetic material between taxa and hybrids will contain a mixture of alleles or chromosomal arrangements from

both parental forms. While some have criticized the over-reliance on genetic data in some cases (Jolly, 2001), they are not without utility for inferring population demographic processes. The parental species can be distinguished on the basis of discrete genetic differences without relying on misleading morphological traits (see discussion above).

Generally, molecular markers have led to numerous discoveries of instances of hybridization, sometimes confirming suspected hybrid zones from morphological studies, and other times revealing cryptic hybridization (Cortes-Ortiz et al., 2007; chapter three). Identification of hybrid individuals can be more reliable with the use of codominant markers (e.g. allozymes, RFLPs, DNA sequences, SNPs, and microsatellites; Boecklen and Howard, 1997; Freeland, 2006). Such markers involve several potential identifiable alleles for particular loci, allowing for the distinction between homozygotes and heterozygotes, as opposed to dominant markers where only the dominant allele can be obtained (Freeland, 2006). Ideally, researchers should aim to find loci with alleles that are fixed in the parental species. For many primate studies, this often entails testing whether loci isolated from humans or other primates can be amplified with success in both of the primate parental species in question, exhibit polymorphisms in both parental species, and contain alleles that are unique to each of them. When different alleles that are fixed in each of the parental species are simultaneously present in the offspring, then the individual is likely a hybrid.

As early as 1981, electrophoretic blood protein variation was used to identify hybrids produced from *Papio anubis* and *P. hamadryas* (Shotake, 1981). Only three markers were effective for distinguishing between the two species although authors concluded in that case that the genetic distance between the

parental taxa was too small to warrant separate biological species status. The authors used the three markers to assign a genetic hybrid index score to identify hybrids. Protein loci also provided evidence of gene flow in Sulawesi macaques (Ciani et al., 1989) and squirrel monkeys (Silva et al., 1992).

Szmulewicz et al. (1999) identified hybrids as those with Alu repeat elements with frequencies intermediate between those of the parental species. Presence and absence of Alu elements (in particular, short intersperced elements, which are retrotransposons found in primates that integrate via an RNA intermediate into the genome) was also used to detect squirrel monkey hybrids (Osterholz et al., 2008). Variation among taxa is represented by the absence or presence of the insertions, and the authors were only able to detect F1 hybrids with certainty. But recently, microsatellite markers are becomingly increasingly popular due to their highly polymorphic nature. Some of the first primate genetic hybrid indices were developed for baboons, such as in 1981 by Shotake et al. based on protein data and in 1999 by Woolley-Barker based on nine autosomal and one Y-chromosome microsatellites. Cortés-Ortiz et al. (2007) were able to identify hybrids produced from mating of *Alouata palliata mexicana* and *Alouatta pigra* using multiple diagnostic markers that included sequence data from the mitochondrial DNA and the Y-chromosome, as well as microsatellite loci to identify hybrids. Similar approaches have been adopted by as Gligor et al. (2009) for mouse lemur hybrids and Merker et al. (2009) for tarsiers.

If microsatellite loci are used alone, this method requires testing a large number of loci, especially if one is interested in determining the generation of the hybrid, or the hybrid genotype class (i.e. F1, F2, backcrosses, etc…) in order to assess the patterns of introgression. Vähä and Primmer (2006) recommend the

use of as few as 12-24 loci for finding F1 hybrids and at least 48 loci for separating backcrosses from parental individuals (although earlier, Boeklen and Howard in 1997 had suggested that 70 markers would be necessary). It is often difficult to determine whether backcrossed individuals represent multigenerational hybrids or members of the parental species possessing some introgressed alleles (Rhymer and Simberloff, 1996). This could prove extremely difficult in primate species where researchers are unable to identify diagnostic loci, in addition to being time consuming and costly for the typical budget of a primatologist. The likelihood of finding completely diagnostic loci is small, especially for taxa with short divergence times (Sanz et al., 2009). Species may be distinguishable on the basis of morphology or behavior but not necessarily "well-behaved genetic markers" (Harrison, 1993). In most cases, loci will exhibit alleles that are present in both species either due to past introgression or because they were retained in both species from their last common ancestor, sometimes even presenting problems for delineating the parental species. However, the use of a large number of semi-diagnostic markers may still allow for the fine-scale inference of hybrid ancestry (Bert and Arnold, 1995). Indeed such markers have been used in assigning ancestry for baboon primate hybrids (Wooly-Barker, 1999; Bergman et al., 2008), but as Tung et al. (2008) recognize, with many limitations.

Conservation studies of invasive species have suggested the use of a large number of loci in conjunction with computational programs that predict the probability of identifying any particular individual as a hybrid (Vähä and Primmer, 2006; Sanz et al., 2009). Some of these programs use likelihood-based assignment tests which require knowledge of the allele frequencies in the parental taxa, such as Whichrun (Banks and Eichert, 2000) and GeneClass

(Cornuet et al., 1999). Others that do not possess such requirements are Bayesian-based methods, such as Structure (Pritchard et al., 2000; Falush et al., 2003), NewHybrids (Anderson and Thompson, 2002), Mr. Bayes (Huelsenbeck and Ronquist, 2001), and Baps (Corander and Marttinen, 2006). Both types of methods are suitable for markers with and without fixed allelic differences in the parental taxa. Some primate studies have begun to take advantage of some of these methods (Charpentier et al., 2008; Tung et al., 2008; Gligor et al., 2009) but these analyses still cannot detect multigenerational backcrossed hybrids with certainty.

Sex-specific, or uniparental, markers can reveal directionality in patterns of gene flow and help to characterize primate hybrid zones. For example, hybrids can be identified when they possess the markers of one species and the external phenotype of another. In 1973, mtDNA data suggested that the anubis-hamadryas hybrid zone was only ~9km in width, whereas phenotypic hybridity suggested 15-30km because males of both species dispersed (Neman, 1997; in Woolley-Barker, 1999). Since then, molecular data have helped to detect hybrids when there is discordance in the species identity among mtDNA, Y-chromosome, nuclear DNA, and/or phenotype in the same individual (Melnick et al., 1993; Evans et al., 2001; Tosi et al., 2002; Wildman et al., 2004; Cortés-Ortiz et al., 2007; Merker et al., 2009; Jolly et al., 2011).

Cytogenetic studies have also contributed to the study of primate hybridization. When hybridizing taxa possess a different number of chromosomes or different chromosomal re-arrangements, hybrids can be identified on the basis of the inheritance of chromosomal elements from both taxa. Researchers examined karyotypes for hybrids born from serendipitous

matings of taxa in captive conditions (Myers and Shafer, 1979; Hirai et al., 2007). Besides their use in hybrid identification, cytogenetic techniques (such as painting, C, G, and R-banding and fish techniques) can also reveal the mechanisms underlying hybrid sterility or infertility. Painting analysis of a Larcon hybrid revealed chromosomal patterns that could likely result in failure of gametogenesis and are therefore unlikely to be fertile (Hirai et al., 2007). Interestingly, in that same study, the authors discuss that lesser apes exhibit many more changes of chromosomal structure than the more deeply divergent papionin genera (baboon and macaque karyotypes are nearly identical, Moore et al., 1999), intergeneric papionins are nevertheless sterile as a result of meiotic arrest that caused a lack of mature spermatozoa.

Primate studies could benefit from considering some limitations of using molecular methods to identify hybrids. There is some disagreement about whether to use hybrid allele frequencies or hybrid genotype classes, as follows. A combination of F1 and backcrossed individuals can yield the same rates of admixture as a group of all F1 individuals, in which case distinguishing between genotype classes is preferable (Allendorf et al., 2001). On the other hand, individuals that are classified as F1 may in fact be backcrosses if additional genotyped loci prove to be homozygous, (Barton, 2001), so genotype classes may not accurately represent hybrids if too few loci are used. Studies should consider both approaches when characterizing hybrid zone patterns to avoid losing the distinction between hybrids of different generational crosses as well as the information that can be gleaned from allelic diversity. Another helpful tool is the potential use of the measurement known as 'D', or gametic disequilibria between pairs of loci to obtain an idea of the age of the hybrid zone (Allendorf et al.,

2001). Nonrandom associations among loci will exist in recently hybridized populations, leading to a high D value, but this value decays over many generations unless loci are closely linked.

Molecular methods have confirmed cases of primate hybridization and provided evidence that hybridization is a more common occurrence in primates. Further technological and methodological advances in genetics will be valuable for identifying hybrids and the genetic basis for phenotypic differences between hybridizing species (Tung et al., 2010). Molecular methods are useful when first generation hybrids do not display readily observable morphological features or when the majority of hybrids are backcrossed. However, studies can benefit from incorporating morphological and molecular data. Ciani et al. (1989) included morphological, molecular, cytogenetic, and behavioral data to provide evidence for gene flow between different species of Sulawesi macaques. Moreover, Ackermann and Bishop (2010) showed that morphological anomalies that have previously been indicative of hybridization correspond with patterns of gene flow between gorilla species. Therefore, a cross-disciplinary approach is often informative and helps to provide strong evidence for testing hybridization hypotheses.

Inferences of ancient hybridization and reticulation using genetic data

Several examples of purported ancient primate hybridization have been based on inferences from molecular data (reviewed in Arnold, 2009). Inferring ancient hybridization from molecular data could be a difficult task. One must first keep in mind that 1) species that are capable of hybridizing may not actually do so, and 2) hybridization events will not result in introgression if hybrids do

not backcross with the parental species due to low fertility or selection against unfit phenotypes. Therefore, examples of hybridization from current hybrid zones or captive crossbreeding do not necessarily provide strong evidence for ancient hybridization.

Conclusions of the possibility of ancient hybridization and introgression are often mentioned in passing as mere speculation. Many methods for inferring ancient hybridization, introgression, and reticulation rely on the presence of discordance among phylogenies created from different genetic data sets, sometimes even suggesting that a primate taxon has evolved as a product of hybridization (marmosets, Tagliaro et al., 1997; macaques, Tosi et al., 2000; Chakraborty et al., 2007; spider monkeys, Arnold and Meyer, 2006). This is because hybridization can result in the movement of some genes between species but not others, and different genes introgress at different rates (Arnold and Meyer, 2006). However, a number of processes could potentially generate incongruent phylogenetic trees, including incomplete lineage sorting, which occurs when alleles do not all sort at a speciation event, leading to the retention of ancestral polymorphisms and discordances between genetic trees and species trees (Gautier and Daubins, 2008; Degnan and Rosenberg, 2009; Meng and Kubatko, 2009). Hybridization and incomplete lineage sorting cause problems for phylogenetic analyses because one involves genetic divergence predating species divergence and the other violates the cladistic assumptions of bifurcating trees (Hennig, 1966). Incomplete lineage sorting has even led to the re-questioning of the gorilla, chimpanzee, and human tree, where some parts of the genome make gorilla and human closer together (Hobolth et al., 2007). Some have proposed tests to distinguish between hybridization and lineage sorting (Sang and Zhong,

2000; Than et al. 2007; Meng and Kubatko, 2009), some of which take into account evidence from currently hybridizing taxa, but they are not without their problems.

Many primate taxonomic studies report discordances in different data sets that could have resulted from ancient hybridization, but for many of them other processes could not be disproven. For example, gene flow has been suggested as a possible source of variation between X-linked and mtDNA sequences in chimpanzee subspecies phylogenies but differences in the effective population size for the two markers can also explain the results (Kaessmann, 1999). Also, an alternative hypothesis to reticulation between lemur taxa is that relationships between chromosomal phylogenetic trees and those based on other data may differ because some chromosomal changes might have occurred repeatedly or that the phylogeny was constructed from only a small number of lemur species (Rumpler et al., 2008), although considering that natural hybrids have been discovered (Wyner et al., 2002), hybridization may have been possible. Another example is where a marmoset phylogeny based on skull morphology suggested that morphological divergence did not reflect genetic divergence (Marroig et al., 2004). In that case, both incomplete lineage sorting and hybridization were plausible explanations.

Different molecular markers may reveal different population genetic structures depending on male and female dispersal patterns. Conflicts between results from different markers may arise because some are uni-parentally inherited while others are bi-parentally inherited (Melnick and Hoelzer, 1993). For example, there was a discordance between microsatellite and mtDNA data in the mouse lemur hybrid zone in Madagascar, where microsatellites showed

mixed ancestry in the hybrid zone but mtDNA "displayed a sharply delimited boundary at the eastern edge of spiny forest" (Gligor et al., 2009), possibly because of only males dispersing from one species into another. Sometimes mtDNA, in comparison with microsatellites, lacks resolution. Morphological evidence has been presented for hybridization in the Awash Valley (Phillips-Conroy and Jolly, 1986), but a mtDNA analysis was unable to detect it (Zinner et al., 2009b). Also, mtDNA has a small effective population size (Kaessmann, 1999). Because effective population size is positively correlated with coalescence times among haplotypes (when there is no selection), lineage sorting for mtDNA takes longer to complete (Evans et al., 2001). Finally mitochondrial lineage sorting could be slower in taxa where males disperse and females do not, also affecting the phylogenies created from mtDNA data (Burrell et al., 2009). Therefore, mtDNA has been problematic for the inference of ancient hybridization in primates. A study on macaque phylogeny found mtDNA patterns suggestive of either ancient polymorphisms or introgression (Hayasaka, 1997). Yet, Y-chromosome and mtDNA analysis presented different trees for the relationship of *Macaca manzala* with other macaque species, while the Y-chromosome results agreed with morphological analysis (Chakraborty et al., 2007). Still, failure to resolve some relationships based on mtDNA leads some to conclude that past introgression may have occurred (Newman et al., 2004; Arnold and Myer, 2006; Zinner et al., 2011). MtDNA is still a preferred type of marker for describing genetic diversity due to its highly polymorphic nature as it accumulates mutations rapidly, because it escapes recombination, and since it is easy to amplify from non-invasive samples (Stoneking, 2003), whereas sequence

data from nuclear genes may not always show differences between species (Cortés-Ortiz et al., 2003).

Geographical distribution of genetic diversity has been used to suggest ancient hybridization in primates (Burrell et al., 2009; Zinner et al., 2009b). As baboons have been shown to hybridize at all their contact zones (Jolly et al., 1997), there has been some debate over baboon taxonomy, the recognition of baboon species, and the role of hybridization in their speciation. In some cases, interpreting molecular data within a geographical context helps to resolve some of these issues. MtDNA from northern and eastern chacma baboons is more closely related to that of yellow and kinda baboons than they are to that of southern chacmas. The discordance between mtDNA and morphotypes for southern African baboons, it was argued, probably did not result from incomplete lineage sorting, a random process, but because of hybridization, mediated by the dispersal of chacma males from the south to the northern yellow baboon species (Keller et al., 2010).

One of the most debated examples of discordance in data sets is the case of *Rungwecebus kipunji*. Owing in part to the controversy over the source of the original specimen, the morphological characteristics of the species, and interpretations of the molecular data, there have been disagreements over the placement of this species and the role of hybridization in its evolution (Jones et al., 2005; Davenport et al., 2006; Olson et al., 2008; Burrell et al., 2009; Singleton et al., 2009; Roberts et al., 2009; Zinner et al., 2009a). Interestingly, sampling from a different locale revealed a complex history including possible hybridization with *Papio* in Northern but not Southern populations. Hybridization has also been proposed in the history of spider monkeys due to the presence of the same

haplotypes in separate geographic areas (Collins and Dubach, 2001; Arnold and Myer, 2006). However, detecting ancient hybridization is difficult precisely because of the challenge of reconstructing ancient events, and likewise, there is no reason to assume that the ancestors of contemporary populations occupied the same geographic areas in the past. Therefore, evidence coming from the ability to map inferred phylogenetic trees onto geography is not necessarily conclusive.

Despite all the challenges with inferring ancient hybridization, introgression, and reticulation, it is likely that these processes took place in primate evolution, especially considering current examples of primate hybridization and the large role that hybridization has been shown to play in the evolution of other organisms (Rieseberg et al., 2003; Seehausen, 2004). The main point of this discussion is that care should be taken before assuming that such processes have occurred when it is difficult to tease out any form of past gene flow from other evolutionary processes. As many have pointed out, acquiring information through different approaches, such as molecular, behavioral, morphological, geographical, and karyological, to strengthen arguments for reticulation is recommended (Boinski and Cropp, 1999; Buckley et al., 2006; Monda et al., 2007), although in some cases, adding more data and utilizing 'democratic vote' methods may lead to incorrect phylogenies (Degnan and Rosenberg, 2006).

As Schwenk (2010) quotes Futuyama and Shapiro (1995): "Arbitrarily chosen molecular and morphological markers have provided abundant insight into hybrid zones, but they have not answered some of the most difficult and important questions: on what genes and characters does selection act, and what

are the agents of selection?...most of the work lies ahead of us." The next step seems to be in the direction of whole genome studies, as has already been done for other organisms (reviewed in Schwenk, 2010). For example, Petit (2009) suggested choosing genome components undergoing high rates of intraspecific gene flow to distinguish between different species. Those studies can help to identify loci that are responsible for speciation and will eventually be useful when more primate genomes are sequenced.

Conclusion

Hybridization in primates has several implications for primate (even human) evolution, conservation management, and speciation more broadly. Tung et al. (2010) believe that hybridization of primates is of interest for investigating "the emergence of genetic and phenotypic differences between divergent groups." Exciting developments in genetics make it possible to better understand the processes of hybridization, gene flow, and introgression. Some approaches that have been utilized are limited in their potential to provide conclusive evidence for the possibility of hybridization, ancient or current. Therefore studies on primate hybridization can benefit from incorporating genetic and morphological data for determining the extent of gene flow between species and the fitness of hybrids in order to better understand the process of hybridization and its role in primate speciation. Taken together, the approaches that have been utilized to study primate hybridization have generated a few conclusions. Certainly, the role of hybridization in primate evolution deserves more attention, considering numerous examples of confirmed instances of primate hybridization. Also, examples of intergeneric as well as interspecific

hybridization between primate taxa with long divergence times show that gene flow is possible regardless of extensive genetic divergence between populations.

It has been argued by some that the "hennegian" way of classifying taxa should be abandoned in favor of a more web-like classification scheme considering the extent of reticulation in evolution (Arnold and Meyer, 2006). New developments in the field of primate genomics will also help to answer questions about hybridization, especially since its effects are genome-wide, and genetic studies overall will provide needed data for cases where hybridization is assumed but not supported by empirical evidence.

CHAPTER THREE: Impact of intra-sexual selection on sexual dimorphism and testes size in the Mexican howler monkeys *Alouatta palliata* and *A. pigra*

Introduction

The theory of sexual selection was proposed to explain the presence of weaponry and/or ornamentation in males in addition to female discrimination of potential reproductive partners (Darwin, 1871). Sexual selection within the sexes, or intra-sexual selection, favors traits that allow males to monopolize mating with receptive females, either by preventing rival males from gaining access to females or by maximizing their chances of fertilization (Kappeler and van Schaik, 2004). Larger body size and canine weaponry can confer a fitness advantage to primate males (e.g. mandrills, in Leigh et al., 2008). One possible consequence of this advantage is the development of sexual dimorphism (or the difference in form between males and females of the same species). Sexual dimorphism in body mass and canine size is common in primate species (Plavcan and van Schaik, 1997). Presumably, the degree of sexual dimorphism would be greater in species in which males fight with each other for direct access to receptive females than in species that exhibit less male-male competition (Clutton-Brock et al., 1977; Alexander et al., 1979). However, the correlation of the level of sexual dimorphism with the intensity of sexual selection in primates is not always clear, partly due to difficulties in finding appropriate measures to estimate the intensity of sexual selection (Plavcan, 2004), which have included

the socionomic sex ratio (Clutton-Brock et al., 1977), mating systems (Harvey et al., 1978; Leutenegger and Cheverud, 1985; Lindonfors, 2002), the operational sex ratio (Mitani et al., 1996), and competition levels (Kay et al., 1988; Plavcan and van Schaik, 1992; Ford, 1994). In addition, many comparative analyses suggest that multiple factors (such as mate choice, allometry, phylogenetic constraints, and natural selection to name a few) can influence the expression of sexual dimorphism in primates (reviewed in Plavcan, 2001).

Intra-sexual selection can also occur after mating via sperm competition, when multiple males copulate with the same female during a reproductive cycle (Birkhead and Kappeler, 2004). Therefore, male fitness depends not only on the ability to mate with females but also on successful fertilization. It has been demonstrated that in many primate species where females mate with more than one male, males have larger testes in relation to body size than in monogamous or polygynous species (Short, 1979; Harcourt et al., 1981; Harcourt, 1997). For example, in chimpanzees (*Pan troglodytes*), which live in multimale-multifemale groups, males have large testes on the order of approximately 120 g of combined weight, whereas the polygynous single-male gorillas (*Gorilla gorilla beringei*) have testes weighing 30 g (Dixson and Brancoft, 1998). Larger relative testes size accommodates greater sperm production and larger ejaculates (Setchell, 1978; as cited in Kenagy and Trombulak, 1986); hence, individuals with larger testes would in turn increase their chances of fertilization.

Mexican howler monkeys, *Alouatta palliata* (mantled howler monkey) and *A. pigra* (Central American black howler monkey) have marked differences in their social systems (Crockett and Eisenberg, 1987; Neville et al., 1988; Treves, 2001) and constitute a good model to explore the differences in sperm

competition, as well as how the intensity of intra-sexual selection affects sexual dimorphism in closely related species. Having diverged around three million years ago (Cortés-Ortiz et al., 2003), *A. palliata* and *A. pigra* are sister species can be clearly distinguished on the basis of genetics (Cortés-Ortiz et al., 2003), cytogenetics (Steinberg et al., 2008), and morphology (Lawrence, 1933; Smith, 1970). Differences in social systems include that *A. pigra* groups usually range from 2–12 individuals, with groups averaging 4–8 individuals (Crockett and Eisenberg, 1987; Treves, 2001; Chapman and Pavelka, 2005; Van Belle and Estrada, 2006; Rosales-Meda et al., 2008). On the other hand, *A. palliata* typically have groups that are much larger than those of *A. pigra*, ranging from 2–45 individuals and averaging 8–23 individuals per group (Crockett and Eisenberg, 1987; Neville et al., 1988; Chapman and Balcomb, 1998; Treves, 2001; Pavelka and Chapman, 2006; Di Fiore and Campbell, 2007). The relative number of females per troop also differs between species: whereas *A. palliata* troops have a sex ratio between 1.37 and 4.11 females per male, the smaller *A. pigra* troops have a sex ratio between 1.2 and 2.1 females per male (Crockett and Eisenberg, 1987; Neville et al., 1988; Treves, 2001; Van Belle and Estrada, 2006). Females of both species are only receptive during 2–6 days of their approximately 16–day cycle (Glander, 1980; Van Belle et al., 2009), during which males must compete to gain reproductive access. In *A. pigra*, females copulate most often with the dominant male (Van Belle et al., 2009), whereas in *A. palliata* copulations with multiple males during a female's estrus cycle are common (Jones and Cortés-Ortiz, 1998; Wang and Milton, 2003).

In both species males and females migrate from their natal groups and join other groups (Van Belle and Estrada, 2006; Clarke and Glander, 2008). In *A.*

palliata male takeovers usually do not involve the ousting of resident males (Glander, 1980), but instead are a way to attain group membership by the invader male (Dias et al., 2010). Although non-alpha *A. palliata* males may face decreased possibilities of monopolizing a receptive female, they can still achieve reproduction through alternative strategies (Jones, 1985; Cortés-Ortiz, 1998; Jones and Cortés-Ortiz, 1998). Small, low-ranking males in these groups would still have an opportunity to reproduce by being able to sneak in copulations and pass on their characteristics to their offspring (i.e., not only large males will sire offspring). Furthermore, a larger number of females in the group implies a higher probability that two or more will be in estrus simultaneously, facilitating the access of multiple males to receptive females (Dunbar, 1988). In contrast, *A. pigra* males are often expelled from the group during a takeover (Brockett et al., 2000). As groups usually have one or two males, the invader male may actually be able to force out all resident males. However, it has been suggested that males in *A. pigra* groups are kin-related and cooperate in the defense of the group (Kitchen, 2004). Therefore, it would be harder for an invader male to defeat a coalition of two or more related resident males. Only large males (and presumably those with large canines that can be useful during battle) would be able to successfully defeat a coalition of resident males, and so it would be expected that large body size and canines would be selected for by being preferentially passed on to the next generation.

In this study I analyze sexual dimorphism and testes size for the two species of Mexican howler monkeys, and explore the connection of these variables with male-male and sperm competition. While sexual dimorphism has been investigated via broad comparative analyses, a closer look at these two

related species with different social systems can help to parse out some of the determinants of sexual dimorphism, at least in platyrrhines. Given the complexity of the social dynamics of these species (presented above) it is difficult to establish straightforward predictions in terms of the expression of sexual dimorphism for each species. The socionomic sex ratio alone suggests that *A. palliata* has more intense male-male competition and reproductive skew than *A. pigra*. This would imply that *A. palliata* should be more sexually dimorphic than *A. pigra*. Yet, since *A. palliata* groups are large and males may have difficulty monopolizing females, reproductive skew may be lower in this species than in *A. pigra*. Furthermore, although many *A. pigra* groups are uni-male, the sex ratio is generally low, and the suggestion that group males are related could mean lower intra-group male-male competition. However, kinship of males in the group and the formation of coalitions may intensify inter-group male-male competition for group takeover. These issues, in addition to the role played by female choice and competition, complicate inferences that can be made about sexual dimorphism in body and canine size.

Alouatta pigra has been reported to be more sexually dimorphic in body size than *A. palliata* (Jungers, 1985; Ford and Davis, 1992; Ford, 1994). However, previous analyses are based on a very small sample size (only two males) for *A. pigra*, so it remains unclear whether a larger sample size supports this difference. Canine data is available for both *A. palliata* and *A. pigra* (Swindler, 2002; Plavcan and Ruff, 2008) although not specifically for *A. palliata mexicana*. Testes size (only in terms of mass, not volume) has only been reported for *A. palliata* (Harcourt et al., 1981). With greater sampling of body mass data and newly acquired testicular volume and dental data from wild-caught individuals of both species,

in this study I examine how body and canine size dimorphism and testicular volume vary between the two species, and discuss how the observed patterns may have been shaped by differences in social systems between *A. pigra* and *A. palliata*.

Methods

Data collection

Between 1998 and 2008 a team collected morphometric measurements, dental molds, and blood samples of howler monkeys from southeastern Mexico (*A. palliata* and *A. pigra*) and Northern Guatemala (*A. pigra*). The capture team followed capturing procedures described in Rodríguez-Luna and Cortés-Ortiz (1994). Sample sizes for the collected data are shown in Table 2.1.

Although *A. pigra* and *A. palliata* are known to naturally hybridize in Mexico (Cortés-Ortiz et al., 2007), individuals in this study are all considered to be purebred. Both pure *A. palliata* and *A. pigra* individuals were collected outside the known hybrid zone in Tabasco (Cortés-Ortiz et al., 2007). I also included individuals from within the hybrid zone after confirming parental species status using 11 microsatellite markers, five of which are diagnostic of hybridization (Cortés-Ortiz et al., 2009). Procedures for capturing and handling primates were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

I used only adults in this study. As the team did not track these individuals from birth, I could not ascertain the exact age and had to rely on other proxies to determine adult status. For both species, I followed dental development and wear patterns of captured individuals according to the criteria

developed in Pope (1966), and I assigned adult status for individuals with fully-erupted dentition and the third molar in functional occlusion, and at least slight wear found on some of the premolars and first molar. Howler monkeys are known to have reached sexual maturity at that stage (DeGusta and Milton, 1998), and although most craniometric studies only use the criterion that all teeth are erupted to determine adult status (Ravosa and Ross, 1994; Jones et al., 2000), I believe that my criteria are more stringent and will include all sexually mature individuals in these species.

Morphometrics. Once animals were captured, mass measurements were collected using a 20 kg Pesola® spring scale to the nearest 100 g. Body mass is commonly used as a marker of overall body size in living primates (e.g., Ford and Davis, 1992). Here I used body mass and a linear body length measurement to estimate sexual size dimorphism in the two species. Mass data for *A. palliata* from different sites throughout their geographic distribution have been reported extensively (17 studies and N >459 individuals: Ford and Davis, 1992; Glander, 2006), but data for *A. pigra* are scarce. Most studies that use body mass data for *A. pigra* relied on the data presented by Murie (1935) and Jungers (1985), with a sample size of two males and three females. My larger *A. pigra* sample provides a more accurate representation of average *A. pigra* body mass (32 females and 37 males).

The body length measurement analyzed in this study is the sitting height (i.e., the length of the head and body excluding the tail, similar to the measurement used by Schultz, 1929). This measurement was taken dorsally from the junction of the last lumbar and first caudal vertebrae to the occipital protuberance of the head using a metallic measuring tape to the nearest 0.1 cm.

Body length measurements are sometimes favored over body mass measurements because they are less subject to variation caused by nutritional and health status (Alexander et al., 1979). I use both measurements in this study to account for possible biases due to such factors.

Dental casting. While the animal was anesthetized, negative dental impressions were made using vinyl polysiloxane material (Exaflex Putty, GC America, Inc., Alsip, IL, USA). Casts were poured using polyester laminating resin thickened with talc and catalyzed with methyl-ethyl-ketone (Eastpointe Fiberglass Sales, Inc., Eastpointe, MI, USA). A paired *t*-test was used to compare upper canine height measurements performed in the field with measurements taken from the casts of the same individuals ($N = 50$). Results of these tests revealed no significant differences ($t = -0.491$, $P = 0.626$), indicating that the casts were representative of live specimens. All measurements were made with Mitutoyo® Digital Calipers to the nearest 0.01 cm.

Canine measurement. I measured upper canine height, mesiodistal length, and labiolingual (aka buccolingual) length from dental casts. My own and L. Cortés-Ortiz's field observations suggest that upper canine height is highly susceptible to wear, and some wear was observed in the mesiodistal dimension as well. However, considering that wear is a continuous process and begins to occur prior to complete eruption of the tooth, I decided to include these data and consider this source of error in my analysis, excluding any teeth that were heavily worn. My measurement of upper canine height is taken from the apex to the buccal-gingival margin, which is slightly above the cementum-enamel junction due to the presence of the gum in live-captured individuals. The mesiodistal and labiolingual dimensions are measured as described in Plavcan

and van Schaik (1992) but are not exactly analogous to those measurements due to the presence of gum tissue in wild-captured individuals. I also included museum dental specimens housed at the University of Michigan Museum of Zoology mammal collection ($N = 9$). Only Mexican *A. palliata* museum samples were included, and all *A. pigra* museum samples (which include those individuals analyzed by Murie, 1935) came from Petén, Guatemala. In the casts of live animals, measurements for upper canine base dimensions were made at the gum line. Museum specimens retained stains on the canines that indicate the location of the gum line when the animals were alive, making it possible to perform analogous measurements in live and museum specimens. I measured left maxillary canines, and in cases where the tooth was broken I used the right maxillary canine ($N = 2$).

Testicular volume. In order to determine testicular volume, I measured testicular breadth and length to the nearest millimeter using Mitutoyo® Digital Calipers, excluding scrotal skin folds. I used the following formula for calculating the volume of a prolate sphere: $\pi LW^2/6$; where L is length and W is width (Harrison et al., 1977). I utilized total testicular volume (sum of left and right testes) to account for any variability that exists between the left and right testes and to have data that are comparable to results presented in the literature. Comparison of testes size across species often involves relating absolute testicular volume with body mass (Short, 1979; Harcourt et al., 1981); here I only present absolute testicular volume, but using relative volume did not affect my results.

Statistical analyses

I used the Shapiro-Wilk test of normality and found that of 22 sample groups, all were normally-distributed except for 4: *A. palliata* male sitting height and *A. pigra* female body mass, sitting height, and canine labiolingual length. For that reason and since some sample sizes are small, I used the Mann-Whitney non-parametric test to determine whether there were significant differences between the sexes (except for testicular volume) and between the species.

To quantify sexual dimorphism, I used the intuitive ratio of average male to female values, which is widely used since the larger sex is preferred in the numerator (Smith, 1999). Because sample sizes and variances are unequal, and because some of the variables are not normally distributed, I utilized a resampling method to avoid making assumptions about how the data were distributed. I pooled males of the two species in one group and females in the other. I randomly selected and averaged a group of males based on the male sample size of one species and divided that value by the average of a randomly selected group of females based on the female sample size of the same species to obtain a value of sexual dimorphism. I repeated this procedure to obtain a random value of sexual dimorphism for the second species, and then subtracted the dimorphism values of the two species from one another. This process was repeated 10,000 times to generate a distribution of randomly sampled sexual dimorphism differences. Then, I tested the null hypothesis that my test statistic, which is the difference between the actual sexual dimorphism values of *A. palliata* and *A. pigra*, fell within the 95% confidence interval (alpha value of 0.025 for a two-tailed test). Statistical analyses were done using SPSS 16.0 and the Resampling Statistics Excel macro.

Results

Table 2.1 shows the descriptive statistics for body mass, sitting height, canine dimensions, and testicular volume for both species. Table 2.1 also shows results for significance testing of all variables for differences between the species for each sex. Males are significantly larger than females for all variables ($P<0.001$). Both male and female *A. pigra* individuals are heavier in body mass and larger in sitting height than their *A. palliata* counterparts. Interestingly, male upper canine dimensions are not significantly different between the two species but female canine mesiodistal length is, and other female dimensions approach significance.

Although *A. pigra* males are the larger of the two, *A. palliata* males have testes that are twice as large as their *A. pigra* counterparts. The difference in absolute testicular volume is great enough that correcting for the effects of body size has no bearing on my results and only serves to increase the difference in the relative testicular volume between the two species.

Table 2.2 shows the sexual dimorphism values for body mass, sitting height, and upper canine dimensions, and the significance values from the resampling test. Upper canines exhibit greater dimorphism than body mass (while canine dimensions are linear, body mass is volumetric, so taking the cube root gives values of 1.10 and 1.09 for *A. pigra* and *A. palliata* respectively). Nevertheless, neither body mass, sitting height, nor canine dimensions showed any significant differences in sexual dimorphism between the species.

Table 3.1 Sample size (N), mean, standard deviation (SD) and range data for *A. pigra* and *A. palliata* morphological variables for both sexes.

	A. palliata				*A. pigra*				*P value*
	N	Mean	SD	Range	N	Mean	SD	Range	
Body mass (kg)									
Female	37	4.39	0.48	3.60 – 5.25	32	5.68	0.63	4.50 – 6.8	*<0.001*
Male	25	5.8	0.69	4.60 – 7.20	37	7.6	1.13	5.50 – 9.60	*<0.001*
Sitting height (cm)									
Female	36	38.8	2.4	33.0 – 43.7	32	43.5	2.9	34.5 – 49.0	*<0.001*
Male	26	41	2.3	37.0 – 45.4	37	48.5	3.2	42.6 – 58.0	*<0.001*
Testicular volume (cm^3)	24	22.66	10.89	11.39 – 61.22	36	11.33	3.79	5.06 – 18.95	*<0.001*
Canine height (mm)									
Female	23	8.14	1.06	6.65 – 10.06	18	8.88	1.39	7.12 – 11.63	0.083
Male	19	14.01	1.75	11.29 – 17.75	20	14.23	2	9.00 – 17.00	0.509
Canine mesiodistal length (mm)									
Female	15	6.15	0.49	5.12 – 7.18	9	6.72	0.43	6.06 – 7.35	*0.006*
Male	10	8.08	0.63	7.00 – 8.93	10	8.29	0.76	7.48 – 9.53	0.597
Canine labiolingual length (mm)									
Female	13	4.76	0.36	4.25 – 5.62	8	5.11	0.39	4.81 – 5.92	0.06
Male	10	6.81	0.97	5.44 – 8.65	9	6.57	0.75	5.64 – 7.62	0.744

Table 3.2 Dimorphism values (mean male/mean female) for *A. pigra* and *A. palliata*, and results of testing for significance of differences in dimorphism using resampling.

	A. pigra	*A. palliata*	*P* value[a]
Body mass dimorphism	1.34	1.31	0.431
Sitting height dimorphism	1.12	1.06	0.053
Canine height	1.60	1.72	0.127
Canine mesiodistal length	1.23	1.31	0.113
Canine labiolingual length	1.29	1.43	0.059

[a]*P* value represents the significance value generated by using resampling statistics.

Discussion

My results show that overall *A. pigra* males and females are bigger than their *A. palliata* counterparts, but have similar upper canine size, and that both species exhibit sexual dimorphism in body mass, sitting height, and upper canine size. My data for *A. palliata mexicana* fall within the ranges in mass reported by other authors for *A. palliata palliata* inhabiting Costa Rica (Ford and Davis, 1992; Glander, 2006), but not for *A. palliata aequatorialis* in Barro Colorado Island, Panama (Scott et al., 1977; Glander, 2006). On the other hand, *A. pigra* average male body mass has been overestimated (11.352 kg: Ford and Davis, 1992), probably because most studies for *A. pigra* relied on the data presented by Murie (1935) and Jungers (1985) using males on the largest end of their size range. Due to the overestimation in male size in previous studies, *A. pigra* has been found to

be highly sexually dimorphic (1.764: Ford and Davis, 1992). However, in my study the degree of sexual dimorphism for all three variables does not differ between *A. palliata* and *A. pigra*. On the other hand, the evidence that *A. palliata* testes are much larger than those of *A. pigra* supports the argument that there is more intense post-copulatory competition in *A. palliata*.

Sexual dimorphism

In most anthropoid primates, males are larger than females (Plavcan, 2001). Although platyrrhines on the whole have been characterized by lesser degrees of body mass dimorphism, some authors claim that *A. pigra* is the exception, with body mass dimorphism comparable to cercopithecoid species (Ford, 1994). My new data do not support that view, and instead place *A. pigra* within similar body mass and length dimorphism ranges as other New World primates with high levels of male-male competition (e.g., *Saimiri* and *Cebus* species), and more specifically, similar to some other howler monkey species (Alexander et al., 1979; Kay et al., 1988; Ford, 1994; Plavcan and Ruff, 2008).

Like in body mass and length, I found that both species exhibit sexual dimorphism in upper canine size. When used as a weapon, a canine is most effective with respect to its height (Greenfield and Washburn, 1991; Plavcan, 1993). While some argue that, in addition to canine height, the basal dimensions are also good indicators of competition (Lucas et al., 1986), others have found them to be weakly correlated with behavioral measures (Plavcan, 2000). While I present upper canine data for *A. palliata mexicana*, and although my measurements on teeth of live-captured animals are not necessarily comparable

to measurements normally conducted on museum specimens (see methods), all dimensions seem to be similar to other *A. palliata* reported values (Swindler, 2002; Plavcan and Ruff, 2008). My sexual dimorphism values are slightly higher for *A. palliata* labiolingual length primarily because I observed larger male measurements. My *A. pigra* values are higher than those reported by Swindler (2002; summarized in Plavcan and Ruff, 2008) of 1.1 for canine mesiodistal length, 1.12 for canine height, and 1.11 for canine labiolingual length, but are in agreement with values reported in Plavcan and van Schaik (listed as *A. villosa*, 1992). All these values fall within the range of canine sexual dimorphism values for many New World Monkeys such as *Ateles*, *Lagothrix*, and other *Alouatta* species, but are not as high as those of many Old World Monkeys like *Macaca* or *Papio* (Plavcan, 2001; Thorén et al., 2006).

Sexual size dimorphism in anthropoids is generally associated with male reproductive skew depending primarily on pre-copulatory competition, in which selection leads to increased male weaponry (e.g., large canines: Plavcan and Kay, 1988; Kay et al., 1988; Plavcan, 2001) and competitive ability (e.g., large body size: Ford, 1994; Mitani et al., 1996; Plavcan and van Schaik, 1997; Plavcan, 2001). The fact that both *A. palliata* and *A. pigra* are dimorphic in both upper canine teeth and body size fits well with the concept that sexual selection has favored these traits because of the advantages they confer in winning fights (Plavcan, 2001). Indeed, there is evidence in both species for aggressive encounters among males that lead to fights, injuries and death (DeGusta and Milton, 1998; Cristóbal-Azkarate et al., 2004; Van Belle et al., 2008; Dias et al., 2010). Body mass dimorphism for these two species appears to be similar to those reported for other howler monkeys (see Ford and Davis, 1992; Plavcan and Ruff, 2008).

However, upper canine dimorphism data is more variable across the genus (Plavcan and Ruff, 2008), though given methodological differences with other studies and without significance testing, the apparent differences in dimorphism values may not reflect real differences among all the species.

For all of the measures of sexual dimorphism considered in this study, I found no statistically significant differences between the two species, despite the differences between the species in the availability of receptive females over space and time and the differences in male and female mating strategies. Females of *A. palliata* will not only mate with the dominant, and presumably largest, male but may also mate with smaller males, when the dominant male is unable to monopolize access to all receptive females (Cortés-Ortiz, 1998). However, *A. palliata* sexual dimorphism is not reduced in comparison with *A. pigra* despite that whenever females copulate with more than one male, sexual dimorphism is typically reduced (Harvey and Harcourt, 1984; Dunbar and Cowlishaw, 1992; Plavcan, 2001). Perhaps greater sexual dimorphism that is otherwise expected in *A. pigra* (since one male is more likely to monopolize reproduction) is tempered by his relatedness to the other group males. While the lack of differences in sexual dimorphism between *A. palliata* and *A. pigra* may result from a similarity in the intensity of male-male competition in the two species, other determinants could also affect male and female body and canine size independently. Phylogenetic factors, especially considering the similarities in body mass sexual dimorphism of the species considered in this study with other howler monkeys (possibly with the exception of *A. caraya*), could restrict changes in sexual dimorphism (Cheverud et al., 1985; Plavcan, 2001). Female-female competition and female choice are also likely to contribute to sexual dimorphism in these

howler monkeys.

Female-female competition may increase female body and canine size, leading to smaller differences between males and females (Plavcan and van Schaik, 1992). Howler monkey females may compete against one another not only for resources, but also to avoid infanticide risk (Ostro et al., 2001). Large groups with many females are good candidates for male takeover (Crockett, 2003), so it would be in a female's interest to keep group size down by evicting other females (Pope, 2000). This would limit the selection on males for larger body and canine size, and would also result in selection on females for those traits (Plavcan, 2001), as the ability of natal females to compete against immigrating females and expelling non-related females from their group may also depend on the development of weaponry and larger body size. In *A. pigra*, where extra-group male takeovers are common and sometimes result in infanticide (Brockett et al., 1999; Horwich et al., 2001), females may choose to limit group size by engaging in aggressive encounters, much like in red howler monkeys (Crockett, 1984). Male takeover, infanticide, and female emigration also occur in *A. palliata* (Clarke and Glander, 1984; Crockett and Eisenberg, 1987; Glander, 1992). However, when many males exist in a large group, one male is unable to monopolize all females, and females may develop less costly strategies to confound paternity and lower risk for infanticide (Crockett and Janson, 2000, see female choice below). The *A. pigra* female canine mesiodistal length is significantly larger than that of *A. palliata* females. Relative canine size of males and females, and not only sexual dimorphism, can be informative on the levels of intrasexual competition (Plavcan, 2004). Therefore, whereas the similarity in sexual size dimorphism between the two species could mean that both have the

same intensity of precopulatory male-male competition, the facts that both male and female *A. pigra* individuals are larger in body size than their *A. palliata* counterparts, and females have larger canines, suggest the alternative possibility that for both sexes, competition is greater in *A. pigra* than it is in *A. palliata*.

Female choice may also play a role in shaping sexual dimorphism (Plavcan, 2004). On the one hand, females may choose to confound paternity by mating with multiple males as a strategy to counteract infanticide (Plavcan, 2001). *A. palliata* females are known to copulate with several males in their group (Cortés-Ortiz, 1998; Jones and Cortés-Ortiz, 1998; Wang and Milton, 2003) and *A. pigra* females sometimes cross the boundaries of their own group and mate with extragroup males (Horwich, 1983; 2000; Van Belle et al., 2009). On the other hand, females may choose to associate with specific males that they select to sire their offspring and protect them (Plavcan, 2001). Van Belle et al. (2009) present evidence that *A. pigra* females direct many of their sexual solicitations specifically towards dominant males. Therefore, female choice is likely to be an important factor in the evolution of sexual dimorphism in howler monkeys.

Additional studies of howler monkey social behavior and genetic data on paternity are needed to further elucidate the correlates of sexual dimorphism in these species. Nevertheless, our knowledge of these howler monkeys' social systems suggests that both male and female reproductive strategies can influence the degree of sexual dimorphism in *A. palliata* and *A. pigra*, and that sexual dimorphism is not necessarily a unique function of male-male competition. Furthermore, these results highlight the complexity of primate social dynamics and the difficulty of drawing simple predictions about the levels of sexual dimorphism based on behavior, warning researchers that make inferences about

behavior from sexual dimorphism data of fossil taxa. Plavcan (2000) points out that modest or low degrees of dimorphism is not unique to any particular social system despite its common use for inferring behavior. Further, while canine dimorphism has recently been shown to be more useful for studying behavior (Leigh et al., 2008), body size dimorphism may not be as reliable. For example, many types of mating systems have been attributed to early hominids based on body size dimorphism that are often in contradiction with inferences based on canine dimorphism, possibly because of other factors affecting body size evolution, such as the development of weapons, reduction in female size, and predator defense, as well as problems with determining body size dimorphism from fossil data (Plavcan and van Schaik, 1997).

Testicular volume

Consistent with the prediction that testes size is larger in species with multi-male groups, *A. palliata* males have larger testes than *A. pigra* males. In fact, the volume of *A. palliata* testes was twice as large as those of *A. pigra*. As these howler monkey species are non-seasonal breeders (Neville et al., 1988), I can assume that there is no seasonal variation in testicular volume (Muehlenbein et al., 2002) and that differences in testes size reflect differences in the intensity of sperm competition (Birkhead and Kappeler, 2004). Thus, sperm competition appears to be more intense in *A. palliata*.

Compared with other anthropoids, the *A. palliata* gonadosomatic index (testicular volume relative to body size) fits within the ranges documented for large group multimale-multifemale breeding systems, such as savanna baboons

(Bercovitch, 1989), though it is not as large as many macaque species, which are known to have the highest levels of sperm competition (Harcourt et al., 1981). The gonadosomatic index of *A. pigra*, on the other hand, is slightly higher than those single male/polygynous species such as gorillas, orangutans, colobus monkeys or hamadrayas baboons (Harcourt et al, 1981). Another howler monkey species, *A. caraya*, exhibits a combined testicular volume of approximately 16 cm^3 (Moreland et al., 2001) and lives in groups that typically have 5-15 individuals (i.e., slightly larger than in *A. pigra*), which can be both unimale and multimale (Juárez et al., 2005). Compared with my measurements of 11 cm^3 and 22 cm^3 for *A. pigra* and *A. palliata*, respectively, these differences suggest more sperm competition in *A. caraya* than in *A. pigra*, but perhaps not as much as in *A. palliata*.

Early studies characterized *A. palliata* as predominantly polygynous with one dominant male monopolizing breeding opportunities with all the females in the troop (Clarke, 1983), and subordinate males copulating with females outside of the peak of the estrus cycle (Jones, 1985). However, the difference in testicular volume suggests that subordinate *A. palliata* males are sometimes successful at fertilizing receptive females. As noted earlier, *A. palliata* groups are large, and there can be up to six males and nine females in a group (Treves, 2001). In larger groups, it is statistically reasonable to assume that many females will be in reproductive synchrony (Dunbar, 1988), and a male must guard all of them against solicitations from other males. Thus, it may not be possible for one male to control access to all females in estrus. Observations from Mexico (Cortes-Ortiz, 1998; Jones and Cortés-Ortiz, 1998), Costa Rica (Jones, 1978) and Panama (Wang and Milton, 2003) indicate that *A. palliata* females may repeatedly copulate with different males during the same estrus cycle. As opposed to engaging in

aggressive combat with the dominant male, subordinate males may instead benefit by sneaking in copulations (Harcourt, 1996). Sneaking males may copulate with a receptive female while the guarding male is momentarily away from the female, eating or chasing away other males (Cortés-Ortiz, 1998). Therefore, the high levels of sperm competition and the strong selection for larger testes observed in my data for *A. palliata* are consistent with the expectations based on what it is known about the socio-sexual behavior of this species.

As I mentioned earlier for *A. pigra*, females copulating with males of neighboring groups have been recorded (Horwich, 1983; 2000) but selection for larger testes would be weak if the dominant male succeeds at fertilizing most receptive females, as suggested by recent studies (Van Belle et al., 2009). In the red howler monkey, *A. seniculus*, where subordinate males may copulate with receptive females (Sekulic, 1983), paternity analysis showed that in nine different troops, only the dominant male sired all the offspring (Pope, 1990). In order to determine the extent of reproductive success and skew among *A. pigra* and *A. palliata* males, it is imperative to conduct long-term behavioral and genetic studies.

Conclusion

The results presented in this study provide strong evidence that corrects the misconception that *A. pigra* is more dimorphic than any other New World primate and sets up new hypotheses to be tested to understand the social systems of howler monkeys.

CHAPTER FOUR: Morphology of genetically-confirmed hybrids of *Alouatta pigra* and *A. palliata* from a natural hybrid zone in Tabasco, Mexico

Introduction

Hybridization, or the production of offspring through the interbreeding between individuals of genetically distinct populations (Harrison, 1990), has been considered to play various roles throughout primate evolution (Arnold and Meyer, 2006) Although there are a number of recent reports of hybridization in the primate literature (Cortés-Ortiz et al., 2007; Aguiar et al., 2008; See Detwiler et al., 2005 for a review of cercopithecines), there is a lack of understanding of the morphological variation associated to the hybridization process in primates. In particular, recent reviews (Arnold and Meyer, 2006; Ackermann, 2010) show the need to conduct long-term studies combining analyses of morphological and genetic traits in hybrid individuals to understand the extent of the variation in phenotypic expression in hybrids, determine the longevity of hybrid traits, and understand the universality of hybrid morphologies.

Much of what is known about variation in primate hybrid morphology comes from studies that utilized individuals of known pedigrees in captivity (Smith and Scott, 1989; Cheverud et al., 1993; Jaquish, 1994; Kohn et al., 2001; Ackermann et al., 2006). However, there is much to be gained from studies of natural hybrid zones (Mayr, 1942; Woodruff, 1973; Barton and Hewitt, 1985; Hewitt, 1988; Arnold and Hodges, 1995) as they can provide natural laboratories

for testing the rate and direction of gene flow, the development of isolating mechanisms, and the relative fitness of hybrid individuals. Further, studies of current natural hybrid zones can generate expectations for understanding hybridization in the fossil record.

In this paper I present data on morphological variation of *Alouatta pigra* and *A. palliata* and their hybrids. Despite earlier lack of consensus on the systematic relationships among *Alouatta* species, Cortés-Ortiz et al. (2003) conducted a molecular phylogenetic analysis based on mitochondrial cytochrome b and ATP-synthase 6 & 8 genes, which established a divergence time of 3 mya between these two taxa. The two species are allopatric in most of their geographic distribution, separated by the highland massif of northern Central America and central highlands of Guatemala, except for one confirmed area of contact in Mexico and one potential area of contact in Guatemala (Baumgarten & Williamson, 2007). Here, I study individuals that live in sympatry in the state of Tabasco, Mexico. This area is characterized by extensive habitat fragmentation and is thought to be a secondary contact zone for the two species (Cortés-Ortiz et al., 2007). The taxonomic distinctness of *A. palliata* and *A. pigra* is supported with different types of evidence, including morphological (Lawrence, 1933, Smith, 1970, Kelaita et al. in 2011), social (reviewed in Kelaita et al., 2011), cytogenetic (Steinberg et al., 2008), and molecular (Cortés-Ortiz et al., 2003). Hybridization between these two species has been confirmed via the use of molecular markers (Cortés-Ortiz et al., 2007).

With the genetic data available, I assess the ancestry of hybrid individuals inhabiting this hybrid zone and how their morphology varies from the parental species. This study evaluates the relative importance of morphological and

molecular data in characterizing hybrid individuals produced from the crosses of two species with a divergence time that is usually long enough for many mammals to establish reproductive isolation (Fitzpatrick, 2004). *A. palliata* and *A. pigra* show differences in discrete morphological characters, such as pelage coloration, cranial, and facial features (Lawrence, 1933; Smith, 1970). However, it is not clear whether such characteristics may be reliable for detecting hybrids between these two species. This may be due to that fact that intermediate features may only be observable in the first generation (F1) of hybrids and that no F1 individuals have been found in this hybrid zone to date (Cortés-Ortiz et al., 2007). Here I use metric (continuous trait) morphological and genetic data that were collected for 224 adult individuals of *A. palliata*, *A. pigra* and their hybrids, both within and outside the putative hybrid zone in Mexico. Differences between the two parental species based on such data were only recently described (Kelaita et al., 2011). The use of molecular markers provides the opportunity to approximate the relative genetic contributions of the parental species to each hybrid, and allows for a morphological analysis of distinct genotypic classes of hybrids in comparison with the parental species.

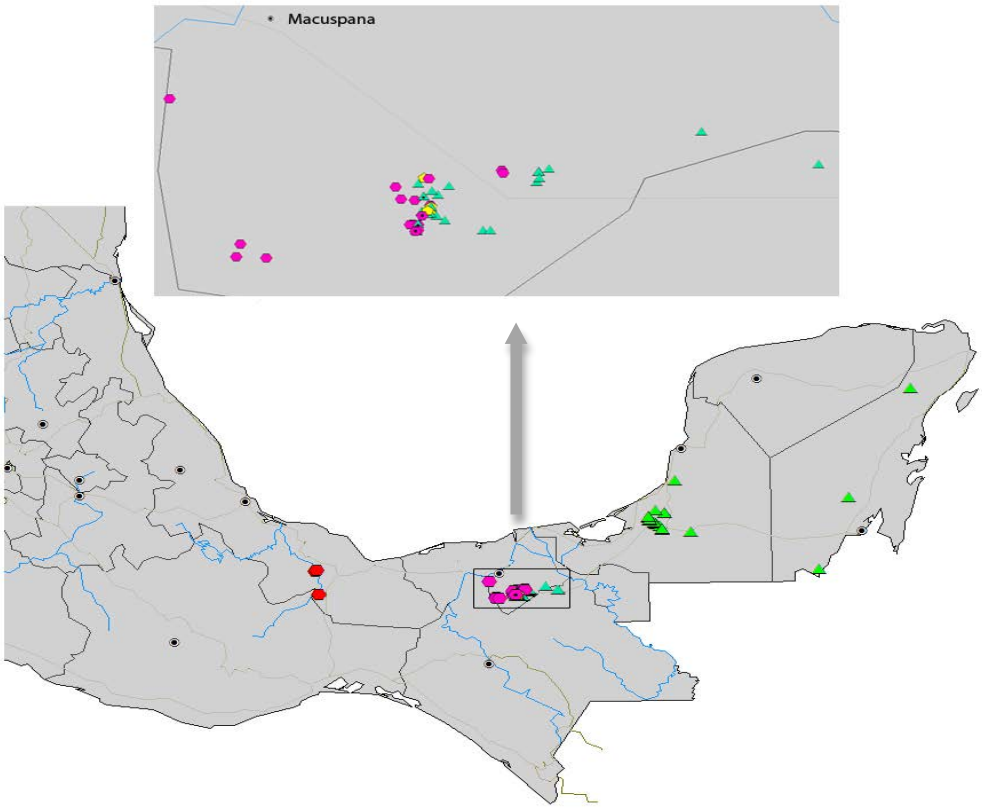
Methods

Data collection and genotyping

For a representative sample of pure *A. palliata* individuals, samples were collected outside the known contact zone from Tabasco (N=25) and Veracruz (N=16). For pure *A. pigra* individuals, samples were collected from Campeche (N=39), Quintana Roo (N=7), Tabasco (N=3), Chiapas (N=1) in Mexico, and from Peten (N=4) in Northern Guatemala.

Candidates for the hybrid analysis (N=129) come from the contact zone in Tabasco, Mexico that was confirmed in 2007 (Cortés-Ortiz et al.). Thus far, the area of contact appears to be 20 km in width, and contains troops of pure and mixed ancestry (see figure 4.1). Both parental species seemed to have a nearly

Figure 4.1 Map of hybrid and pure group distributions. The inset represents the hybrid zone, with *A. pigra* and *A. pigra* backcrossed hybrid mixed groups in blue triangles and *A. palliata* and *A. palliata* backcrossed hybrid mixed groups in pink circles. A. palliata purebred groups are in red circles and *A. pigra* purebred groups are in green triangles outside the hybrid zone.



equal number of pure individuals within the hybrid zone (Cortés-Ortiz et al., 2007).

Individuals were captured as described in Rodríguez-Luna and Cortés-Ortiz (1994). While anesthetized, the field team collected blood, hair, and morphometric measurements. Several morphometric variables were measured to determine individual morphology, including overall body size, limb

measurements, and reproductive morphology. Differences between *A. palliata* and *A. pigra* were previously established for body mass, sitting height, and testicular volume (Kelaita et al., 2011). I measured 14 additional variables to describe overall morphology in the species and their hybrids. Table 4.1 shows each measure used in this study and explains how these measures were collected in the field.

Table 4.1: List of morphometric measurements and their definitions

Variable Name	**Measurement Definition (using flexible metalic measuring tape unless otherwise noted)**
Body mass	Measured using a 20 kg Pesola® scale to the nearest 100 g.
Sitting height	Includes the head and trunk but not the tail. Measured dorsally from the junction of the last lumbar and first caudal vertebrae to the occipital protuberance of the head using a metallic measuring tape to the nearest 0.1 cm.
Tail length	Measured dorsally from the first caudal vertebrae to the tip of the tail w/o including the hair to the nearest 0.1 cm.
Leg length	Measured on the outside of the leg w/o the foot from the greater trochanter of the femur to the heel with the leg fully extended to the nearest 0.1 cm.
Foot length	Measured from the pternion to the tip of the longest toe (usually the middle one) to the nearest 0.1 cm.
Arm length	Measured dorsally from the articulation of the humerus with the clavicle to the distal point of the longest finger (usually the middle) excluding the nail to the nearest 0.1 cm.
Hand length	Measured dorsally from the carpale to the distal point of the longest finger (usually the middle) excluding the nail to the nearest 0.1 cm.
Thorax	While the animal is held by the armpits and sitting upright on a table measured at the widest part of the rib cage to the nearest 0.1 cm.
Abdomen	While the animal is held by the armpits and sitting upright on a table measured at the widest part of the abdomen (usually at the navel) to the nearest 0.1 cm.
Cranial circumference	While the animal's head is held upright at the chin measured from the brow ridge around the head to the occipital protuberance and back to the nearest 0.1 cm.
Vertical cranial length	Linear distance measured from the top of one ear, over the top half of the skull to the top of the other ear using a vernier caliper to the nearest 0.1 cm.
Horizontal cranial length	Linear distance measured from the glabella over the top half of the skull to the occipital protuberance using a vernier caliper to the nearest 0.1 cm.
Mandible	Measured from the center of the chin at the height of the space between the two front incisors on the left until the end of the jaw where it makes a square to the nearest 0.1 cm.
Interorbital distance	Distance between the orbits measured at their medial margins to the nearest 0.1 cm.
Internasal distance	Measured from medial end to medial end of the nose slits to the nearest 0.1 cm.
Ear length	Measured from the distal helix to the lobe tip using a vernier caliper to the nearest 0.1 cm.
Testicular volume	Total of the right and left testes, volume calculated using formula for the prolate sphere: $\pi LW2/6$ where L is length and W is width (Harrison et al., 1977), measured using a vernier caliper to the nearest 0.01 cm.

Sequence and genotype data were obtained using diagnostic haplotypes and alleles that are unique to each species outside the hybrid zone. Molecular markers included the control region of mitochondrial DNA, a fragment of the sex determining gene on the Y-chromosome (Cortés-Ortiz et al., 2007), and 16 microsatellite loci (Apm68, D5S111, D6S260, D8S165, D17S804, PEPC8, Ab20, Apm1, Apm4, Ab12, Ab16, Apm9, Api06, Api07, Api09, Api11, Api14, Cortés-Ortiz et al., 2007; 2010). DNA extraction and amplification procedures are described elsewhere (Cortés-Ortiz et al., 2009). During collection of field data, individuals were identified as either *A. palliata*-like or *A. pigra*-like based on pelage coloration, overall size, and facial features. Those with some evidence of mixed characteristics, such as unexpected variations in pelage coloration and the presence of often diagnostic facial features of both parental species in the same individual were noted as questionable until microsatellite data further shed light on their ancestry. Individuals were considered "hybrids" whenever discordance between mtDNA, SRY, and/or microsatellites occurred or when microsatellite loci in the same individual contained combinations of alleles diagnostic of each species. Bayesian statistical methods (Pritchard et al., 2004) were not reliable for identifying hybrids that we found strong evidence for based on fully diagnostic alleles. These analyses, although useful in some cases, do not always have enough power to recognize the hybrid identity of multigenerational backcrossed individuals (Anderson and Thompson, 2002; Tung et al., 2008). Some individuals were confirmed as hybrids based on the discordance between SRY or mtDNA data with the autosomal data, possibly a result of multiple generations of backcrossing with one of the parental species.

Statistical Analyses

Genetic data revealed that the genotype of the majority of hybrids was predominantly composed by alleles diagnostic for one of the species and only a small fraction being diagnostic to other species; indicating that most individuals are multigenerational backcrossed hybrids. Of all the hybrids identified (N=129), only a few had a more equal share of genes from both species. However, none could be clearly identified as F1 individuals, since no males had discordant mtDNA and SRY haplotypes, and no individuals were heterozygous at all of the diagnostic microsatellite loci. Since the majority of hybrids detected are multigenerational backcrossed hybrids, individual hybrids were divided into three artificially established genotypic classes based on the number of diagnostic alleles likely to be found in each class: 1) *A. palliata* – backcrossed hybrids (ApaH) for those individuals with alleles predominantly characteristic of *A. palliata* (only 1-4 alleles diagnostic for *A. pigra*), 2) *A. pigra* – backcrossed hybrids (ApiH) for individuals that have mostly *A. pigra* alleles (only 1-4 alleles diagnostic for *A. palliata*), and 3) intermediate hybrids (Int) for individuals with 5 – 28 diagnostic alleles of one species and the remaining of the other species.

Descriptive statistics for all variables were calculated separately for each group and sex. Univariate nonparametric statistical comparisons were conducted for each variable, including a Kruskal Wallis test for comparing hybrid and pure groups overall and Mann-Whitney tests for pairwise comparisons. I applied a sequential bonferroni correction (Holm, 1979) when conducting multiple tests of the same hypothesis to reduce the probability of committing Type I error.

Other authors have combined male and female samples (by adjusting male mean to female mean) to increase their sample size (Ackermann et al.,

2006). Here, males and females were analyzed separately because comparisons of morphometric variables between hybrid and parental groups for females produced different results from those for males.

To gain an understanding of differences in overall morphology between hybrid and parental individuals, I employed principal component analysis (PCA), a multivariate data reduction method. This process takes potentially related variables and reduces them to a few uncorrelated components (Sokal and Rohlf, 1988). Only variables that showed statistically significant differences between the parental species (N=12 for males and N=11 for females) were used in this analysis. First, the data were log-transformed in order to decouple the variance from the means and to equalize variables that are on different scales. Missing values were handled using the "mi" package in R (R Development Core Team 2009) for multiple imputation which utilizes a regression model to predict the missing values. The PCA was conducted in R using the average of the three imputed data sets, using a correlation matrix, and scores were extracted to create a bivariate plot of the first two components, a procedure that is helpful for visualizing whether individuals group according to a certain set of variables. 90% confidence interval ellipses were constructed around the parental species and the multigenerational backcrossed hybrids.

Results

The descriptive statistics for male and female adult raw variables are presented in Tables 4.2 and 4.3. Male and female results for formal statistical comparisons are displayed separately (Tables 4.4 and 4.5).

Results from the Kruskal-Wallis analyses show an overall difference among males of the different groups (*A. palliata*, *A. pigra*, and hybrids) for all variables, except for vertical cranial distance, horizontal cranial distance, and interorbital distance. For females, differences among groups were also observed for most variables, but not for leg length, arm length, hand length, and horizontal cranial distance. Furthermore, for both males and females some measurements of cranial morphology remain similar between the two species and their hybrids.

Mann-Whitney statistical comparisons (see Tables 4.4 and 4.5) indicate that body mass, sitting height, and testicular volumes are significantly different between the two species, as found previously (Kelaita et al., 2011). In addition, males are significantly different between the two species for all other variables measured here except foot length, hand length, horizontal cranial length, vertical cranial length, and interorbital distance. Females, while also not significantly different between the two species for foot length, hand length and horizontal cranial distance, they also do not show differences for leg length, arm length, or cranial circumference.

Multigenerational hybrids are not significantly different in most traits when compared to the parental species with which they share most of their genotype. For males, there were no differences between species in 12 variables. For females, there were no differences between species in 11 variables. However, although mostly not significant, there is an interesting trend that ApiH individuals are slightly larger than pure *A. pigra* and ApaH are slightly smaller than pure *A. palliata* individuals. This could be a result of transgressive segregation, in which extreme trait values not observed in either parental taxon

appear in the hybrids, ultimately increasing phenotypic variation in the hybrid population (Rieseberg et al., 1999).

Table 4.2 Male raw data for morphometric variables of pure and hybrid groups

Males	*A. palliata*			*A. palliata* backcrosses			*A. pigra* backcrosses			*A. pigra*			Intermediates		
	N	Mean (Range)	SD	N	Mean (Range)	SD	N	Mean (Range)	SD	N	Mean (Range)	SD	N	Mean (Range)	SD
Weight (g)	16	6,069 (5,100 – 7,200)	664	15	5,543 (4,600 – 6,400)	496	33	7,956 (6,250 – 10,000)	938	27	7,424 (5,500 – 9,600)	1,093	5	7,310 (5,600 – 9,050)	1,228
Total Length (cm)	16	103.6 (97.6 – 111.0)	4.5	16	99.3 (90.4 – 105.4)	4.0	33	114.0 (100.0 – 127.0)	5.8	27	114.0 (100.0 – 122.0)	5.9	6	100.2 (89.8 – 113.0)	7.7
Sitting height (cm)	16	42.3 (39.0 – 46.0)	2.3	16	40.0 (36.6 – 43.2)	2.1	32	48.2 (41.8 – 56.0)	3.0	27	48.9 (41.0 – 58.0)	3.2	6	41.8 (35.2 – 47.5)	4.2
Tail length (cm)	16	61.4 (57.4 – 69.0)	3.1	16	58.1 (53.2 – 62.7)	2.8	33	64.7 (54.5 – 72.5)	3.7	27	65.1 (55.5 – 75.0)	4.6	6	57.4 (54.2 – 63.4)	3.4
Leg length (cm)	14	30.4 (29.5 – 31.5)	0.7	16	30.1 (27.8 – 31.7)	0.9	30	33.0 (30.0 – 35.8)	1.6	16	32.3 (28.5 – 34.2)	1.6	6	30.5 (29.0 – 33.0)	1.8
Foot length (cm)	16	14.5 (13.4 – 15.4)	0.5	15	14.1 (13.6 – 14.7)	0.3	32	15.4 (14.1 – 16.8)	0.6	26	14.7 (12.5 – 16.0)	0.8	6	14.6 (13.6 – 17.3)	1.4
Arm length (cm)	16	39.2 (37.2 – 43.7)	1.9	15	37.8 (36.3 – 39.2)	0.9	32	41.2 (37.0 – 46.5)	2.4	26	41.0 (37.3 – 44.5)	2.1	6	38.8 (36.4 – 42.2)	2.7
Hand length (cm)	16	12.2 (11.0 – 13.8)	0.7	16	11.7 (10.8 – 12.5)	0.5	32	12.6 (11.8 – 14.7)	0.7	27	11.8 (9.5 – 13.8)	1.0	6	11.6 (10.2 – 13.7)	1.2
Thorax (cm)	16	36.1 (33.5 – 39.0)	2.0	16	35.7 (32.4 – 38.1)	1.6	33	42.4 (37.5 – 47.4)	2.8	27	39.3 (34.0 – 45.0)	2.8	6	38.7 (35.4 – 41.8)	2.7
Abdomen (cm)	15	35.7 (31.0 – 38.5)	2.0	16	36.2 (31.7 – 40.6)	2.8	33	41.9 (35.2 – 49.8)	3.8	27	42.9 (36.0 – 50.5)	4.0	5	38.7 (34.7 – 43.9)	3.9
Cranial circumference (cm)	14	26.0 (24.3 – 27.7)	1.0	16	25.0 (23.6 – 26.2)	0.7	29	27.8 (24.5 – 31.0)	1.7	17	27.0 (24.0 – 30.0)	1.7	6	27.4 (25.1 – 29.5)	1.8
Cranial vertical (cm)	13	9.6 (8.5 – 10.6)	0.5	16	9.3 (8.7 – 9.9)	0.4	26	9.4 (8.2 – 10.5)	0.7	13	9.1 (8.0 – 10.5)	0.8	6	9.4 (8.5 – 10.8)	0.8
Cranial horizontal (cm)	13	8.9 (8.0 – 10.0)	0.8	16	8.2 (6.6 – 10.0)	0.9	28	9.0 (7.6 – 10.4)	0.8	16	9.0 (7.8 – 10.3)	0.8	6	9.0 (8.2 – 9.5)	0.5
Mandible (cm)	15	9.7 (8.2 – 11.5)	1.0	15	10.0 (8.4 – 10.8)	0.6	27	11.2 (9.5 – 12.9)	0.9	13	10.7 (9.5 – 12.0)	1.8	6	10.6 (9.6 – 12.5)	1.1
Interorbital (cm)	14	1.7 (1.4 – 2.1)	0.20	16	1.6 (1.4 – 1.9)	0.2	31	1.8 (1.4 – 2.1)	0.1	21	1.7 (1.3 – 2.0)	0.2	6	1.7 (1.5 – 2.1)	0.2
Internasal (cm)	14	1.4 (0.9 – 2.4)	0.44	15	1.18 (0.93 – 1.32)	0.3	33	0.9 (0.5 – 2.1)	0.3	26	0.8 (0.6 – 1.2)	0.1	6	1.0 (0.6 – 2.0)	0.5
Ear (cm)	15	3.2 (2.7 – 3.8)	0.26	16	3.3 (2.94 – 3.56)	0.2	32	3.8 (3.3 – 4.8)	0.4	26	3.6 (3.2 – 4.3)	0.3	6	3.7 (2.8 – 4.4)	0.5
Testicular Volume (mm^3)	13	20.3 (9.8 – 30.4)	5.9	13	21.0 (11.4 – 31.7)	5.7	33	13.2 (4.9 – 26.1)	5.3	24	9.6 (5.1 – 13.8)	2.3	5	15.1 (4.4 – 28.0)	10.1

Table 4.3 Female raw data for morphometric variables of pure and hybrid groups

Females	*A. palliata*			*A. palliata* backcrosses			*A. pigra* backcrosses			*A. pigra*			Intermediates		
	N	Mean (Range)	SD	N	Mean (Range)	SD	N	Mean (Range)	SD	N	Mean (Range)	SD	N	Mean (Range)	SD
Weight (g)	22	4,549 (3,850 – 5,500)	428	26	4,388 (3,600 – 5,500)	539	39	5,909 (4,900 – 7,300)	643	24	5,606 (4,500 – 7,000)	744	6	5,858 (4,900 – 7,700)	1,050
Total Length (cm)	24	99.6 (93.0 – 106.5)	3.6	25	97.3 (88.4 – 105.0)	4.0	40	106.9 (96.0 – 119.0)	4.9	25	107.8 (96.0 – 116.0)	4.9	7	101.4 (91.4 – 111.0)	7.2
Sitting height (cm)	24	39.8 (35.2 – 43.7)	2.1	26	38.4 (33.0 – 41.0)	2.0	39	42.7 (34.5 – 48.0)	2.8	26	44.7 (39.0 – 49.0)	2.7	7	41.0 (36.8 – 44.6)	3.1
Tail length (cm)	24	59.7 (56.0 – 67.3)	2.8	26	57.6 (51.6 – 62.5)	3.0	39	62.9 (57.1 – 68.6)	3.1	26	62.1 (54.8 – 68.2)	3.7	7	59.6 (53.0 – 63.5)	4.2
Leg length (cm)	24	30.1 (27.3 – 32.0)	1.2	25	30.1 (28.0 – 33.0)	3.0	38	31.2 (27.5 – 34.2)	1.7	13	30.8 (28.0 – 33.6)	1.6	7	30.5 (28.2 – 32.8)	1.7
Foot length (cm)	24	13.5 (12.4 – 14.7)	0.6	26	13.3 (12.3 – 14.2)	0.6	40	14.2 (12.4 – 16.5)	0.9	26	13.5 (11.5 – 15.1)	0.9	7	14.3 (12.8 – 15.4)	1.1
Arm length (cm)	25	37.8 (34.5 – 40.7)	1.5	26	37.7 (34.7 – 41.0)	1.4	40	37.9 (32.6 – 43.0)	2.2	24	37.9 (34.7 – 41.5)	1.6	7	37.1 (34.8 – 39.2)	1.4
Hand length (cm)	23	11.4 (10.5 – 12.8)	0.5	26	11.1 (10.1 – 12.6)	0.7	39	11.6 (10.2 – 14.5)	0.9	26	11.1 (10.0 – 14.5)	1.0	7	11.5 (10.4 – 12.0)	0.6
Thorax (cm)	24	32.0 (28.5 – 36.0)	1.9	25	31.4 (25.6 – 34.6)	2.1	40	35.9 (30.4 – 42.0)	3.1	25	34.2 (27.9 – 40.0)	3.0	7	33.7 (29.8 – 38.0)	3.2
Abdomen (cm)	22	34.0 (29.0 – 41.0)	4.0	25	33.8 (26.9 – 45.0)	3.9	39	39.1 (30.5 – 46.0)	3.6	22	39.9 (34.0 – 45.3)	3.3	6	38.0 (30.2 – 46.5)	6.4
Cranial circumference (cm)	24	23.2 (20.7 – 25.0)	1.0	26	22.9 (21.0 – 24.8)	0.9	38	24.2 (20.0 – 26.5)	1.5	12	23.9 (21.5 – 26.0)	1.5	7	23.9 (22.8 – 25.8)	1.2
Cranial vertical (cm)	24	9.1 (7.9 – 10.0)	0.5	25	8.7 (8.0 – 9.2)	0.4	35	8.6 (7.0 – 10.0)	0.6	9	8.4 (7.8 – 8.7)	0.3	7	8.5 (8.0 – 9.2)	0.4
Cranial horizontal (cm)	25	8.2 (7.3 – 9.5)	0.6	26	7.8 (7.0 – 8.8)	0.5	35	8.3 (6.6 – 9.5)	0.7	15	8.0 (7.0 – 9.0)	0.5	7	8.2 (7.2 – 9.6)	0.9
Mandible (cm)	24	8.0 (6.8 – 9.5)	0.8	25	8.7 (7.5 – 9.8)	0.6	34	9.2 (7.3 – 10.4)	0.8	14	8.9 (8.0 – 9.7)	0.5	7	9.2 (8.6 – 10.8)	0.8
Interorbital (cm)	18	1.4 (1.2 – 1.7)	0.1	25	1.5 (1.2 – 2.0)	0.2	36	1.5 (1.2 – 1.8)	0.1	23	1.5 (1.2 -1.7)	0.1	7	1.5 (1.3 – 1.7)	0.2
Internasal (cm)	19	1.2 (0.8 – 1.9)	0.2	26	1.2 (0.9 – 1.9)	0.3	39	0.8 (0.5 – 1.9)	0.2	24	0.8 (0.6 – 1.2)	0.1	7	0.8 (0.6 – 1.1)	0.1
Ear (cm)	20	3.0 (2.8 – 3.3)	0.2	26	2.9 (2.6 – 3.4)	0.2	40	3.6 (3.0 – 4.3)	0.3	26	3.4 (2.8 – 4.0)	0.3	7	3.4 (3.2 – 3.6)	0.2

Table 4.4 P-values for multiple comparisons for male groups using non-parametric tests*

Males	Kruskal-Wallis	Multiple Comparisons									
		A. palliata x *A. pigra*	*A. palliata* x *A. palliata* backcrossed hybrids	*A. pigra* x *A. pigra* backcrossed hybrids	*A. palliata* x intermediate hybrids	*A. pigra* x intermediate hybrids	*A. palliata* backcrossed hybrids x intermediate hybrids	*A. pigra* backcrossed hybrids x intermediate hybrids	*A. palliata* backcrossed hybrids x *A. pigra* backcrossed hybrids	*A. pigra* x *A. palliata* backcrossed hybrids	*A. palliata* x *A. pigra* backcrossed hybrids
Weight (g)	**P < 0.001**	**P < 0.001**	P = 0.034	P = 0.045	P = 0.018	P = 0.795	**P = 0.006**	P = 0.243	**P < 0.001**	**P < 0.001**	**P < 0.001**
Total Length (cm)	**P < 0.001**	**P < 0.001**	P = 0.023	P = 0.683	P = 0.197	**P = 0.001**	P = 0.912	**P = 0.001**	**P < 0.001**	**P < 0.001**	**P < 0.001**
Sitting height (cm)	**P < 0.001**	**P < 0.001**	**P = 0.015**	P = 0.483	P = 0.825	**P = 0.001**	P = 0.210	**P = 0.001**	**P < 0.001**	**P < 0.001**	**P < 0.001**
Tail length (cm)	**P < 0.001**	**P = 0.007**	**P = 0.007**	P = 0.587	**P = 0.015**	**P = 0.002**	P = 0.460	**P = 0.001**	**P < 0.001**	**P < 0.001**	**P = 0.003**
Leg length (cm)	**P < 0.001**	**P = 0.001**	P = 0.451	P = 0.226	P = 0.431	P = 0.060	P = 0.710	**P = 0.010**	**P < 0.001**	**P < 0.001**	**P < 0.001**
Foot length (cm)	**P < 0.001**	P = 0.148	**P = 0.008**	**P = 0.002**	P = 0.352	P = 0.174	P = 0.784	**P = 0.020**	**P < 0.001**	**P = 0.001**	**P < 0.001**
Arm length (cm)	**P < 0.001**	**P = 0.007**	P = 0.044	P = 0.667	P = 0.483	P = 0.050	P = 1.000	P = 0.043	**P < 0.001**	**P < 0.001**	**P = 0.006**
Hand length (cm)	**P < 0.001**	P = 0.221	**P = 0.016**	**P = 0.002**	P = 0.095	P = 0.386	P = 0.580	**P = 0.012**	**P < 0.001**	P = 0.365	P = 0.079
Thorax (cm)	**P < 0.001**	**P < 0.001**	P = 0.664	**P < 0.001**	P = 0.076	P = 0.726	**P = 0.020**	**P = 0.009**	**P < 0.001**	**P < 0.001**	**P < 0.001**
Abdomen (cm)	**P < 0.001**	**P < 0.001**	P = 0.553	P = 0.426	P = 0.149	P = 0.061	P = 0.283	P = 0.084	**P < 0.001**	**P < 0.001**	**P < 0.001**
Cranial circumference (cm)	**P < 0.001**	P = 0.054	**P = 0.011**	P = 0.255	P = 0.078	P = 0.724	**P = 0.008**	P = 0.827	**P < 0.001**	**P = 0.001**	**P = 0.001**
Cranial vertical (cm)	P = 0.174	P = 0.085	P = 0.039	P = 0.243	P = 0.378	P = 0.310	P = 0.738	P = 0.884	P = 0.658	P = 0.218	P = 0.238
Cranial horizontal (cm)	P = 0.023	P = 0.808	P = 0.024	P = 0.903	P = 0.605	P = 0.970	P = 0.050	P = 0.803	**P = 0.006**	**P = 0.018**	P = 0.448
Mandible (cm)	**P < 0.001**	**P = 0.009**	P = 0.191	P = 0.066	P = 0.109	P = 0.567	P = 0.369	P = 0.088	**P < 0.001**	P = 0.065	**P < 0.001**
Interorbital (cm)	P = 0.014	P = 0.567	P = 0.298	P = 0.185	P = 0.710	P = 0.930	P = 0.184	P = 0.173	**P = 0.001**	P = 0.070	P = 0.064
Internasal (cm)	**P < 0.001**	**P < 0.001**	P = 0.190	P = 0.625	P = 0.026	P = 0.717	P = 0.024	P = 0.953	**P < 0.001**	**P < 0.001**	**P < 0.001**
Ear (cm)	**P < 0.001**	**P < 0.001**	P = 0.058	**P = 0.021**	P = 0.039	P = 0.514	P = 0.071	P = 0.496	**P < 0.001**	**P = 0.001**	**P < 0.001**
Testicular Volume (mm^3)	**P < 0.001**	**P < 0.001**	P = 0.930	**P = 0.004**	P = 0.301	P = 0.419	P = 0.215	P = 0.948	**P < 0.001**	**P < 0.001**	**P = 0.001**

*Numbers in bold represent statistically significant relationships

Table 4.5 P-values for multiple comparisons of female groups using nonparametric tests*

	Kruskall-Wallis	*A. palliata* x *A. pigra*	*A. palliata* x *A. palliata* backcrossed hybrids	*A. pigra* x *A. pigra* backcrossed hybrids	*A. palliata* x intermediate hybrids	*A. pigra* x intermediate hybrids	*A. palliata* backcrossed hybrids x intermediate hybrids	*A. pigra* backcrossed hybrids x intermediate hybrids	*A. palliata* backcrossed hybrids x *A. pigra* backcrossed hybrids	*A. pigra* x *A. palliata* backcrossed hybrids	*A. palliata* x *A. pigra* backcrossed hybrids
Weight (g)	**P < 0.001**	**P < 0.001**	P = 0.238	P = 0.084	**P = 0.001**	P = 0.550	**P = 0.001**	P = 0.422	**P < 0.001**	**P < 0.001**	**P < 0.001**
Total Length (cm)	**P < 0.001**	**P < 0.001**	P = 0.119	P = 0.315	P = 0.478	P = 0.038	P = 0.164	P = 0.066	**P < 0.001**	**P < 0.001**	**P < 0.001**
Sitting height (cm)	**P < 0.001**	**P < 0.001**	P = 0.033	**P = 0.010**	P = 0.219	**P = 0.011**	P = 0.040	P = 0.192	**P < 0.001**	**P < 0.001**	**P < 0.001**
Tail length (cm)	**P < 0.001**	**P = 0.011**	P = 0.034	P = 0.524	P = 0.887	P = 0.158	P = 0.209	P = 0.052	**P < 0.001**	**P < 0.001**	**P < 0.001**
Leg length (cm)	P = 0.013	P = 0.171	P = 0.711	P = 0.405	P = 0.493	P = 0.812	P = 0.386	P = 0.286	**P = 0.003**	P = 0.106	**P = 0.005**
Foot length (cm)	**P < 0.001**	P = 0.807	P = 0.922	**P = 0.003**	P = 0.097	P = 0.111	P = 0.070	P = 0.719	**P < 0.001**	P = 0.673	**P < 0.001**
Arm length (cm)	P = 0.662	P = 0.689	P = 0.865	P = 0.895	P = 0.274	P = 0.277	P = 0.270	P = 0.169	P = 0.478	P = 0.466	P = 0.594
Hand length (cm)	P = 0.060	P = 0.070	P = 0.060	P = 0.040	P = 0.711	P = 0.184	P = 0.208	P = 0.830	P = 0.020	P = 0.720	P = 0.629
Thorax (cm)	**P < 0.001**	**P = 0.004**	P = 0.490	P = 0.054	P = 0.237	P = 0.615	P = 0.126	P = 0.113	**P < 0.001**	**P = 0.001**	**P < 0.001**
Abdomen (cm)	**P < 0.001**	**P < 0.001**	P = 0.983	P = 0.528	P = 0.123	P = 0.466	P = 0.121	P = 0.713	**P < 0.001**	**P < 0.001**	**P < 0.001**
Cranial circumference (cm)	**P = 0.001**	P = 0.126	P = 0.335	P = 0.474	P = 0.192	P = 0.933	P = 0.055	P = 0.490	**P < 0.001**	P = 0.031	**P = 0.001**
Cranial vertical (cm)	**P = 0.001**	**P = 0.001**	**P = 0.003**	P = 0.272	**P = 0.013**	P = 0.421	P = 0.383	P = 0.932	P = 0.231	P = 0.054	**P < 0.001**
Cranial horizontal (cm)	P = 0.046	P = 0.297	**P = 0.009**	P = 0.184	P = 0.598	P = 0.972	P = 0.426	P = 0.659	**P = 0.007**	P = 0.152	P = 0.724
Mandible (cm)	**P < 0.001**	**P = 0.001**	**P = 0.002**	P = 0.070	**P = 0.009**	P = 0.652	P = 0.252	P = 0.690	**P = 0.006**	P = 0.419	**P < 0.001**
Interorbital (cm)	**P = 0.008**	**P = 0.003**	P = 0.730	P = 0.658	P = 0.565	P = 0.202	P = 0.855	P = 0.263	P = 0.023	P = 0.022	**P = 0.004**
Internasal (cm)	**P < 0.001**	**P < 0.001**	P = 0.756	P = 0.538	**P = 0.001**	P = 0.776	**P < 0.001**	P = 0.890	**P < 0.001**	**P < 0.001**	**P < 0.001**
Ear (cm)	**P < 0.001**	**P < 0.001**	P = 0.485	**P = 0.003**	**P < 0.001**	P = 0.441	**P < 0.001**	P = 0.151	**P < 0.001**	**P < 0.001**	**P < 0.001**

*Numbers in bold represent statistically significant relationships

Among hybrid groups, results indicate that the two different backcrossed hybrid groups (ApaH and ApiH) are significantly different from each other (for 16 out of 17 variables for males and 12 out of 16 variables for females). This is expected given the results described above. Interestingly, intermediate hybrids were not significantly different from either the backcrossed hybrids or the parental species for most variables (13 – 16), likely because mean values for this group tended to be intermediate between the two parental species means and because intermediates showed a great deal of variability. Even differences between males and females could not be observed in the intermediate hybrids.

The first component (PC1) of the principal component analysis for males explains 53% of the overall variation while the second (PC2) explained 17%. For females, PC1 explained 43% of the overall variation while PC2 explained 17%. PC1, for both sexes, does the best job of sorting the two parental species out. Figures 4.2 and 4.3 show that for both males and females, there are two distinct groupings where each group has individuals belonging to the parental species and overlap with multigenerational hybrids backcrossed with the respective parental species. Intermediate hybrids generally overlap at the edges of both groups, indicating variable phenotypes that span the distribution of phenotypes for the two species and their hybrids. Results from the PCA were concordant with those from the univariate analyses, in that backcrossed hybrids cannot be distinguished from the species with which they share most of their alleles, and that intermediate individuals are highly variable. For males, all variables load negatively and roughly equally on PC1 except for testes. Therefore, the first component reflects size differences and would distinguish males with large testes compared to males with larger overall size. For females, PC1 also reflects

size differences and distinguishes individuals with large vertical cranial length from those with larger overall size. Male and female variables load to different extents on PC2, and therefore PC2 is likely to reflect shape differences between groups.

Figure 4.2 Male bivariate plot of scores for PC1 and PC2. Ellipses represent 90% confidence interval around each genotypic class.

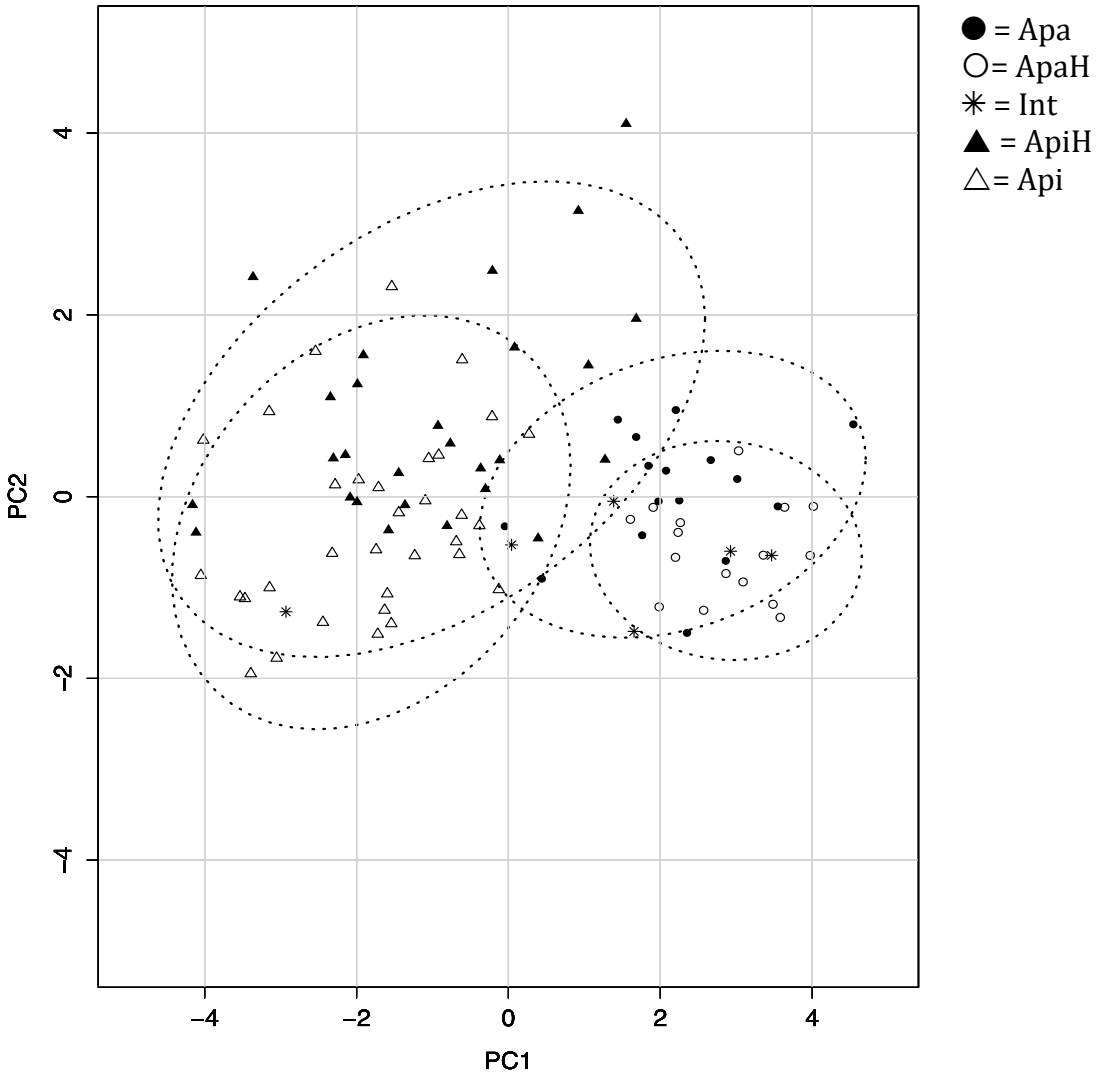


Figure 4.3 Female bivariate plot of scores for PC1 and PC2. Ellipses represent 90% confidence interval around each genotypic class.

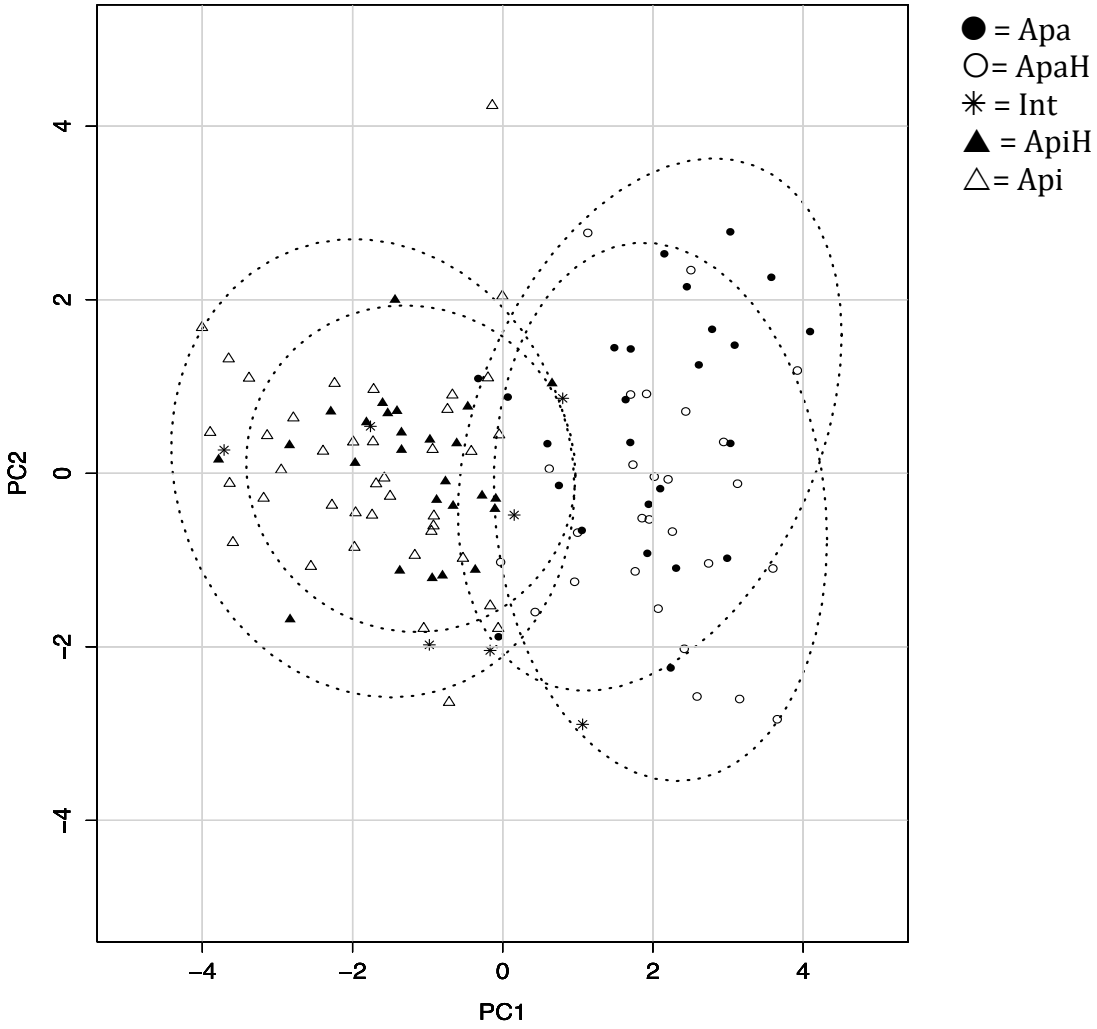


Table 4.6 Male eigenvector loadings for the principal component analysis

	PC1	PC2
Weight	-0.398	0.016
Sitting Height	-0.332	0.423
Tail Length	-0.322	0.216
Leg Length	-0.343	-0.272
Arm Length	-0.317	0.287
Thorax	-0.360	-0.296
Abdomen	-0.342	0.148
Mandible	-0.224	-0.691
Testes	0.156	-0.305
Ear	-0.302	-0.158

Table 4.7 Female eigenvector loadings for the principal component analysis

	PC1	PC2
Weight	-0.436	0.116
Sitting Height	-0.301	0.319
Tail Length	-0.294	0.342
Thorax	-0.392	---
Abdomen	-0.360	---
Mandible	-0.260	-0.305
Vertical Cranial Length	0.174	0.495
Interorbital Distance	-0.101	0.555
Internasal Distance	0.301	0.342
Ear	-0.39	---

Discussion

The main goal of this study was to analyze data from this unique primate hybrid system with a relatively long divergence time for the two parental species to understand variation in the morphology of hybrid individuals. The use of molecular markers provided the information necessary to approximate the relative genetic contributions of the parental species, allowing for a morphological analysis of distinct genotypic classes of hybrids. Univariate and principal component analyses provided evidence that multigenerational backcrossed hybrids morphologically resemble individuals of the species with which they share most of their genetic makeup. Only differences between the two parental species and between the two groups of backcrossed hybrids exist and are primarily accounted for by differences in size. Intermediate hybrids, on the other hand, exhibited a good deal of variation in morphology. Therefore, the morphology of the hybrid individual may differ depending on its genetic background, reflecting the extent of backcrossing and/or the interbreeding among hybrids for subsequent generations.

Preliminary findings of this hybrid system based on five males and six females showed that male hybrids exhibited particularly large body size compared to both parental species, while females were somewhat intermediate (Kelaita and Cortés-Ortiz, 2009). However, the initial small sample size for the analysis contained hybrid individuals of different genotypic backgrounds. My results here show that hybrids of different genotypic classes exhibit different morphological patterns and therefore should not be grouped together in morphological analyses. Otherwise, morphological values can exhibit a great

deal of variation. This is likely true not only for morphology but for any phenotype in general. For example, studies of hybrid fitness can be misleading if all hybrid genotypic classes are grouped together, as studies of plants grouping all hybrids in a single category showed them to have lower than actual fitness (Arnold and Hodges, 1995).

The large degree of morphological variation in intermediate hybrid individuals was also found in other primate studies (Phillips-Conroy and Jolly, 1986; Froehlich and Supriatna, 1996; Peres et al., 1996; Bynum et al., 1997; Bynum, 2002, Ackermann et al., 2006). Some of those authors have tested for heterosis or dysgenesis in hybrid individuals. Heterosis, or hybrid vigor, results due to an increase in heterozygosity, such as when two populations that differ in gene frequencies and dominance deviations interbreed (Falconer and Mackay, 1996). On the other hand, dysgenesis occurs when hybridization causes the breakdown of two separately "coadapted gene complexes" (Templeton, 1987). Heterosis and dysgenesis are often measured as the departures of the hybrid morphological trait value from that of the parental species' midpoint (Turner and Young, 1969; Falconer and Mackay, 1996). Primate hybrid individuals were often found to express heterosis (macaques: Smith and Scott, 1989; Schillaci et al., 2005; tamarins: Cheverud et al., 1993; Kohn et al., 2001), although not for all morphometric variables (Schillaci et al., 2005; Ackermann et al., 2006). Studies that performed heterosis/dysgenesis analyses relied on known pedigrees where F1 individuals can be found, especially since subsequent backcrossing will temper the effects of such phenomena (Ackermann et al., 2006). None of the hybrids in this study are F1 individuals; therefore it would not have been appropriate to conduct an analysis to test for heterosis and/or dysgenesis in

morphology. Here, intermediates show phenotypic values with means at, below, and above the midpoints of *A. palliata* and *A. pigra* means but also can range below and above the overall range of variation for the two species. The presence of such extreme phenotypes, which is sometimes referred to as transgressive segregation (Rieseberg et al., 1999), is expected for relatively divergent and genetically differentiated taxa (see Ackermann, 2010 for a discussion). Interestingly, in our hybrid sample there was no detectable evidence of developmental instability, such as supernumerary teeth, despite the fact that such evidence was more readily observable than heterosis in baboon hybrids (Ackermann et al., 2006).

Multigenerational backcrossed hybrids that have predominantly *A. pigra* genetic background are quite different morphologically from ones that have a predominantly *A. palliata* genetic background. Further, both of these multigenerational hybrids are overall morphologically indistinguishable based on continuous trait data from the parental species with which they share most of their nuclear alleles. Therefore, in natural studies of wild animals where specific pedigrees are not known, both for extant primate systems or fossil specimens, morphology may not be reliable for discriminating between hybrid and parental lines. Several mammalian studies have revealed cryptic hybridization, where molecular methods identified hybrid individuals that could not be distinguished morphologically from the parental species (Davison et al., 1999; Randi et al., 2001; Thulin & Tegelström, 2002; Pierpaoli et al., 2003; Gaubert et al., 2005; Norén et al., 2005). Along with my findings here, and as suggested by Ackermann (2010) and these studies, the presence and extent of hybridization can be underestimated when morphology is used alone for detection of admixture.

In the current study, with an increased sample size for each sex, males and females show some similar morphological patterns when compared among groups, such as the fact that multigenerational backcrossed hybrids do not differ in overall morphology from the parental species with which they share most of their alleles. The exception is that a trend that emerged from the principal component analysis, whereby intermediate males seem to group more so with *A. palliata* males than *A. pigra* males, and females overlap both groups somewhat equally. Nonetheless, this trend should be treated with caution because of the small sample size of intermediate individuals. However, there are some differences between male and female morphological patterns. For example, there are no statistically significant differences between *A. palliata* and *A. pigra* females in limb lengths and vertical cranial length, whereas males do show differences in those variables between the two species. Additionally, females show differences in interorbital length but males do not. It is unclear why some of these patterns exist, but trends in limb variation suggest that males require longer limbs to accommodate locomotive requirements for larger body sizes in *A. pigra*. Males and females also differ in which variables showed statistically significant differences between the multigenerational backcrossed hybrids and the parental species they share most of their with.

The results from this study have interesting implications for hybrid fitness. In particular, some morphological traits may be advantageous for males when they compete for access to reproduction with receptive females (Leigh et al., 2008; Kelaita et al., 2011). Hybrid males may inherit morphological features from one of the parental species that aid them in competing with males from the other parental species. For example, intermediate hybrid males that join *A.*

palliata groups will have a large body size advantage in competition compared to smaller *A. palliata* males. Likewise, hybrid males joining *A. pigra* groups could benefit from having larger testes in the event of sperm competition with *A. pigra* males (see Kelaita et al., 2011, for a discussion of testicular volume and sperm competition in these two species). Hybrid fitness advantages in this case could explain the existence of a large number of multigenerational backcrossed hybrids even though only a few intermediate hybrids are found. In other words, several incompatibilities and obstacles may need to be overcome to produce a first generation hybrids, but hybrid fitness advantages promote hybrid reproductive success and subsequent backcrossing with purebred individuals. This can be tested with further studies on the reproductive success of hybrid individuals as well as the behavior of both *A. palliata* and *A. pigra* individuals in response to attempts of hybrids and non-conspecifics to join their groups.

While some studies find a strong correlation between morphological and genetic indices for identifying hybrids (baboons: Tung et al., 2008; wild cats: Beaumont et al., 2001), the morphological traits used in such studies were non-metric (e.g. pelage coloration, head shape, body shape, etc.), measured by assigning discrete phenotypic scores to each trait. In the two species I consider here, hybrids were difficult to identify in the field based on external features. Some intermediate hybrids showed unusual and unpredictable variation in pelage coloration, particularly around the face and on the flanks (personal observations and those made by other researchers at the field site), but which could not be used with any reliability to detect hybrids. During data collection, individuals were assigned to one of the two species based on overall appearance. All of the genetically identified intermediate male individuals were recorded as

members of *A. pigra*. Yet, results from the principal component analysis shows them to be more similar to *A. palliata*, suggesting that metric traits may be expressed differently in hybrids from non-metric traits.

Results from this study suggest similarities with other howler monkey hybrids. Aguiar et al. (2008) have suggested that hybridization is taking place between *A. caraya* and *A. clamitans* in southern Brazil. The authors provide as evidence the presence of mixed species groups, the wide array of color polymorphisms, and the female-biased sex ratio that could be explained by Haldane's rule (that the heterogametic sex is often absent or sterile, Haldane, 1922). Hybrids were identified based on the presence of mosaic pelage color patterns, some of which were earlier described by Gregorin (2006) based on museum specimens as evidence for hybridization. The authors recommend that genetic data from hybrids and individuals outside the hybrid zone are necessary to confirm that hybridization is taking place, and that the apparent mosaic/intermediate color polymorphisms are not instead due to existing variation within *A. clamitans*. Results from the Mexican howler monkey hybrid study, where morphological features may not be reliable for detecting hybrid individuals, are in agreement with their final recommendation. Nevertheless, if the proposed *A. clamitans* x *A. caraya* hybrids are in fact true hybrids, and considering the fact that in this study, intermediate individuals show the greatest variability and are the most likely to exhibit some variable pelage color patterns, then it is likely that individuals identified in Aguiar et al. (2008) are also genetically intermediate hybrids and that backcrossed individuals are not distinguishable in the Brazilian howler monkeys either. Intermediates comprise approximately 12% of all the individuals in the *A. palliata*/*A. pigra* hybrid zone,

which is consistent with Aguiar et al. (2008)'s estimate that hybrids comprise 14% of the total number of individuals in their sampled fragment. This small percentage of intermediate hybrids remains in contrast to those of Old World primate hybrid zones such as macaque (Bynum, 2002) and baboon (Bergman and Beehner, 2004) zones, where intermediate forms can be found in greater numbers.

Further investigation is needed for documenting hybrid behavior in this and other primate systems. The morphological and behavioral phenotypes of hybrids may either reinforce reproductive barriers or promote the introduction of novel adaptations (Holiday, 2003). Bergman et al. (2008) found that hybrid behavior was correlated with their phenotypic hybridity index, where some hybrid males employed hamadryas strategies and others anubis strategies. Relative ancestry from the parental taxa can also influence ages of natal dispersal and attainment of adult rank (Alberts and Altmann, 2001). Development is intimately tied to life history variables, such as maturation rate, which are in turn tied to reproductive behaviors and strategies (Charpentier et al., 2008). One possibility for further research is to obtain data from howler monkey hybrid individuals on frequency of interspecific copulations and reproductive success to assess whether hybrid have behaviorial fitness advantages.

Thus far, it has been difficult to confirm instances of hybridization in the primate (including hominin) fossil record. This is due, in part, to the lack of clear expectations for what a hybrid should look like (Ackermann, 2010). In addition, as results suggest here, many hybrids may go undetected when relying on morphological features for identifying them. However, the lack of strong evidence

for hybridization in the fossil record does not negate the role it could have played in human evolution. Firstly, fossils are rare, making the discovery of hybrid fossils even more unlikely, with the exception of fossil rich sites that simultaneously yield fossils from more than one recognized species, such as the Levantine early human sites (Arensburg and Belfer-Cohen, 1998). Secondly, contact zones are likely to contain a mixture of purebred individuals and first generation, backcrossed, and multigenerational hybrids, so many of the hybrids may not exhibit any clear morphological features indicative of hybridization and can be confused as part of the intraspecific variation. Considering the extensive evidence for hybridization in primates, despite long divergence times, and the fact that hybridization is most likely underestimated in the fossil record, hybridization should not be dismissed definitively as it is in some research circles (Schwartz and Tattersall, 2010).

In cases where researchers have been able to identify wild primate hybrids, external non-metric morphological features relating to pelage coloration or soft tissue were used and are therefore not useful for studying fossilized specimens (Ackermann, 2010; Schwartz and Tattersall, 2010). Studies based on quantitative metric traits found evidence of heterosis and dysgenesis in hybrid individuals (Cheverud et al., 1993; Ackermann et al., 2006) but those studies were limited to known-pedigree first generation or backcrossed second generation individuals, which could be rare in natural hybrid zones (as is the case for howler monkeys). While Ackermann (2010) questioned the likelihood that sufficient data on the longevity of a morphological signature for long evolutionary time frames would ever exist, this howler monkey hybrid study provides evidence suggesting that hybrid

morphological signatures in this system are ephemeral. The main findings of this study are that in the howler monkey hybrid zone, few hybrid individuals are genetically intermediate and those individuals have a high degree of variation in morphology. The majority of hybrids are the result multigenerational backcrossing with the parental species and are morphologically similar to them. In this case, the morphological signatures of hybridization are short-lived, suggesting that only intermediate hybrids may experience fitness benefits or disadvantages. Despite the occurrence of hybridization in this area of sympatry, the species boundary between *A. palliata* and *A. pigra* seems to be relatively well-maintained but not completely impermeable to gene flow. Additional studies of hybrid fitness will help to shed light on the correlates of reproductive success and the extent that advantageous genes introgress through repeated hybridization events.

CHAPTER FIVE: Conclusion

Despite having a common ancestor around 3mya, *A. palliata* and *A. pigra* are able to hybridize. Studying the morphology of these distinct but closely-related taxa and their hybrids, I have presented some valuable findings.

The differences between the two howler species in their social systems have been clearly documented (see chapter three). The results of my study reveal that differences in testicular volume are also marked, which combined with the knowledge of differences in the two species social systems, suggest that the differences reflect the mating systems of the two species.

Interestingly, sexual dimorphism in canines and body size is not different, between the two species, which could either be due to the possibility that this measurement is not a good correlate of competition, sexual selection has operated equally on male body and canine size, or that both male and female competition could be operating such that ultimately, there are no differences between the two species. Based on these findings, and the fact that documenting correlates of competition has not been without its obstacles, I recommend that caution is taken when interpreting the causes of sexual dimorphism found in fossil specimens.

In addition, this study suggests the next step in research on the behavior of these two species and their hybrids. Specifically, molecular techniques can be employed to confirm relationships between individuals in order to determine paternity, and therefore reproductive success. This information can be used to

study the benefits of phenotypic adaptations confer for fitness and the extent that natural and sexual selection are operating on them.

The morphological and further genetic characterization of the howler monkeys in this study have revealed some of the dynamics of the hybrid zone as well as the possible variation in phenotypes that can be exhibited by different primate hybrids. One of the only other well-studied primate hybrid zones is that of the *P. anubis* x *P. hamadryas* hybrids. Thus, we have had a limited understanding of hybrid morphology from natural hybrid zones. Unlike baboon hybrids, not all hybrids are viable, and previous work on this system suggests that these howler monkey hybrids follow Haldane's rule, where only females are born or are fertile in the first generation, and males can only be produced by backcrossing with the parental species.

Of all the hybrids we detected, none were first generation, and most were hybrids backcrossed for several generations. A few scenarios could explain this pattern. Since F1's are presumably rare, then it is possible that several incompatibilities must be overcome for them to be viable. Another possibility is that interspecific mating is rare, although mixed species groups do exist. In addition to these possibilities, the number of multigenerational hybrids suggest that backcrossing has been occurring for some time, and that hybrids may enjoy greater fitness after backcrossing. It appears that based on the number of hybrids sampled here, those that contain many diagnostic alleles from both species and are therefore considered more intermediate (less backcrossed) show a great deal of variation in morphology. On the other hand, multigenerational backcrosses cannot be distinguished morphologically from the parental species they share most of their alleles with. This has implications for the ability to identify

backcrossed hybrids in the fossil record, and suggest the likelihood that hybridization may have been underestimated in paleoanthropological studies.

Overall this work highlights that in some primate hybrid zones, without using molecular data, most of the hybrids would not have been detectable with certainty, unlike others where many hybrids are intermediate and can be easily identified. Therefore, not all primate hybrid zones will show the same patterns, especially when isolation mechanisms, the genetic distance between the hybridizing species, and other ecological factors contribute to the specific dynamics of each hybrid zone. This study also shows that gene flow can be possible between species with fairly maintained boundaries, as evidenced by the number of multigenerational backcrossed hybrids. Therefore, it seems that hybridization may have played a role in primate evolution.

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