Morphological Variation of Genetically Confirmed *Alouatta pigra* × *A. palliata* Hybrids From a Natural Hybrid Zone in Tabasco, Mexico

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KEY WORDS howler monkeys; hybridization; neotropical primates

ABSTRACT While hybridization has been reported for a large number of primate taxa, there is a general lack of data on hybrid morphology for wild individuals with known genetic ancestry. A confirmed hybrid zone for the closely related Neotropical primates *Alouatta palliata* and *A. pigra* has provided a unique opportunity to study primate hybrid morphological variation. Here we used molecular evidence based on mitochondrial, Y-chromosome, and autosomal data to assess hybrid ancestry. We conducted univariate and multivariate statistical comparisons of morphometric data collected from individuals both outside and within the hybrid zone in Tabasco, Mexico. Our results show that of all the hybrids detected (N = 128), only 12% of them were approximately genetically intermediate, and none of them were first generation hybrids. Univariate pairwise comparisons among parental individuals, multigenerational backcrossed hybrids, and intermediate hybrids showed that overall, multigenerational backcrossed hybrids resemble the parental species with which they share most of their alleles. Conversely, intermediates were highly variable. Similarly, principal component analysis depicts an overlap between the parental species and their backcrosses when considering overall morphological differences. Finally, discriminant function analysis of the morphological variables was overall unreliable for classifying individuals into their assigned genotypic classes. Taken together, our results suggest that primate natural hybridization studies should incorporate molecular methods for determining ancestry, because morphology may not always be a reliable indicator of hybrid status. Hybrid zones could comprise a large number of multigenerational backcrossed hybrids that are indistinguishable from the parental species. The implications for studying hybridization in the primate fossil record are discussed. Am J Phys Anthropol 150:223–234, 2013.

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, sometimes facilitating gene flow between populations and other times reinforcing reproductive barriers (Arnold and Meyer, 2006). Although there are a number of recent reports of hybridization in the primate literature (Detwiler et al., 2005 for a review of cercopithecine hybridization; Cortés-Ortiz et al., 2007; Aguiar et al., 2008; Merker et al., 2009), there is a lack of understanding of the morphological variation associated with 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 variation, longevity, and universality of hybrid phenotypic patterns across the primate order.

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. Natural hybrid zones could illustrate not only whether hybridization is theoretically possible among taxa but also the likelihood of its occurrence given the existence of any postzygotic isolating mechanisms. As many primate taxa studies have implications for understanding human evolution (e.g., Jolly, 2001), primate natural hybrid zone studies can establish the basis for understanding the likelihood of hybridization between extinct hominin lineages. In particular, morphological studies within and outside hybrid zones can provide the framework for assessing the

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: National Science Foundation; Grant numbers: DEB-0640519 and BCS-0962807. Grant sponsor: PROMEP; Grant numbers: 103.5/03/1154EXB-9. Grant sponsors: Museum of Zoology, Department of Anthropology and Rackham Graduate School at the University of Michigan.

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Received 1 June 2012; accepted 24 October 2012

DOI 10.1002/ajpa.22196
Published online 7 December 2012 in Wiley Online Library (wileyonlinelibrary.com).

reliability of using metric and nonmetric morphological traits to identify hybrid individuals, considering the extent of multigenerational hybrid backcrossing and the frequencies of hybrid individuals with varying genetic backgrounds within the hybrid zone.

In this article, we analyze morphometric data from a Neotropical primate hybrid zone to evaluate the variation in hybrid morphology and whether we can use morphometric traits to identify hybrid individuals. To do this, we first present data on the morphological variation of two related species, *Alouatta pigra* and *A. palliata*, and their hybrids. These species are sister taxa that diverged approximately 3 mya (Cortés-Ortiz et al., 2003). They are allopatric in most of their geographic distribution, except for one confirmed area of contact in Mexico and one potential area of contact in Guatemala (Baumgarten and Williamson, 2007). Here, we study individuals that live in sympatry in the state of Tabasco, Mexico, an area that 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. 2011), social (reviewed in Kelaita et al., 2011), cytogenetic (Steinberg et al., 2008), and molecular (Cortés-Ortiz et al., 2003). Further, hybridization between these two species has been confirmed via the use of molecular markers (Cortés-Ortiz et al., 2007).

This study is unique in that we assess the genetic ancestry of hybrid individuals inhabiting this hybrid zone using molecular data before attempting to address morphological variation. 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. In doing this, we evaluate the relative reliability of morphological and molecular data in characterizing hybrid individuals produced from the crosses of two species with a divergence time that is often 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 shape, and facial features (Lawrence, 1933; Smith, 1970). However, it is not clear whether such characteristics are reliable for detecting hybrids between these two species in the wild. 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; this study). We, therefore, collect metric (continuous trait) morphological data to quantify differences between hybrid and pure individuals more objectively. Kelaita et al. (2011) confirmed that differences exist and are statistically significant for many variables between the two parental species, which allows for our comparative analysis of hybrid morphological patterns.

METHODS

Data collection and genotyping

Samples for this study were collected from within and outside the *A. palliata*/*A. pigra* contact zone in Tabasco, Mexico, where hybridization was previously genetically confirmed (Cortés-Ortiz et al., 2007). Thus far, the area of contact appears to be ~20 km in width (Cortés-Ortiz, unpublished) and contains groups of pure *A. palliata* and *A. pigra* individuals, as well as individuals of mixed ancestry. Between 1998 and 2008, we sampled adult individuals from several locations in Tabasco ($N = 135$), Veracruz ($N = 13$), Campeche ($N = 51$), Chiapas ($N = 3$), and Quintana Roo ($N = 7$) in Mexico and Peten ($N = 2$) in Guatemala. Figure 1 displays the locations of the sampled individuals and the species/hybrid compositions of adults in the sampled groups.

Adult individuals were captured as described in Rodríguez-Luna and Cortés-Ortiz (1994). The field team collected blood, hair, and morphometric measurements from the anesthetized animals. Sample collection from wild monkeys was carried out in accordance with UCUCA protocol #09319 at the University of Michigan. Differences between *A. palliata* and *A. pigra* were previously established for body mass, sitting height (here called trunk length), and testicular volume (Kelaita et al., 2011). We measured 14 additional variables and increased our sample size to describe overall morphology in the species and their hybrids. All measurements were performed and recorded by LCO and/or her field assistant (M. S. Aguilar-Cucurachi). Given the intrinsic difficulties of obtaining accurate morphological data from wild primates by more than one person, during different collecting expeditions LCO and MSAC independently collected data from the same individuals and a paired *t*-test was performed to check for interobserver error. No statistically significant differences were found (not reported).

The animals were weighed on a 20 kg Pesola® scale to the nearest 100 g. Measurements represent the right side unless otherwise noted. The following measurements were taken with a flexible metallic tape to the nearest 0.1 cm: (1) trunk length: external occipital protuberance to base (articulation of last sacral and first caudal vertebra) of tail, dorsally, with animal laying on the side and fully extended; (2) tail length: base of tail to tip of tail without hair, dorsally, with tail fully extended; (3) leg length: most lateral extent of greater trochanter of femur to landing point of foot, laterally, with leg fully extended; (4) foot length: pternion along plantar surface to tip of middle digit, excluding nail; (5) arm length: glenohumeral joint to tip of middle finger, excluding nail, dorsally, with arm fully extended; (6) hand length: base of thenar/hypothenar pad (i.e., palm) to tip of middle finger, excluding nail; (7) chest girth: circumference of chest at the middle of sternum, animal carried by the axilla with body fully extended and with no support of legs; (8) abdominal girth: greatest circumference of abdomen, animal in same position as for chest girth; and (9) head circumference: circumference including the frontal (at level of glabella) and the occipital (at most posterior protrusion of head) regions. The following measurements represent linear distances and were taken using a digital vernier caliper, to the nearest 0.1 cm: (10) head breadth: eurion to eurion, measured right above ears; (11) head length: glabella to occipital protuberance; (12) mandibular length: left, infradentale to gonion; (13) interorbital breadth: distance between the orbits measured at their medial margins; (14) internasal distance: medial to medial end of nostrils; (15) ear length: distal extent of helix to tip of lobule, and (16) testicular volume: total of the right and left testes, calculated using formula $\pi LW2/6$ (Harrison et al., 1977).

During collection of field data, individuals were identified as either *A. palliata*-like or *A. pigra*-like based on

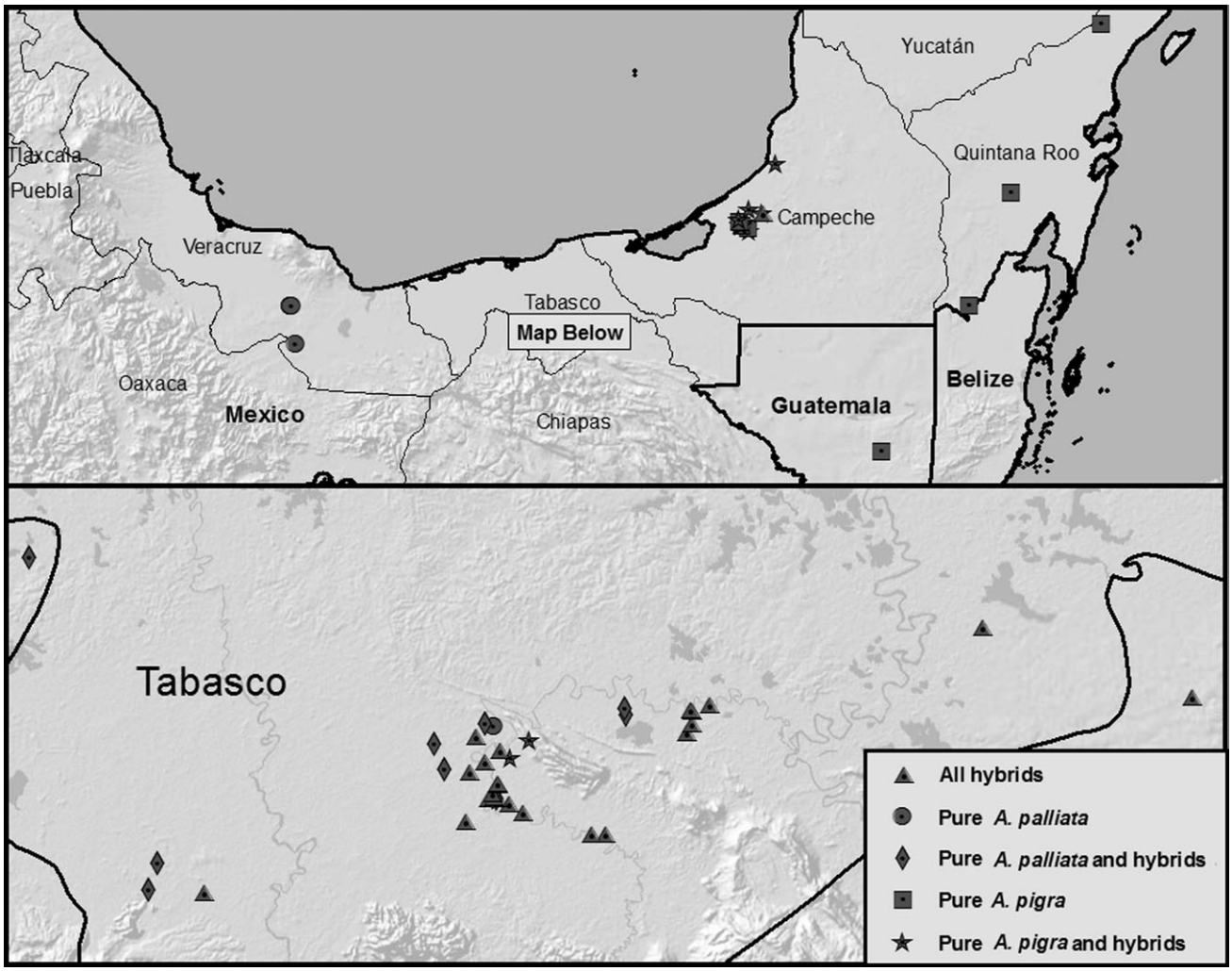

Fig. 1. Map of the sample collection locations.

pelage coloration, overall size, and facial features (for photos representative of pure and hybrid individuals, see Cortés-Ortiz et al., 2007). Those with some evidence of mixed characteristics, such as unexpected variations in pelage coloration and the presence of facial features of both parental species in the same individual were noted as questionable until microsatellite data further shed light on their ancestry.

DNA extraction and amplification procedures are described elsewhere (Cortés-Ortiz et al., 2007, 2010). Sequence and genotype data were obtained using markers with haplotypes and alleles that are unique to each species outside the hybrid zone. Sequence data included a fragment of the mitochondrial cytochrome *b* (Cyt *b*) gene for all individuals and a fragment of the Y-chromosome sex determination gene (SRY) for all males as described by Cortés-Ortiz et al. (2007). Diagnostic haplotypes for these markers are available in GenBank (Cyt *b* for *A. pigra*: DQ875685 and DQ875698; Cyt *b* for *A. palliata mexicana*: DQ875714; SRY for *A. pigra*: DQ875683 and DQ875678; and SRY for *A. palliata*: DQ875674). All individuals were also genotyped with a panel of 16 microsatellite loci (Apm68, D5S111, D6S260, D8S165, PEPC8, Ab20, Apm1, Apm4, Ab12, Ab16, Apm9, Api06, Api08, Api09, Api11, Api14), all of which had diagnostic alleles for one or both of the parental species (Cortés-Ortiz et al., 2010). Table 1 shows the diagnostic alleles for each locus used in this study and their respective frequencies in parental species outside the hybrid zone. We considered alleles to be diagnostic when they were exclusively found in individuals of one species outside the hybrid zone at frequencies > 0.15 but not in individuals of the other species outside the hybrid zone. Five additional alleles were considered diagnostic because they had high frequencies (above 0.5) in one of the parental species outside the hybrid zone and low frequencies (<0.05) in the other parental species (i.e., presence may be due to introgression; e.g., allele 261 of locus Api11 was fixed in our sample of *A. palliata* outside the hybrid zone and was present in one individual of *A. pigra* outside the hybrid zone, see Table 1). To determine diagnostic alleles, we excluded populations living in the proximity of the hybrid zone (within 40 km), as it is possible that they possess diagnostic alleles from the other parental species due to gene introgression. Previous analyses also showed admixed individual in some *A. pigra* groups in a particular population in Campeche (likely due to either gene introgression or past hybridization; Cortés-Ortiz, unpublished data). These groups were excluded from the assays to determine diagnostic loci,

TABLE 1. Diagnostic microsatellite alleles and their frequencies in populations of A. palliata and A. pigra populations outside the putative hybrid zone

Locus	No. of total alleles	No. diagnostic alleles	Allele size	ApaO	ApiO
Ap68	5	4	187[a]	0.000	**0.220**
			191	0.000	**0.240**
			193	**1.000**	0.000
			197	0.000	**0.540**
D5S111	6	2	163	**1.000**	0.000
			180[a]	0.000	**0.800**
D6S260	10	5	177	**0.577**	0.000
			179	**0.423**	0.020
			181	0.000	**0.200**
			183	0.000	**0.220**
			187	0.000	**0.480**
D8S165	3	2	119	0.000	**1.000**
			143	**1.000**	0.000
PEPC8	5	1	239	0.000	**0.340**
Ab20	9	4	236	**1.000**	0.000
			244	0.000	**0.380**
			262[a]	0.000	**0.320**
			266[a]	0.000	**0.180**
Apm1	11	4	183[a]	0.000	**0.260**
			199	0.000	**0.400**
			201[a]	0.000	**0.280**
			208	**0.846**	0.020
Apm4	7	3	239	0.000	**0.260**
			243	0.000	**0.240**
			249	**0.308**	0.000
Ab12	5	3	219	0.000	**0.340**
			233[a]	0.000	**0.560**
			234	**1.000**	0.000
Ab16	4	3	168[a]	0.000	**0.780**
			170	**0.962**	0.000
			177	0.038	**0.220**[b]
Apm9	5	4	170[a]	0.000	**0.180**
			172	0.000	**0.600**
			174[a]	0.000	**0.220**
			176	**0.923**	0.000
Api06	7	4	250	0.000	**0.180**
			252	0.000	**0.620**
			254[a]	0.000	**0.160**
			277	**0.923**	0.000
Api08	6	3	271[a]	0.000	**0.500**
			275[a]	0.000	**0.280**
			279	**0.231**	0.000
Api09	6	4	459[a]	0.000	**0.180**
			461	0.000	**0.460**
			463	0.000	**0.300**
			467	**1.000**	0.040
Api11	5	2	253	0.000	**0.960**
			261	**1.000**	0.020
Api14	8	4	181	**1.000**	0.000
			202[a]	0.000	**0.420**
			204[a]	0.000	**0.280**
			210	0.000	**0.200**

Frequencies in bold show the species for which that allele is diagnostic (see text for explanation).
[a] ApaO, *A. palliata* from outside the putative hybrid zone; ApiO, *A. pigra* from outside the putative hybrid zone.
[b] Identified as diagnostic but not observed in hybrid individuals. Although this represents an exception in our way of identifying diagnostic alleles, a larger sample of individuals outside the hybrid zone (including juveniles and adult individuals for which we do not have morphological data available) present a higher frequency of this allele in ApiO.

but all the individuals were included in further analyses in this study.

Individuals were considered "hybrids" whenever discordance between mtDNA, SRY and/or microsatellite data occurred or when a combination of microsatellite alleles that are diagnostic of each species were present in the same individual. Model-based methods used to infer the population of origin of alleles within an individual (e.g., Pritchard et al., 2000; Anderson and Thompson, 2002) have been increasingly used to identify the presence of hybrid individuals (e.g., Vähä and Primmer, 2006; Merker et al., 2009). However, these methods were not reliable for identifying hybrids (data not shown), for which we had found strong evidence for their hybrid status based on fully diagnostic haplotypes or alleles. For example, some individuals classified as non-hybrid by these programs were confirmed as hybrids based on the discordance between SRY and mtDNA data or with either of those markers and the autosomal data, possibly a result of multiple generations of backcrossing with one of the parental species. These assignment analyses, although useful in some cases, do not always have enough power to recognize the hybrid identity of multigenerational backcrossed individuals (Tung et al., 2008; Anderson, 2009). Therefore, we omit the use of these programs in our analyses.

Statistical analyses

Genetic data revealed that the genotype of the majority of hybrids was predominantly composed of alleles diagnostic for one of the parental species and only a small fraction being diagnostic of the other species, indicating that most individuals are multigenerational backcrossed hybrids. Of all the hybrids identified ($N = 128$), only a few had a more equal share of genes from both parental species. However, none was identified as an F1 individual. As the majority of hybrids detected are multigenerational backcrossed hybrids of widely varying genetic backgrounds, hybrid individuals were divided into three artificially established genotypic classes based on the number of diagnostic alleles present in each individual. Individuals with alleles predominantly characteristic of *A. palliata* (only 1–4 alleles diagnostic for *A. pigra*) are considered *A. palliata*–backcrossed hybrids (ApaH). Individuals that have mostly *A. pigra* alleles (only 1–4 alleles diagnostic for *A. palliata*) are considered *A. pigra*–backcrossed hybrids (ApiH). Intermediate hybrids (Int) are individuals with ≥ 5 diagnostic alleles of one species and the remaining alleles 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. To correct for multiple comparisons, we used a procedure for controlling the false discovery rate (FDR; Benjamini and Hochberg, 1995), which controls the expected proportion of incorrectly rejected null hypotheses, making it less conservative and more powerful than familywise error rates (FWER) (García, 2004).

Other authors have combined male and female samples (by adjusting male mean to female mean) to increase their sample size (e.g., 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, we used two multivariable methods, principal component analysis (PCA) and discriminant function analysis (DFA). PCA 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 ($N = 11$ for males and $N = 7$ for females) were used in this analysis. First, the data were log-transformed to decouple the variance from the means and to equalize variables that are on different scales. Missing values were replaced using a regression model with the multiple imputation function in IBM SPSS Statistics (Version 20, Armonk, NY). The PCA was conducted in SPSS on the imputed data sets using a correlation matrix, and the scores were extracted to create a bivariate plot of the first two components. This procedure is helpful for visualizing whether individuals group according to a particular set of variables. The PCA bivariate plots, including 90% confidence ellipses around the parental species and the multigenerational backcrossed hybrids, were constructed in R (R Development Core Team, 2010). DFA (stepwise, in SPSS, see Collard and Lycett, 2008) was used to determine whether individuals could be assigned using morphological variables to groups known a priori, based on molecular data. The same variables used in the PCA were included in the DFA model after they were log-transformed and missing values were replaced using the methods described above. As we used the principal components to provide a visual representation of overall morphological comparisons across the genotypic classes, we only presented the DFA results pertaining to the DFA model fit and its classification power.

RESULTS

The descriptive statistics for male and female raw variables are presented in the Supporting Information. Male and female results for formal statistical univariate comparisons are displayed separately (Tables 2 and 3, respectively). 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 head length. For females, differences among groups were also observed for most variables, but not for arm length, hand length, head breadth, or interorbital breadth.

Mann-Whitney statistical comparisons (see Tables 2 and 3) indicate that weight, trunk length, and testicular volume 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 leg length, foot length, hand length, head circumference, head breadth, and interorbital breadth. Significant differences between females of the parental species were only found for weight, trunk length, abdominal girth, head length, mandibular length, internasal distance, and ear length. Multigenerational hybrids (ApiH and ApaH) are not significantly different in most traits when compared to the parental species with which they share most of their alleles.

Among hybrid groups, the two different backcrossed hybrid groups (ApaH and ApiH) are significantly different from one another (for 16 of 17 variables for males and 11 of 16 variables for females). This is expected given the reported difference between species and the lack of significant differences found between each class of multigenerational backcrossed hybrid with their most genetically similar parental taxa. For many variables, intermediate hybrids were not significantly different from either the backcrossed hybrids or the parental species, 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 (see Supporting Information). It is notable that none of the differences among *A. palliata* or *A. palliata* backcrossed hybrids and intermediate males were statistically significant. Conversely, for females, none of the differences among *A. pigra*- or *A. pigra*-backcrossed hybrids and intermediates were statistically significant.

The first component (PC1) of the principal component analysis for males explains 45% of the overall variation while the second (PC2) explained 13.5%. For females, PC1 explained 45% of the overall variation while PC2 explained 17%. PC1, for both sexes, is the best component for discriminating between the two parental species. Figures 2 and 3 show that for both males and females, there are two distinct groupings where each group has individuals belonging to the parental species and overlapping with those multigenerational hybrids highly 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 microsatellite alleles, and that intermediate individuals are highly variable. For males, all variables load positively and roughly equally on PC1 except for internasal distance and testicular volume (Table 4). Therefore, the first component reflects size differences and would distinguish males with large testicular volume and internasal distance compared to males with larger overall size. For females, PC1 also reflects size differences and distinguishes individuals with large head length and internasal distance values from those with larger overall size (Table 4). Male and female variables load to different extents on PC2, and, therefore, PC2 is likely to reflect shape differences between groups.

The DFA produced four canonical discriminant functions (because five groups were compared). The first function accounts for most of the total among-group variability (see Table 5). The model fit for the data was significant for the first three functions for both males and females (see Table 6). The classification results (or how well morphological variables can help predict group membership) are presented in Table 7 for each genotypic class and both the sexes. However, overall the DFA correctly classified 72% of the males and 64% of the females.

DISCUSSION

The main goal of this study was to assess morphological variation of hybrids produced from interbreeding between sister primate species with relatively long divergence times and measurable quantitative morphological differences. The use of molecular markers provided the information necessary to approximate the relative genetic contributions of the parental species, allowing

TABLE 2. P values for multiple comparisons for male groups using nonparametric tests

		Multiple comparisons									
Males	Kruskal-Wallis	*A. palliata* × *A. pigra*	*A. palliata* × *A. palliata* backcrossed hybrids	*A. pigra* × *A. pigra* backcrossed hybrids	*A. palliata* × intermediate hybrids	*A. pigra* × intermediate hybrids	*A. palliata* backcrossed hybrids × intermediate hybrids	*A. pigra* backcrossed hybrids × intermediate hybrids	*A. palliata* backcrossed hybrids × *A. pigra* backcrossed hybrids	*A. pigra* × *A. palliata* backcrossed hybrids	*A. palliata* × *A. pigra* backcrossed hybrids
Weight	< **0.001**	<**0.001**	0.092	0.121	0.359	0.141	0.250	0.033	< **0.001**	< **0.001**	< **0.001**
Trunk length	< **0.001**	< **0.001**	0.073	0.561	0.647	<**0.001**	0.455	<**0.001**	< **0.001**	< **0.001**	< **0.001**
Tail length	< **0.001**	<**0.001**	**0.007**	0.100	0.022	<**0.001**	0.709	**0.001**	< **0.001**	< **0.001**	**0.001**
Leg length	< **0.001**	0.075	0.281	0.049	0.457	0.332	0.910	**0.014**	< **0.001**	0.024	< **0.001**
Foot length	< **0.001**	0.102	0.115	**0.009**	0.336	0.139	0.576	**0.008**	< **0.001**	**0.020**	< **0.001**
Arm length	< **0.001**	**0.015**	0.097	0.402	0.289	0.037	0.781	**0.016**	< **0.001**	< **0.001**	**0.002**
Hand length	< **0.001**	0.445	0.149	**0.005**	0.089	0.290	0.218	**0.006**	< **0.001**	0.730	0.050
Chest girth	< **0.001**	< **0.001**	0.570	< **0.001**	0.169	0.432	0.167	**0.003**	< **0.001**	**0.001**	< **0.001**
Abdominal girth	< **0.001**	< **0.001**	0.295	0.616	0.525	**0.013**	0.823	**0.008**	< **0.001**	< **0.001**	< **0.001**
Head circumference	< **0.001**	0.611	0.006	0.071	0.293	0.553	0.043	0.649	< **0.001**	0.042	**0.004**
Head length	0.056	**0.010**	0.032	0.051	0.191	0.130	0.910	0.920	0.576	0.064	0.233
Head breadth	**0.009**	0.693	**0.004**	0.319	0.396	0.203	0.079	0.535	**0.005**	**0.002**	0.449
Mandibular length	< **0.001**	**0.010**	0.025	0.117	0.072	0.445	0.842	0.041	**0.001**	0.264	< **0.001**
Interorbital distance	**0.016**	0.179	0.961	0.401	0.427	0.506	0.351	0.080	**0.002**	0.066	0.023
Internasal distance	< **0.001**	< **0.001**	0.130	0.345	0.036	0.345	0.068	0.685	< **0.001**	< **0.001**	< **0.001**
Ear length	< **0.001**	< **0.001**	0.154	0.050	0.023	0.787	0.048	0.367	< **0.001**	< **0.001**	< **0.001**
Testicular Volume	< **0.001**	< **0.001**	0.963	0.027	0.296	0.176	0.296	0.601	< **0.001**	< **0.001**	< **0.001**

Values in bold represent statistically significant differences.

TABLE 3. P values for multiple comparisons of female groups using nonparametric tests

		Multiple comparisons									
Females	Kruskal-Wallis	*A. palliata* × *A. pigra*	*A. palliata* × *A. palliata* backcrossed hybrids	*A. pigra* × *A. pigra* backcrossed hybrids	*A. palliata* × *A. palliata* intermediate hybrids	*A. pigra* × *A. pigra* intermediate hybrids	*A. palliata* backcrossed hybrids × *A. palliata* intermediate hybrids	*A. pigra* backcrossed hybrids × *A. pigra* intermediate hybrids	*A. palliata* backcrossed hybrids × *A. pigra* backcrossed hybrids	*A. pigra* × *A. palliata* backcrossed hybrids	*A. palliata* × *A. pigra* backcrossed hybrids
Weight	< **0.001**	< **0.001**	0.079	**0.012**	**0.002**	0.333	<**0.001**	0.398	< **0.001**	< **0.001**	< **0.001**
Trunk length	< **0.001**	< **0.001**	0.030	0.062	0.050	0.048	**0.006**	0.341	< **0.001**	< **0.001**	< **0.001**
Tail length	< **0.001**	0.017	0.099	0.218	0.479	0.319	0.105	0.042	< **0.001**	< **0.001**	< **0.001**
Leg length	**0.002**	0.172	0.901	**0.001**	0.504	0.167	0.407	0.162	**0.006**	0.139	**0.005**
Foot length	< **0.001**	0.587	0.488	**0.008**	0.027	0.054	**0.013**	0.647	< **0.001**	0.868	< **0.001**
Arm length	0.238	0.864	0.936	0.163	0.293	0.313	0.191	0.054	0.159	0.924	0.199
Hand length	0.148	0.070	0.315	0.036	0.810	0.179	0.422	0.636	0.094	0.336	0.509
Chest girth	< **0.001**	0.032	0.421	0.038	0.163	1.000	0.050	0.150	< **0.001**	**0.006**	< **0.001**
Abdominal girth	< **0.001**	**0.001**	0.433	0.757	0.151	0.299	0.045	0.223	< **0.001**	< **0.001**	< **0.001**
Head circumference	**0.016**	0.523	0.857	0.025	0.026	0.058	0.017	0.688	**0.015**	0.559	0.025
Head length	**0.001**	**0.001**	**0.009**	0.096	**0.005**	0.340	0.127	0.603	0.199	0.026	**0.002**
Head breadth	0.820	0.452	0.038	0.389	0.597	0.145	0.336	0.948	0.054	0.389	0.851
Mandibular length	< **0.001**	**0.016**	0.021	0.026	**0.009**	0.346	0.144	0.385	**0.002**	0.663	< **0.001**
Interorbital distance	0.062	0.033	0.820	0.890	0.218	0.817	0.450	0.679	0.037	0.151	**0.010**
Internasal distance	< **0.001**	< **0.001**	0.704	0.473	**0.002**	0.524	**0.001**	0.919	< **0.001**	< **0.001**	< **0.001**
Ear length	< **0.001**	< **0.001**	0.829	**0.002**	< **0.001**	0.124	< **0.001**	0.274	< **0.001**	< **0.001**	< **0.001**

Values in bold represent statistically significant differences.

Fig. 2. Male bivariate plot of scores for PC1 and PC2. Ellipses represent 90% confidence intervals around each genotypic class. The solid and striped ellipses on the right represent *A. pigra* and *A. pigra*-like hybrids, respectively, whereas the solid and striped ellipses on the left represent *A. palliata*- and *A. palliata*-like hybrids, respectively.

for a morphological analysis of distinct genotypic classes of hybrids. Both univariate and multivariate analyses provided evidence that multigenerational backcrossed hybrids morphologically resemble individuals of the species with which they share most of their genetic makeup. Conversely, intermediate hybrids exhibited great variation in morphology. Therefore, hybrid morphology may vary depending on the extent of backcrossing and/or the interbreeding among hybrids of subsequent generations.

Our results suggest that in studies of wild populations where pedigrees are unavailable, morphological characteristics 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 and Tegelström, 2002; Pierpaoli et al., 2003; Gaubert et al., 2005; Norén et al., 2005). Along with our findings here and as suggested by Ackermann (2010) and these studies, the existence and extent of hybridization can be underestimated when relying solely on morphology.

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. Results in this study reveal that hybrids of different genotypic classes exhibit different morphological patterns and, therefore, should not be grouped together in morphological analyses. Similarly, plant hybrids of different genotypic classes have been shown to possess varying levels

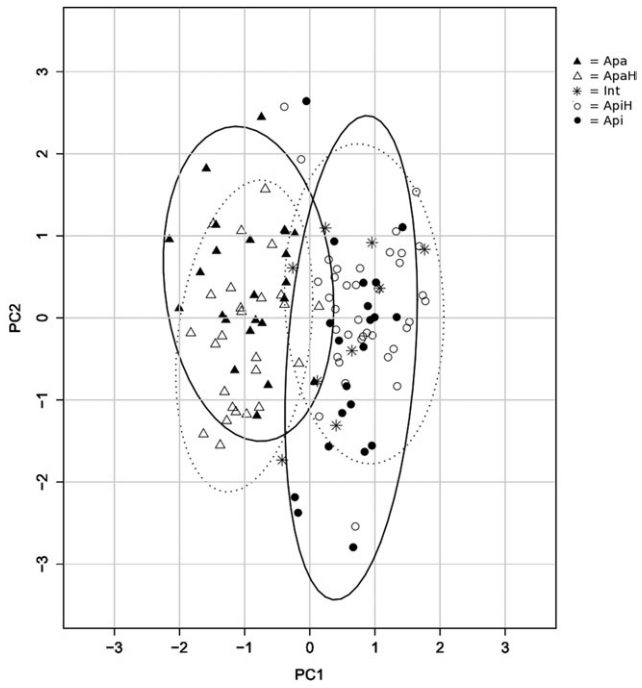

Fig. 3. Female bivariate plot of scores for PC1 and PC2. Ellipses represent 90% confidence intervals around each genotypic class. The solid and striped ellipses on the right represent *A. pigra* and *A. pigra*-like hybrids, respectively, whereas the solid and striped ellipses on the left represent *A. palliata* and *A. palliata*-like hybrids, respectively.

TABLE 4. Eigenvector loadings for the principal component analysis

	Male PC1	Male PC2	Female PC1	Female PC2
Weight	0.891	0.176	0.846	0.400
Trunk length	0.788	0.071	0.628	0.240
Tail length	0.739	0.222	—	—
Arm length	0.692	0.211	—	—
Hand length	0.480	0.588	—	—
Chest girth	0.830	-0.056	—	—
Abdominal girth	0.791	-0.141	0.740	0.265
Head length	—	—	−0.426	0.764
Internasal distance	−0.500	0.653	−0.633	0.533
Mandibular length	0.524	−0.190	0.471	-0.131
Ear length	0.708	−0.091	0.836	0.046
Testicular volume	−0.337	0.739	—	—

TABLE 5. Canonical discriminant function eigenvalues and percentage of variance explained by that function, for both males and females

	Males		Females	
Function	Eigenvalues	% Variance	Eigenvalues	% Variance
1	5.948	89.4	3.610	89.6
2	0.581	8.7	0.330	8.2
3	0.126	1.9	0.089	2.2
4	0.001	0.0	0.002	0.0

of fitness relative to their parental taxa (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). Conversely, dysgenesis occurs when hybridization causes the breakdown of two separately "coadapted gene complexes" (Templeton, 1987). Primate hybrids have been 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 as subsequent backcrossing will temper the effects of such phenomena (Ackermann et al., 2006). As our sample does not contain any F1 individuals, we did not test for heterosis and/or dysgenesis. Nevertheless, intermediates show phenotypic values with means at, below and above the midpoints of *A. palliata* and *A. pigra* means but can also fall below or above the overall range of variation for the two species. The presence of such extreme phenotypes 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 this phenomenon was more readily observable than heterosis in baboon hybrids (Ackermann et al., 2006).

When morphological data were analyzed separately by sex, results revealed some differences in morphological patterns among males and females. Of note, in the multivariate analyses, intermediate males group more closely with *A. palliata* males than with *A. pigra* males, while females overlap more with *A. pigra* females. In fact, the DFA analysis had poor predictability for intermediate males and females and incorrectly classified 42.3% of the intermediate males as *A. palliata*-backcrossed hybrids and 88.9% of intermediates as *A. pigra*-backcrossed hybrids.

Our results indicate interesting implications for hybrid fitness. The morphological and behavioral phenotypes of hybrids may either reinforce reproductive barriers or promote the introduction of novel adaptations (Holliday, 2003). The latter can be achieved by having beneficial traits that confer an added fitness advantage. 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 when competing with smaller *A. palliata* males. Likewise, hybrid males joining *A. pigra* groups could benefit from having larger testes for sperm competition with *A. pigra* males (see Kelaita et al., 2011). Therefore, despite the potential genetic, demographic, or social obstacles for producing first generation hybrids, viable and fertile F1 individuals may have a fitness advantage and continue to backcross, resulting in the observed large number of multigenerational backcrossed individuals. This hypothesis can be tested with combined genetic and behavioral studies on the reproductive success of hybrid individuals and the

TABLE 6. Significance of the DFA model fit, showing Wilks' Lambda, χ^2 statistic, degrees of freedom (df), and significance for both males and females

	Males				Females			
Test of functions	Wilks' Lambda	χ^2	df	Significance	Wilks' Lambda	χ^2	df	Significance
1 through 4	0.081	221.371	20	<0.001	0.150	211.861	16	<0.001
2 through 4	0.562	50.784	12	<0.001	0.689	41.471	9	<0.001
3 through 4	0.888	10.483	6	0.106	0.917	9.692	4	0.046
4	0.999	0.077	2	0.962	0.998	0.224	1	0.636

TABLE 7. DFA classification results for both males and females, showing counts and percentages of individuals' predicted group memberships based on morphology for each genotypic class (see methods for genotypic class abbreviations)

		Predicted group membership based on morphology											
		Males						Females					
Genotypic class		Apa	ApaH	Int	ApiH	Api	Total	Apa	ApaH	Int	ApiH	Api	Total
Count	Apa	10	3	2	0	0	15	14	8	1	0	1	24
	ApaH	5	9	0	0	0	14	8	18	0	0	0	26
	Int	1	3	2	1	0	7	0	1	0	8	0	9
	ApiH	0	0	0	31	4	35	2	1	0	28	6	37
	Api	0	0	0	7	16	23	1	0	0	5	15	21
Percentage	Apa	66.7	20.0	13.3	0.0	0.0	100.0	58.3	33.3	4.2	0.0	4.2	100.0
	ApaH	35.7	64.3	0.0	0.0	0.0	100.0	30.8	69.2	0.0	0.0	0.0	100.0
	Int	14.3	42.9	28.6	14.3	0.0	100.0	0.0	11.1	0.0	88.9	0.0	100.0
	ApiH	0.0	0.0	0.0	88.6	11.4	100.0	5.4	2.7	0.0	75.7	17.2	100.0
	Api	0.0	0.0	0.0	30.4	69.6	100.0	4.8	0.0	0.0	23.8	71.4	100.0

behavior of both *A. palliata* and *A. pigra* individuals in response to attempts of hybrids and nonconspecifics to join their groups.

Some investigations of wild primates have assumed hybrid status for individuals collected in the hybrid zone without a predetermined phenotype (e.g., Schillaci et al., 2005) and others used only phenotypic criteria for determining hybrid ancestry (Froehlich and Supriatna, 1996; Bynum, 1997; Alberts and Altmann, 2001; Bynum, 2002; Bergman and Beehner, 2004; Agostini et al., 2008; Aguiar et al., 2008; Bicca-Marques et al., 2008; Delmore et al., 2011). While some studies find certain levels of 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 nonmetric (e.g., pelage coloration, head shape, body shape, etc.), measured by assigning discrete phenotypic scores to each trait. In the howler monkey species considered here, hybrids were sometimes 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 (LCO and MAK personal observations), but many highly backcrossed individuals in this study were considered "pure" based on these characteristics, so further work is needed to assess the utility of nonmetric traits for identifying hybrids in these populations. Based on overall appearance, most of the genetically identified intermediate male individuals were initially recorded as having *A. pigra*-like appearance. Yet, results from the principal component analysis show them to be more similar to *A. palliata*, suggesting that metric traits may be expressed differently in hybrids from nonmetric traits.

Results from this study suggest similar patterns to another howler monkey hybrid system. Aguiar et al. (2008) concluded that hybridization is taking place between *A. caraya* and *A. clamitans* in southern Brazil (also suggested by Bicca-Marques et al., 2008). The authors provide as evidence the presence of mixed species groups, the wide array of pelage color polymorphisms, and the female-biased sex ratio that could be explained by Haldane's rule (that when hybridization takes place the heterogametic sex is often absent or sterile, Haldane, 1922) as formerly proposed for the *A. palliata* x *A. pigra* hybrid system (Cortés-Ortiz et al., 2007). Hybrids were identified based on unique 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 despite the fact that the apparent mosaic/intermediate pelage color polymorphisms are not encompassed within the range of variation documented for *A. clamitans* pelage coloration. Considering that in our study, intermediate individuals show the greatest variability, it is possible that the purported hybrids identified in Aguiar et al. (2008) are also genetically intermediate hybrids and that backcrossed individuals are not morphologically distinct based on overall appearance in the Brazilian howler monkeys. Intermediates comprise ~12% of all the individuals in the *A. palliata*/*A. pigra* hybrid zone, which is consistent with the estimate by Aguiar et al. (2008) 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. Many howler monkey species are known to be sympatric [e.g., *A. palliata* and *A. seniculus* in northwestern Colombia (Defler, 1994); *A. belzebul* and *A. seniculus* in Brazil (Pinto and Setz, 2000); *A. caraya* and *A. sara* in Brazil (Iwanaga and Ferrari, 2002)], and genetic analysis in those areas could reveal that hybridization is more common in howler monkeys than initially considered and may serve to identify genus wide hybridization patterns.

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). Ackermann (2010) questioned the likelihood that sufficient data on the longevity of a hybrid morphological signature for long evolutionary time frames would ever exist, but we believe our study helps shed some light on this issue. The main findings of this study are that in this 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 of multigenerational backcrossing with the parental species and are, for the most part, morphologically similar to them. In this case, the morphological signatures of hybridization are relatively short-lived and the species boundaries seem to be relatively well maintained though not completely impermeable to gene flow. Therefore, hybridization could have led to gene introgression in human evolution even if fossil morphological evidence is sparse. In addition, as results suggest here, many hybrids may go undetected when solely relying on morphological features for identification. However, the lack of strong evidence for hybridization in the fossil record does not negate the role it could have played in human evolution. First, fossils are rare, making the discovery of hybrid fossils even more unlikely. Even in 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), finding a significant number of fossils of hybrid individuals may be unlikely. Second, contact zones are likely to contain a mixture of purebred individuals and first generation, backcrossed, and multigenerational hybrids, and many of the hybrids may not exhibit any clear morphological features indicative of hybridization and can be confused as being on the continuum of intraspecific variation. Third, in cases where researchers have been able to identify wild primate hybrids based on morphology, external nonmetric 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 have 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 our study and for lemurs (Delmore et al., 2011). 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, the role of hybridization in human evolution should be given greater consideration than it has been thus far in some reviews (e.g. Schwartz and Tattersall, 2010). The suggestion based on genome sequence data that non-African humans and Neanderthals may have hybridized before the divergence of Eurasian groups from each other (Green et al., 2010) supports our argument.

ACKNOWLEDGMENTS

The authors appreciate the helpful feedback provided by Milford Wolpoff, John Mitani, Laura MacLatchy, and Thore Bergman on the earlier stages of this manuscript. The authors would also like to thank our collaborators Domingo Canales Espinosa, Pedro A. Dias Duarte, Javier Hermida Lagunes and Francisco García Orduña for their essential support during field expeditions that made possible this research. The authors would also like to thank Ma. del Socorro Aguilar Cucurachi, Antonio Jauregui, Antonio Jauregui Jr., Ariadna Rangel Negrin, Alba Rodas Martinez, María de Jesús Rovirosa, and Paulo Quintana for their assistance during field work. Data from Guatemalan howler monkeys were collected during a project headed by Gabriela Ponce and supported by WCS-Guatemala and Primate Conservation Inc. Molecular methods were conducted at the Genomic Diversity Laboratory of the Museum of Zoology, University of Michigan. Official collecting, exportation and importation permits for samples used in this research were obtained from SEMARNAT Mexico, CONAP Guatemala, and USFWS.

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