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Authors: Harris, Eugene E., Gifalli-Iughetti, Cristiani, Braga, Zelinda Hirano, and Koiffmann, Célia P.

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Cytochrome *b* Sequences Show Subdivision between Populations of the Brown Howler Monkey (*Alouatta guariba*) from Rio de Janeiro and Santa Catarina, Brazil

Eugene E. Harris, Cristiani Gifalli-Iughetti Zelinda Hirano Braga, Célia P. Koiffmann

Introduction

The brown howler monkey (*Alouatta guariba*) is a medium-sized and fully arboreal monkey that inhabits the Atlantic Forest of South America. Its geographic distribution extends from southern Bahia through the coastal Brazilian states south to the province of Misiones in northernmost Argentina (Kinzey, 1982; Di Bitetti *et al.*, 1994; Rylands *et al.*, 1988, 1996). Traditionally, two subspecies have been recognized, *A. guariba guariba* in the north and *A. guariba clamitans* in the south, although their exact distributions remain unclear. Kinzey (1982) reported that the transition from one subspecies to the other occurs in Espírito Santo and Minas Gerais, in regions flanking the Rio Doce, while Rylands *et al.* (1988) found evidence suggesting that *A. guariba clamitans* extends as far north as the Rio Jequitinhonha in northern Minas Gerais, and that *A. guariba guariba* may be restricted to southern Bahia (see Rylands *et al.*, 1996).

In general, *A. guariba* has been little studied, although several studies have described variation in pelage coloration (Kinzey, 1982) and in cranial and hyoid dimensions (Gregorin, 1996) that for the most part appear to be clinal in nature. The southern populations tend to be larger in body size, with larger cranial and hyoid dimensions, and display greater sexual dichromatism (see Kinzey, 1982; Gregorin, 1996). Chromosomal variation is extensive in *A. guariba*: Koiffmann (1977), Oliveira *et al.* (1995, 1996, 1998, 2000, 2001, 2002), and Gifalli (2003) have reported large differences among populations, including chromosomal rearrangements and differences in diploid number (ranging from 2N = 45 to 52). To date very little information exists about genetic variation within *A. guariba*.

Here we present a preliminary assessment of levels of genetic variation and inferred population structure in *A. guariba*, based on mtDNA encoded cytochrome *b* (cyt-*b*) sequences collected from populations in the Brazilian states of Rio de Janeiro, São Paulo and Santa Catarina.

Materials and Methods

We analyzed a total of 19 DNA sequences from *A. guariba*; details are provided in Appendix I. We collected cyt-*b* sequences from a total of 15 samples, including eight individuals from Santa Catarina, five from São Paulo, and two from Rio de Janeiro (Fig. 1). All samples from Santa Catarina were collected from frozen specimens preserved by Projeto Bugio at the Universidade Federal e Regional do Blumenau (FURB). Projeto Bugio collected some of these monkeys after they died in accidents (e.g., crossing the road); the project also rescues monkeys that have been captured by local residents. To sample these animals, a small (1 cm^2) section of frozen muscle was collected under the direction of one of the authors (ZHB). The samples from São Paulo were collected by another author (CG-I) and derive from animals held in captivity at DEPAVE (Departamento de Parques e Áreas Verdes do Estado de São Paulo, Divisão Técnica de Medicina Veterinária e Manejo da Fauna Silvestre – São Paulo, SP, Brazil) or at CEMAS (Centro de Estudo e Manejo de Animais Silvestres, Instituto Florestal, Fundação Florestal, São Paulo). Of the five sequences derived from samples from Rio de Janeiro, three were downloaded from GenBank and have been previously published (see Appendix I). Of these samples, one was derived from a specimen at the Centro de Primatologia do Rio de Janeiro (CPRJ), and two others from the Universidade Federal do Pará, Belém (UFPA). Another sample from Rio de Janeiro was collected under the auspices of CEMAS from an individual from Seropedica, RJ. The final sample from Rio de Janeiro was from an individual rescued from a fire by IBAMA in Poço das Antas, RJ; although the individual subsequently died, its body has been frozen and is stored by Projeto Bugio at FURB.

Our analysis also included an additional four sequences of cyt-*b* from *A. guariba* that were downloaded from GenBank at the National Center for Biotechnology Information. One sequence comes from the São Paulo Zoo (GenBank Accession No. AF289987; see Bonvicino *et al.*, 2001) and three sequences came from the Centro de Primatologia do Rio de Janeiro (AY065898 and AY065899 from Cortés-Ortiz *et*

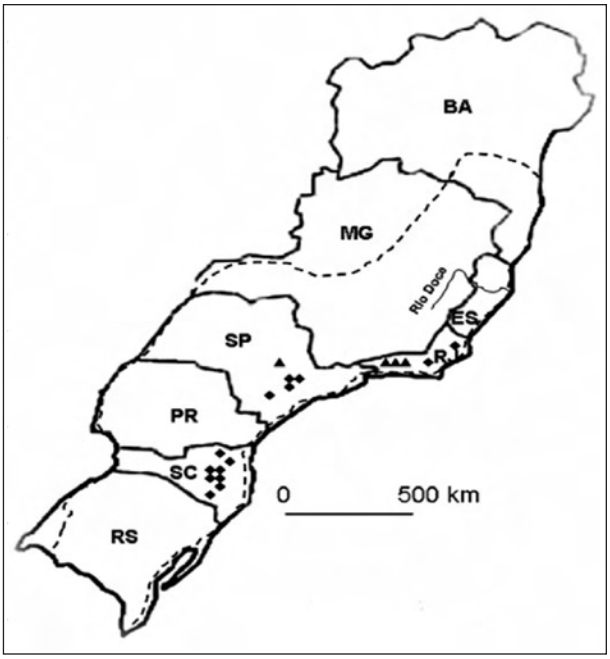

Figure 1. Map of southeastern Brazil showing the distribution of *A. guariba*. ◆ = Localities of samples of *A. guariba* collected and sequenced in this study: Rio de Janeiro (RJ), São Paulo (SP), Paraná (PR), Santa Catarina (SP). ▲ = Additional sequences from Rio de Janeiro and São Paulo downloaded from GenBank, National Center for Biotechnology Information. In Appendix I we list all individuals analyzed in this study.

al., 2003, and AF289986 from Bonvicino *et al.*, 2001). Fig. 1 provides more detailed information on the geographical origin of individual monkeys.

DNA was extracted either from frozen blood using a GFX Mini Blood Kit (Amersham) or from frozen muscle samples using a standard phenol-chloroform protocol (Maniatis *et al.*, 1982). An mtDNA region of nearly 1.2 kb, including the cyt-*b* gene, was amplified by PCR and sequenced using the oligonucleotide primers citb1, citb2, cit-alo (for primer sequences see Bonvicino *et al.*, 2001; Nascimento *et al.*, 2005) and CB1-5', CB2-3', CB-435L (see Cortés-Ortiz *et al.*, 2003). The sequences from our samples of *Alouatta guariba* are available in GenBank under the accession numbers reported in Appendix 1.

We used BioEdit v.7.0.1.4 (Hall, 1999) to align the sequences. Phylogenetic analyses were performed in MEGA v.3.1 (Kumar *et al.*, 2004) and PAUP* (beta version 10) (Swofford, 2000), while population genetic parameters were estimated in SITES (Hey and Wakeley, 1997) and ARLEQUIN v.2.0 (Schneider *et al.*, 1997). Translations of cyt-*b* nucleotides to amino acids were done using the EMBOSS Transeq application (Rice *et al.*, 2000). We used ModelTest 3.06 (Posada and Crandall, 1998) to estimate the best model of sequence evolution for our distance-based estimates of divergence dates.

Results

The cyt-*b* DNA sequences show 28 polymorphic sites and 8 unique mtDNA sequences (haplotypes). Transitions outnumber transversions by a ratio of 2.5 to 1.0. There are no indels in the sequences and the amino acid sequences (determined in the EMBOSS Transeq application) are not interrupted by premature stop codons, indicating they are functional cyt-*b* sequences and confirming they are not Numts (i.e., mitochondrial sequences inserted into the nuclear genome; see Mundy *et al.*, 2000).

Genealogical analysis and population genetics

Identical genealogical trees were generated using the Neighbor-Joining algorithm based on either p-distances or NrT + G (gamma = 0.2441) distances, selected by ModelTest 3.06 and rooted with published sequences from *A. belzebul* and *A. caraya* (Bonvicino *et al.*, 2001; Nascimento *et al.*, 2005). The deepest branch of the tree (Fig. 2) leads to two distinct haplogroups, labeled Haplogroups 1 and 2. All individuals from Rio de Janeiro fall into Haplogroup 1, whereas all individuals from Santa Catarina fall into Haplogroup 2. Individuals from São Paulo have cyt-*b* haplotypes that fall into either haplogroup. These same two haplogroups were found in the strict consensus Maximum Parsimony Tree and in the Maximum Likelihood Tree (-ln likelihood = 1955.66419) using the TrN + G model of sequence evolution (Posada and Crandall, 1998). These trees include sequences from *A. belzebul* (Ab-1001 and Ab-1088) and *A. caraya* (Ac-592 and Ac-XO51). Each pair represents the two most divergent haplotypes within each of these two species (see Bonvicino *et al.,* 2001; Nascimento *et al.*, 2005). As can be seen, the deepest branch in the *A. guariba* tree (occurring between Haplogroup 1 and 2) is slightly older than the divergence between the two *A. belzebul* haplotypes, and is nearly the same age as the deepest branch between the two *A. caraya* haplotypes. This presumably indicates that the divergence between Haplogroups 1 and 2 in *A. guariba* took place earlier than the split between the two most divergent haplotypes of *A. belzebul,* and appears to be as old as the split between the two most divergent *A. caraya* haplotypes.

Since individuals from Rio de Janeiro and Santa Catarina are found exclusively in either Haplogroups 1 or 2, respectively, we describe parameters estimating their degree of population differentiation as compared with estimates of differentiation between other pairs of populations. First, we note that there are nine fixed differences between individuals from Rio de Janeiro and Santa Catarina. Fixed nucleotide differences are sites at which all samples from one population show a different nucleotide compared to the nucleotide at that site in all samples from another population. Such differences are not expected if two populations are panmictic (i.e. freely interbreeding). In contrast, there is only one fixed difference between São Paulo and Santa Catarina, and none between Rio de Janeiro and São Paulo. Second, we found that F_{st} values, which measure population differentiation, are notably high when comparing samples from Santa Catarina and Rio de Janeiro (0.933, $p < 0.05$). This is almost double the value found when comparing samples

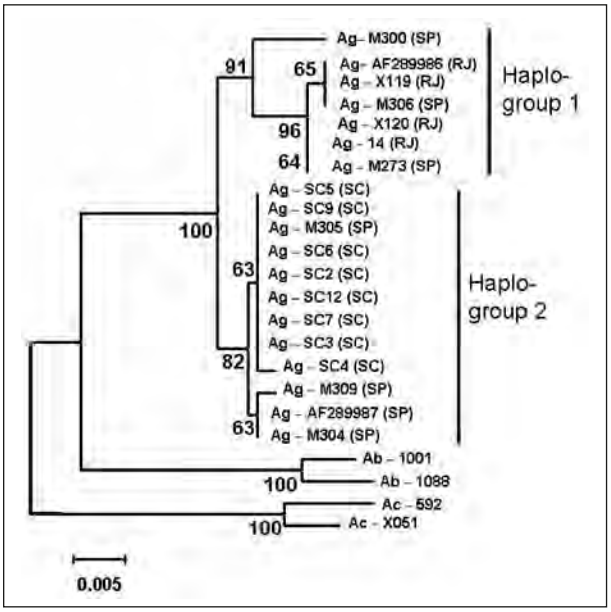

Figure 2. Neighbor-Joining Tree based on p-distances with bootstrap values from 1000 replications printed at nodes. RJ = Rio de Janeiro, SP = São Paulo, and SC = Santa Catarina. All samples of *A. guariba* in the gene tree are labeled beginning with the prefix "Ag." More information on the specimens of *Alouatta belzebul* and *A. caraya* is available via their sample numbers: Ab-1001 and Ab-1088 (*A. belzebul,* Tucuruí, Pará, Brazil; see Nascimento *et al.*, 2005), Ac-592 (*A. caraya,* Rio Casca, Manso Dam Reservoir, Chapada dos Guimarães, Mato Grosso, Brazil; see Bonvicino *et al.*, 2001) and Ac-X051 (*A. caraya,* Bolivia; see Nascimento *et al.*, 2005). Scale units are percent nucleotide divergence.

from Rio de Janeiro and São Paulo (0.532) and four times greater than the value when comparing São Paulo and Santa Catarina (0.221).

Tajima's (1993) relative rate test (as employed in MEGA), using *Brachyteles* as an outgroup, indicated no significant departures from equal rates of evolution along ingroup lineages. Since the Santa Catarina and Rio de Janeiro populations are exclusive to different haplogroups, we estimated the divergence time between them using two different calibration points: 12.9 Myrs (Goodman, 1996) and 16 Myrs (Cortés-Ortiz *et al.*, 2003) for divergence between *Brachyteles* and *Alouatta*. Distances were estimated using a TrN + G (gamma = 0.2411) model of sequence evolution (Posada and Crandall, 1998). We used these distances instead of p-distances, since they correct for multiple hits and among-site rate variation that, if left unconsidered, could produce large overestimates of the actual dates of divergence between haplotypes (Arbogast *et al.*, 2002). We used net distances in order to subtract the time it takes for the coalescence of sequences within ancestral species. We solved Formula 5.13 in Li and Graur (1991) using the distance between *Brachyteles* and *A. guariba* from Rio de Janeiro (0.36212), the distance between *Brachyteles* and *A. guariba* from Santa Catarina (0.37158), and the distance between *A. guariba* from Rio de Janeiro and *A. guariba* from Santa Catarina (0.01254). This yielded divergence dates of ~441 Kyrs and ~532 Kyrs for the respective calibration points. These dates were as old as the estimated dates between the most divergent sequences within *A. belzebul* (~326 Kyrs) and within *A. caraya* (~511 Kyrs).

Measures of genetic diversity

We compared the levels of variation in *A. guariba* in two ways: by comparing *A. guariba* with the closely related species *A. belzebul* and *A. caraya*, for which comparable numbers of sequences are available (23 and 27 samples, respectively; see Nascimento *et al.*, 2005), and by comparing levels of variation within the three geographic samples of *A. guariba* (Fig. 2). While mean p-distances are very similar in these three species, average pairwise diversity (π/bp) in *A. guariba* (0.00778) is over twice the value in *A. caraya* (0.0038) and about one-third greater than in *A. belzebul* (0.00579).

The maximum p-distance in *A. guariba* (1.7%) was considerably greater than the maximum p-distances in both *A. caraya* and *A. belzebul*. Furthermore, p-distances compared across Haplogroups 1 and 2 (ranging from 1.2% to 1.7%) mostly exceed the largest within-species distances in *A. belzebul* or *A. caraya* (1.0% and 1.3%, respectively). In fact, the maximum p-distances within *A. guariba* (1.7%) are nearly twice the genetic distances between *A. caraya* individuals from the geographically disparate localities of Santa Cruz, Bolivia and Serra da Mesa in the state of Goiás, Brazil (~0.9%) (see Nascimento *et al.*, 2005).

Within *A. guariba*, the Santa Catarina population is notably depauperate in mtDNA diversity. It is between 4 to 15 times less diverse in its π/bp and mean p-distance measures compared with populations from Rio de Janeiro and São Paulo. Conversely, the São Paulo population shows very high levels of diversity in all measures, due to the fact that it alone possesses haplotypes found in both Haplogroups 1 and 2.

Discussion

Our samples of cyt-*b* diversity in *Alouatta guariba*, drawn from populations in Rio de Janeiro, São Paulo, and Santa Catarina, are only representative of the southern portion of the full distribution of *A. guariba*, which extends from southern Bahia to northern Argentina (Rylands *et al.*, 1994). The region we sampled is usually ascribed to the southern subspecies *A. guariba clamitans,* reported by Kinzey (1982) to range as far north as the south bank of the Rio Doce in Espírito Santo, or, as more recent observations by Rylands *et al.* (1988) indicate, as far north as the Rio Jequitinhonha in Minas Gerais. The primary distinguishing feature of *A. guariba clamitans* is its sexual dichromatism, in which the male pelage is a dark rufous-red and the female is generally dark to light brown, although considerable variation is recognized (Kinzey, 1982). Gregorin (1996), however, reported a north-to-south cline in sexual dichromatism, as well as in measurements of the cranium and hyoid, which are generally larger in southern populations. Gregorin (1996) found that these clines weakened the value of these characters for distinguishing the two subspecies.

Table 1. Population genetic parameters. V = number of variable sites; Hapl. = number of haplotypes; π = nucleotide diversity (average proportion of nucleotide differences between all possible pairs of DNA sequences).

	N	Mean P-Distance	Range P-Distance	V	Hapl.	π/bp
A. guariba	19	0.70%	0.0–1.7%	28	8	0.00778
Rio de Janeiro	5	0.02%	0.0–0.4%	5	5	0.00196
São Paulo	6	0.92%	0.0–1.7%	20	3	0.00876
Santa Catarina	8	0.05%	0.0–0.2%	2	2	0.00047
Between Haplogroups 1 & 2	—	1.40%	1.2–1.7%	—	—	—
A. caraya	27	0.50%	0.0–1.3%	22	13	0.00338
A. belzebul	23	0.60%	0.0–1.1%	37	17	0.00579

Although we studied samples only from the range of the southern subspecies, *A. g. clamitans*, we found evidence in the cyt-*b* sequences suggesting a strong population subdivision between *A. g. clamitans* from Rio de Janeiro and those from Santa Catarina. This subdivision is evident in several aspects of the data: (1) samples from the two states fall exclusively into different haplogroups; (2) the samples show a considerable number of fixed nucleotide differences between them, which would not be expected if the populations were panmictic; and (3) the samples show statistically significant F_{st} values, indicating differentiation.

Howler monkeys show the greatest degree of karyological variation, both between and within species, of any platyrrhine genus (Koiffmann, 1977; Gifalli, 2003). Within *Alouatta*, *A. guariba* shows a notable degree of geographic chromosomal variation (Koiffmann, 1977; Oliveira *et al.*, 1995, 1998, 2000, 2002) that appears to be consistent with our findings of geographic differentiation. Individuals of *A. guariba clamitans* from the southern states of Santa Catarina and Paraná contrast with *A. guariba clamitans* from Rio de Janeiro in their diploid number (2N = 45 [♂♂] or 46 [♀♀] versus 2N = 49 [♂♂] or 50 [♀♀]), as well as in several Robertsonian rearrangements, pericentric inversions, and chromosomal translocations (Oliveira *et al.*, 1995, 2000, 2002). Our ongoing efforts to karyotype all individuals in the preliminary cyt-*b* genealogy presented here should help to clarify the association between chromosomal and cyt-*b* results.

The population differentiation we observe within *A. guariba clamitans,* along with the chromosomal differences previously described by Koiffmann (1977), Oliveira *et al.* (1995, 1996, 1998, 2000, 2001, 2002), and Gifalli (2003), may indicate that *A. g. clamitans* is actually representative of two distinct subspecies, or possibly even two separate species. Oliveira (2000) suggested that the large chromosomal differences characterizing these populations may indicate they are reproductively isolated from each other.

This pattern of population subdivision may have arisen during the late middle Pleistocene (over 400,000 years ago, based on our estimate) following forest fragmentation, and/or the formation of distinct ecoregions that became centers of endemism. One such region might have formed in the southern Atlantic Forest, including Santa Catarina, and another in the middle northern Atlantic Forest, including Rio de Janeiro. Müller (1973) and Kinzey (1982) speculated on refuges in the Atlantic Forest, but neither recognized a distinct refuge in the southern Atlantic Forest that would account for the differentiation of the Santa Catarina population. A more recent study of possible centers of endemism in the Atlantic Forest (Costa and Leite, 2000) likewise did not identify a center of endemism as far south as Santa Catarina. Nevertheless, the polymorphic São Paulo population—which shares haplotypes with both the Rio de Janeiro and Santa Catarina populations—may have formed as the forests themselves expanded (in the case of refuges), or as populations expanded from centers of endemism, and animals carrying divergent haplotypes came into renewed contact with each other.

Interestingly, we found that the maximum genetic distances between individuals of *A. guariba* were considerably greater than those found in either *A. caraya* or *A. belzebul*. For example, even the distances between geographically widespread individuals of *A. caraya*, from Bolivia and from Goiás in Brazil (see Nascimento *et al.*, 2005), are only half the maximum distances we found between *A. guariba* in Rio de Janeiro and Santa Catarina. The reasons for this are not clear, but may be related to the topographic differences in habitat occupied by these three species. *A. guariba* inhabits mountainous forests of the Serra do Mar and Serra da Mantiquiera of the Atlantic Forest, while the respective habitats of *A. caraya* (ranging across southern Brazil, Paraguay and northern Argentina) and *A. belzebul* (in the south-eastern Amazon and far northeastern Atlantic Forest) are generally devoid of mountainous terrain. Altitudinal variation has likewise been suggested to have played a role in the population differentiation of the genus *Brachyteles*, also endemic to the Atlantic Forest (see Rylands *et al.*, 1996).

Populations of *A. guariba* show remarkable variation in mtDNA. Although these differences need to be confirmed with other genetic markers, the Santa Catarina population demonstrates 4 to 15 times less variation in cyt-*b* than the populations in Rio de Janeiro or São Paulo, respectively, while the São Paulo population shows extreme variability. Apart from the insights it may allow into the evolution of these populations, this information is also relevant for conservation efforts, as *A. guariba* has been listed as Vulnerable (*A. guariba clamitans*) or Critically Endangered (*A. guariba guariba*) (Rylands *et al.*, 1994; Hilton-Taylor *et al.*, 2004).

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Eugene E. Harris, Department of Biological Sciences and Geology, Queensborough Community College, City University of New York, New York, USA, **Cristiani Gifalli-Iughetti,** Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Brazil, **Zelinda Hirano Braga,** Fundação Universidade Regional de Blumenau, Santa Catarina, Brazil, and **Célia P. Koiffmann,** Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Brazil. Corresponding author: Eugene E. Harris, e-mail <EHarris@qcc.cuny.edu>.

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Appendix I. List of all *Alouatta guariba* samples analyzed in this study.

Sample ID[a]	Geographic Origin[b]	GenBank Accession Number[c]	Organization[d]	Reference[e]
SC2	Blumenau, SC	DQ679777	Projeto Bugio, FURB	This study
SC3	Brusque, SC	DQ679778	Projeto Bugio, FURB	This study
SC4	Jaraguá do Sul, SC	DQ679779	Projeto Bugio, FURB	This study
SC5	Indial, SC	DQ679780	Projeto Bugio, FURB	This study
SC6	São Bento de Sul, SC	DQ679781	Projeto Bugio, FURB	This study
SC7	Blumenau, SC	DQ679782	Projeto Bugio, FURB	This study
SC9	Indial, SC	DQ679783	Projeto Bugio, FURB	This study
SC12	Lages, SC	DQ679784	Projeto Bugio, FURB	This study
M273	Mairiporã, SP	DQ679776	DEPAVE	This study
M300	Serra de Cantereira, SP	DQ679773	CEMAS	This study
M304	Serra de Cantereira, SP	DQ679774	CEMAS	This study
M305	Reserva Florestal in Campinas, SP	DQ679772	CEMAS	This study
M309	Serra de Cantereira, SP	DQ679775	CEMAS	This study
AF289987	SP	AF289987	ZSP	Bonvicino *et al.* (2001)
AF289986	RJ	AF289986	CPRJ	Bonvicino *et al.* (2001)
M306	Seropedica, RJ	DQ679771	CEMAS	This study
X119	RJ	AY065898	UFPA	Cortés-Ortiz *et al.* (2003)
X120	RJ	AY065899	UFPA	Cortés-Ortiz *et al.* (2003)
14	Poço das Antas, RJ	DQ679770	Projeto Bugio, FURB	This study

[a] The sample ID is the code assigned to a specific animal and that is used to label the cyt-*b* gene tree in Figure 2. Exceptions are AF289987 and AF 289986, which are also GenBank accession numbers.
[b] SC = Santa Catarina; SP = São Paulo; RJ = Rio de Janeiro.
[c] The GenBank database may be accessed at <http://www.ncbi.gov>.
[d] Name of organization where the individual is kept, either as a living specimen or preserved. See Materials and Methods for more information.
[e] References to consult for more detailed information about sample and sequence.