Marmoset Phylogenetics, Conservation Perspectives, and Evolution of the mtDNA Control Region

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Marmosets (genus *Callithrix*) are a diverse group of platyrrhine primates with 13–15 purported taxa, many of them considered endangered. Morphological analyses constitute most of the basis for recognition of these forms as distinct taxa. The purpose of this study was to provide a molecular view, based on mitochondrial control region sequences, of the evolutionary history of the marmosets, concomitant with a molecular phylogenetic perspective on species diversity within the group. An additional purpose was to provide the first comparative examination of a complete New World monkey control region sequence with those of other mammals. The phylogenetic analyses provide convincing support for a split between the Atlantic forest and Amazonian marmosets, with the inclusion of the pygmy marmoset (*Cebuella pygmaea*) at the base of the Amazonian clade. The earliest branch of the Atlantic forest group was *C. aurita.* In the Amazonian group, the analyses do not support the recognition of *C. humeralifer* and the recently described *C. mauesi* as distinct taxa. They do, however, support a clear distinction between *C. argentata* and a strongly supported mixed clade of *C. humeralifer* and *C. mauesi.* In the Atlantic forest group, the phylogenetic tree suggests mixing between *C. penicillata, C. kuhli,* and possibly *C. jacchus.* Most of the sequence features characteristic of other mammal control regions were also evident in marmosets, with the exception that conserved sequence blocks (CSBs) 2 and 3 were not clearly identifiable. Tandem repeat units often associated with heteroplasmy in a variety of other mammals were not evident in the marmoset sequences.

Introduction

The marmosets are a diverse group of platyrrhine primates within the subfamily Callitrichinae, genus *Callithrix.* Depending on the authority, there are currently 13–15 distinct taxa listed as members of this genus (reviewed in Rylands, Coimbra-Filho, and Mittermeier 1993). This includes recent discoveries of two new species of marmoset, *C. mauesi* (Mittermeier, Schwarz, and Ayres 1992) and *C. nigriceps* (Ferrari and Lopes 1992), both from the state of Amazonas, in central Brazilian Amazonia. Most of the recent and current systematic discussion regarding this group has centered around whether variously recognized taxa are distinct species or subspecies (reviewed in Rylands, Coimbra-Filho, and Mittermeier 1993). There is, however, no convincing molecular genetic evidence indicating that the various forms are even distinct evolutionary entities, whether you regard them as species or subspecies. There is no published account of a molecular phylogenetic analysis of this issue at the DNA sequence level, and much of the protein electrophoretic data have been inconclusive, with only a few of the taxa represented (e.g., Meireles et al. 1992). Morphological analyses constitute most, if not all (in some cases), of the basis for recognition of these forms as distinct taxa.

Most of the recognized marmoset taxa are vulnerable or endangered, many being currently classified within Appendix 1 or 2 of the CITES (Rylands, Coimbra-Filho, and Mittermeier 1993). From a conservation perspective, it is clearly of fundamental importance to have a confident understanding of which forms repres distinct evolutionary entities, based on a reliable pl logenetic framework, before effective conservati management programs can be implemented. One proach for the establishment of such a phylogene framework should be through the principles and te niques of molecular evolutionary genetics. The abser of such information has been shown to result in seri conservation management mistakes, the most wid documented example being the case of the dusky seas sparrow (Avise and Nelson 1989; Avise 1994).

In addition to the concerns related to conservati there is a wide range of phylogenetic issues in mar sets that remain virtually unexplored at the DNA s quence level. The only widely accepted phylogene hypothesis is that the Atlantic forest and Amazonian est marmosets represent two distinct clades; howev even this view has no published support at the DN sequence level. Relative relationships within each of t Atlantic and Amazonian forest groups remain highly u certain. The longstanding view has been to place *C buella,* the pygmy marmoset, at the base of the m moset clade, no more closely related to either the A azonian or Atlantic group; however, even this idea h little convincing support.

The mitochondrial control region, in the major of taxa so far investigated, is the most rapidly evolvi region of the mtDNA molecule (see, e.g., Aquadro a Greenberg 1983; Horai and Hayasaka 1990; Brow Beckenbach, and Smith 1993; Zhu et al. 1994). It therefore a putatively informative region for addressi evolutionary relationships of closely related species and subspecies. Complete control region sequences a available from a number of hominid primates (see, e. Anderson et al. 1981; Foran, Hixson, and Brown 198 but there are no complete sequences available from a

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New World monkeys. Comparisons of various mammalian control region sequences have already identified several sequence features broadly characteristic of the control region including a conserved central domain, a divergent left (L; adjacent to the $tRNA^{Pro}$) and right (R; adjacent to the $tRNA^{Phe}$) domain, the presence of termination-associated sequences (TAS elements) in the L domain, and several conserved sequence blocks (CSBs) in the R domain which have been implicated in the initiation of H strand replication (Saccone, Attimonelli, and Sbisá 1987; Saccone, Pesole, and Sbisá 1991; Gemmell et al. 1996). Since no similar comparisons have been made involving New World monkeys, it is not clear to what extent the hominid features are also characteristic of other groups of primates. For example, there is an insertion sequence present in chimpanzee, gorilla, and human (Foran, Hixson, and Brown 1988; Saccone, Pesole, and Sbisá 1991) that is not present in other mammals; however, it is not known more specifically when this feature might have arisen in the evolution of the mammalian control region or whether it may have any conserved features representing a possible functional role unique to the group possessing it. Intraspecifc and interspecific mitochondrial DNA length variants are now widely documented from a diversity of animal groups (reviewed in Rand 1993), including Japanese monkeys (Hayasaka, Ishida, and Horai 1991), often due to variation in copy number of tandemly repeated sequences in the control region. It is not known whether this is a feature also characteristic of New World monkey mtDNAs.

The purpose of this study is to provide a mtDNA view of the evolutionary history of the marmosets concomitant with a molecular phylogenetic perspective of subspecies/species diversity in the group. This latter issue employs a consideration of the congruence or lack of congruence between purported morphological taxa and a molecular phylogenetic species concept (i.e., strongly supported monophyletic groups). An additional purpose is to provide a comparative examination of a New World monkey control region sequence with those of other vertebrates and in particular other mammals.

Materials and Methods

DNA sequences of the mtDNA control region were determined by direct sequencing of PCR-amplified fragments. Primers for amplification of this region were L15174 (5′-TGAGGACAAATATCATTCTGAGGGGC-3′), located in the cytochrome *b* gene, and H00651 (Kocher et al. 1989). A second PCR was performed on the resulting fragment using L15926 (Kocher et al. 1989) as an internal primer. This internal PCR eliminated any false priming products that occasionally arose in the original genomic DNA PCR. The DNA sequences were determined using dye terminator cycle sequencing reactions that were subsequently loaded on an Applied Biosystems 373A automatic sequencer, following the manufacturer's protocols. Additional sequencing primers were designed as necessary. All sequences were obtained on both strands. The scientific names of the taxa included in the analyses, as well as the geographic origins of the various individuals from each taxon, appear in table 1; their relative distributions are depicted in figure 1.

Table 1
The Origins and Identifications of the Various Marmosets for Which Control Region Sequence Was Determined

Taxonomic Identification	Origin
Callithrix argentata 21 (Car21)	Rio Anauera, Cameta, Pará
Callithrix argentata 23 (Car23)	Rio Anauera, Cameta, Pará
Callithrix argentata 98 (Car98)	Santarem, Pará
Callithrix aurita 120 (Cau120)	Iquaquecetuba–Suzano, São Paulo
Callithrix aurita 121 (Cau121)	Mogi das Cruzes, São Paulo
Callithrix geoffroyi 81 (Cge81)	Criadouro Barbuse Leal, Brasilia
Callithrix geoffroyi 83 (Cge83)	Criadouro Barbuse Leal, Brasilia
Callithrix geoffroyi 85 (Cge85)	Criadouro Barbuse Leal, Brasilia
Callithrix geoffroyi 87 (Cge87)	Criadouro Barbuse Leal, Brasilia
Callithrix humeralifer 29 (Chu29)	Rio Arapiuns, Santarem, Pará
Callithrix humeralifer 31 (Chu31)	Rio Arapiuns, Santarem, Pará
Callithrix jacchus 33 (Cja33)	Extremos, Rio Grande do Norte
Callithrix jacchus 43 (Cja43)	Extremos, Rio Grande do Norte
Callithrix kuhli 94 (Cku94)	Ilhéus, Bahia
Callithrix kuhli 95 (Cku95)	Una, Bahia
Callithrix kuhli 96 (Cku96)	Ilhéus, Bahia
Callithrix kuhli 122 (Cku122)	Una, Bahia
Callithrix kuhli 123 (Cku123)	Ilhéus, Bahia
Callithrix mauesi 09 (Cma09)	Municipality of Nova Olinda do Norte, Rio Abacaxis, Amazonas
Callithrix mauesi 10 (Cma10)	Municipality of Nova Olinda do Norte, Rio Abacaxis, Amazonas
Callithrix mauesi 11 (Cam11)	Municipality of Nova Olinda do Norte, Rio Abacaxis, Amazonas
Callithrix penicillata 89 (Cpe89)	Bioterio da Universidade de Brasilia
Callithrix penicillata 129 (Cpe129)	Bioterio da Universidade de Brasilia
Cebuella pygmaea 104 (Cpy104)	Centro Nacional de Primatas, Belem, Pará (unknown origin)
Cebuella pygmaea 105 (Cpy105)	Centro Nacional de Primatas, Belem, Pará (unknown origin)
Leontopithecus chrysomelas 108 (Lch108)	Centro de Primatologia do Rio de Janeiro (born in captivity)

Initial sequence alignments were constructed using the Clustal algorithm within the MEGALIGN program of the DNASTAR package and were subsequently perfected by eye using the eyeball sequence editor (Cabot and Beckenbach 1989). A few gaps were evident, and they were included in the analyses (i.e., the PHYLIP DNAPARS algorithm assumes that each nucleotide gap represents a single change). The best alignment was taken as the one that yielded the lowest parsimony score.

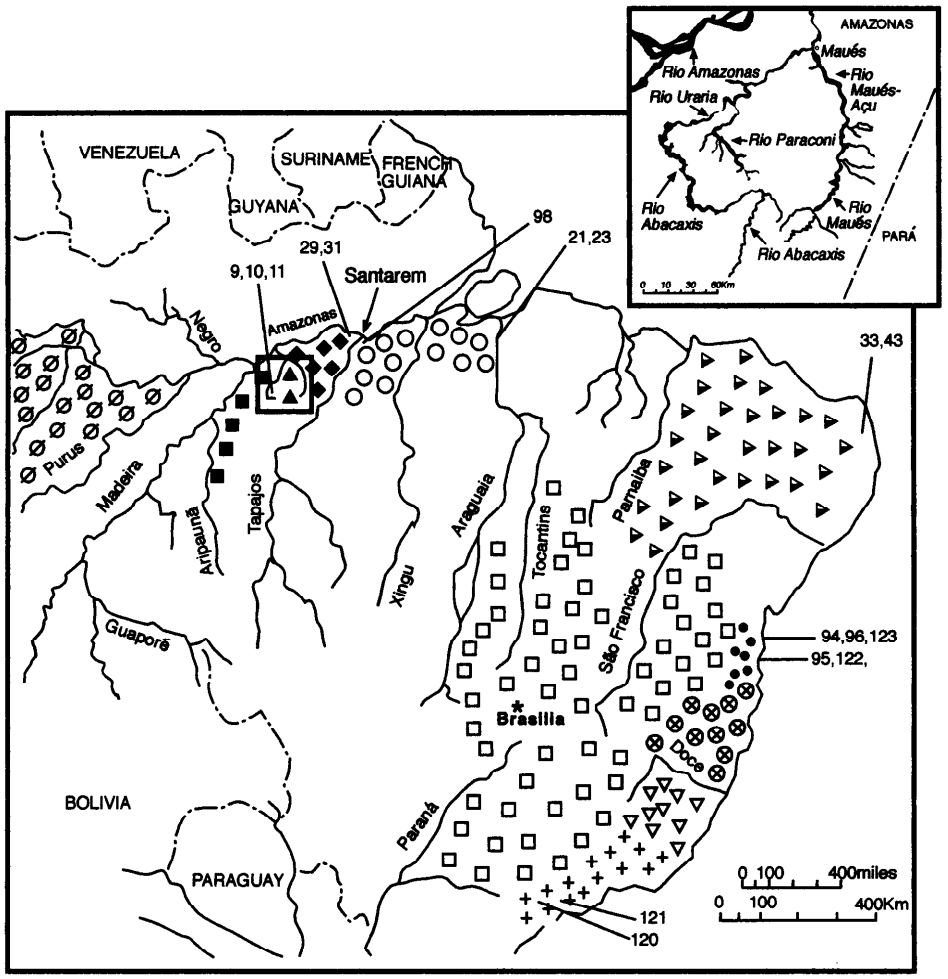

FIG. 1.—Distribution and sampling locations of the marmoset species and individuals discussed in this paper. Numbers refer to the marmoset sample designations listed in table 1. The inset in the top right-hand corner is a magnified view of the squared region from which *Callithrix mauesi* samples 9, 10, and 11 arise. Species are coded and their approximate distributions are represented with the following symbols: *Callithrix jacchus*, half-filled triangle; *Callithrix kuhli*, ●; *Callithrix penicillata*, □; *Callithrix aurita*, +; *Callithrix geoffroyi*, ⊗; *Callithrix humeralifer*, ♦; *Callithrix mauesi*, ▲; *Callithrix argentata*, ○; *Callithrix chrysoleuca*, ■; *Callithrix flaviceps*, ▽; *Cebuella pygmaea*, Ø.

Data were analyzed by neighbor-joining (NJ; Saitou and Nei 1987), maximum-parsimony (MP; Wagner parsimony), and maximum-likelihood (DNAML; Felsenstein 1981) methods using PHYLIP (Felsenstein 1993). The robustness of the phylogenetic hypotheses was tested by bootstrapping (Felsenstein 1985*a*). All bootstrap analyses of DNA sequence data involved 1,000 replications of the data. Neighbor-joining analyses of the DNA sequence data were performed using different distance calculations as input: Jukes and Cantor (1969); Kimura two-parameter (Kimura 1980) with 1.5, 2.0, 2.5, 3.0, 5.0, and 10.0 transition/transversion ratios; and maximum-likelihood with the same range of transition transition/transversion ratios (Felsenstein 1993). Maximum-parsimony analyses of the DNA sequence data were performed with the total data unweighted, and with transversions only. Most-parsimonious trees were determined by randomizing the input order 50 times. In several instances, significance tests were performed between user-defined or constrained trees and the maximum-parsimony tree; such tests were conducted using the method proposed by Templeton (1983) and Felsenstein (1985*b*) available in PHYLIP (Felsenstein 1993), in which the mean and variance of step differences between trees are evaluated. Maximum-likelihood analyses of the DNA sequence data were performed with the global branch-swapping option and expected transition/transversion ratios of 1.5, 2.0, 2.5, 3.0, and 5.0. All trees were rooted at the lion tamarin, *Leontopithecus chrysomelas*.

Results

Sequence Characteristics and Patterns of Substitution

The marmoset control region ranged from 1,081 to 1,142 bp in length. Complete control region sequences were obtained from *C. penicillata* (sample 89), *C. argentata* (sample 21), *C. mauesi* (sample 10), and *Cebuella* (sample 104); these four complete sequences (presented in fig. 2) were used in all the sequence characteristic comparisons that involved the 3′ quarter (most of the R domain) of the control region, because data for all other taxa were obtained solely from the remaining 75% of the D loop (i.e., L and central domains). This was because approximately 300 bp of the region adja-

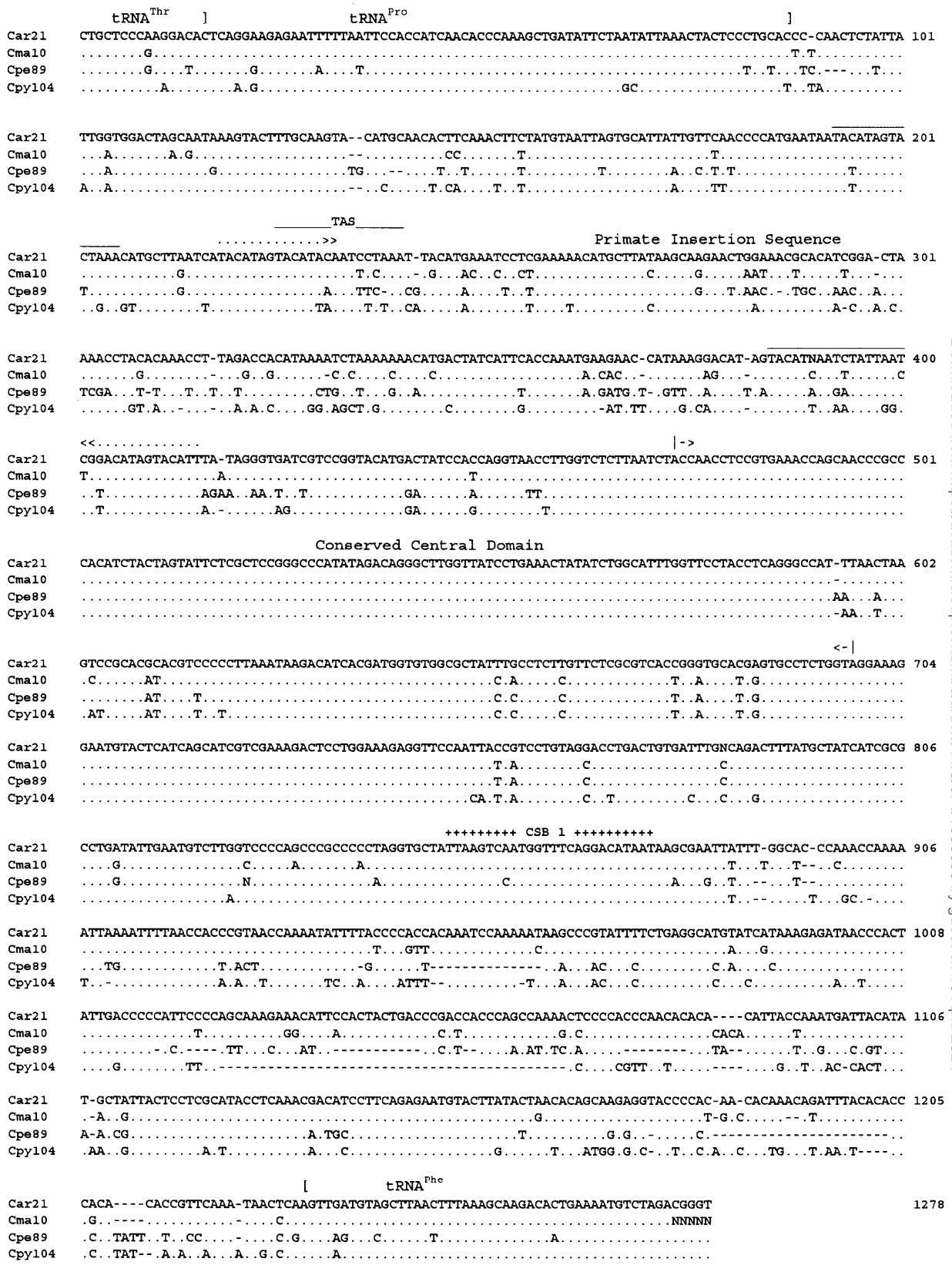

FIG. 2.—Alignment of four complete marmoset control region sequences. Car = *Callithrix argentata,* Cma = *Callithrix mauesi,* Cpe = *Callithrix penicillata,* Cpy = *Cebuella pygmaea.* The 3′ ends of the tRNA^{Thr} and tRNA^{Pro} are indicated by]; the 5′ end of the tRNA^{Phe} is indicated by [. The sequence orthologous to the human termination-associated sequence (TAS) is indicated as _TAS_; other sequences homologous to the TAS are indicated with a solid line. The direct repeats flanking the "primate insertion sequence" are indicated by dots followed by ≫ and ≪ for 5′ and 3′, respectively. The boundaries of the conserved central domain as demarcated by Saccone, Pesole, and Sbisá (1991) are indicated by |-> and <-| for 5′ and 3′, respectively. CSB 1 is indicated by a stretch of +'s. Gaps are indicated by a dash.

Table 2
Divergence Matrix for the Marmoset Control Region Sequences

	1	2	3	4	5	6	7	8	9	10	11	12
1. Cja33....		0.001	0.065	0.061	0.064	0.055	0.065	0.073	0.075	0.075	0.074	0.062
2. Cja43....	*		0.064	0.060	0.062	0.054	0.064	0.074	0.076	0.076	0.075	0.061
3. Cku94....	4.86	5.67		0.065	0.001	0.056	0.000	0.065	0.065	0.066	0.065	0.056
4. Cku95....	4.25	4.86	3.78		0.064	0.025	0.065	0.066	0.070	0.069	0.069	0.060
5. Cku96....	4.86	5.67	*	3.78		0.055	0.001	0.065	0.068	0.067	0.066	0.058
6. Cku122...	4.57	5.33	4.83	5.33	4.83		0.056	0.061	0.064	0.062	0.063	0.047
7. Cku123...	4.86	5.67	*	3.78	*	4.83		0.065	0.066	0.067	0.066	0.058
8. Cge81....	5.00	4.44	2.91	2.21	2.91	2.08	2.91		0.003	0.002	0.000	0.061
9. Cge83....	5.00	4.44	2.91	2.21	2.91	2.08	2.91	*		0.001	0.003	0.060
10. Cge85....	5.00	4.44	2.91	2.21	2.91	2.08	2.91	*	*		0.002	0.060
11. Cge87....	5.00	4.44	2.91	2.21	2.91	2.08	2.91	*	*	*		0.061
12. Cpe89....	4.37	5.00	2.78	3.00	2.78	2.56	2.78	2.80	2.70	2.70	2.80	
13. Cpe129...	4.17	5.00	6.40	4.75	6.40	7.40	6.40	3.42	3.42	3.42	3.42	4.50
14. Cau120...	4.92	4.57	3.19	4.38	3.19	4.17	3.19	2.63	2.63	2.63	2.63	3.60
15. Cau121...	4.43	4.13	2.88	3.93	2.88	3.69	2.88	2.40	2.40	2.40	2.40	3.25
16. Car21....	2.10	2.03	2.04	1.97	2.04	1.80	2.04	1.68	1.68	1.68	1.68	1.61
17. Car23....	2.10	2.03	2.04	1.97	2.04	1.80	2.04	1.68	1.68	1.68	1.68	1.61
18. Car98....	2.14	2.07	2.07	1.97	2.07	1.93	2.07	1.68	1.68	1.68	1.68	1.67
19. Cma09....	1.73	1.69	1.61	1.61	1.61	1.51	1.61	1.31	1.31	1.31	1.31	1.28
20. Cma10....	1.73	1.69	1.61	1.61	1.61	1.51	1.61	1.31	1.31	1.31	1.31	1.28
21. Cma11....	1.59	1.54	1.67	1.58	1.67	1.43	1.67	1.19	1.19	1.19	1.19	1.39
22. Chu29....	1.66	1.61	1.73	1.59	1.73	1.44	1.73	1.35	1.35	1.35	1.35	1.49
23. Chu31....	1.64	1.59	1.72	1.51	1.72	1.47	1.72	1.37	1.37	1.37	1.37	1.49
24. Cpy104...	2.03	2.11	2.29	1.97	2.29	1.90	2.29	1.67	1.67	1.67	1.67	1.73
25. Cpy105...	1.52	1.56	1.54	1.50	1.54	1.49	1.54	1.37	1.37	1.37	1.37	1.43
26. Lch108...	1.26	1.29	1.23	1.24	1.23	1.25	1.23	1.13	1.11	1.11	1.13	1.22

NOTE.—Jukes and Cantor divergences are above the diagonal and transition/transversion ratios are below the diagonal. * refers to transition/transversion with transversions equal to 0.

cent to the 12S gene could not be unambiguously aligned with the outgroup and therefore were eliminated from the phylogenetic sequence comparisons. The resulting phylogenetic sequence alignment was 920 bp for all individuals, which included 14 bp of the 3′ end of the $tRNA^{Thr}$ gene, the $tRNA^{Pro}$ gene, and 830 bp of the subsequent control region.

The nucleotide sequence composition of the marmoset control region was 33% A, 27% T, 25% C, and 15% G (average of the four complete sequences). Guanine was particularly underrepresented in the L and R domains, but exhibited a significant increase in the middle third, accompanied by a comparable drop in adenine for this same region. Most of the sequence homology with human (outside the tRNAs) occurred in the 5′ 550 bp of the marmoset control region and, in particular, between 16319 and 1 of the published human sequence (Anderson et al. 1981). In this middle 250-bp region, there were several highly conserved sequence blocks, some of them as long as 30 bp, with an overall marmoset/human sequence divergence of approximately 30%. After bp 1 in human (representing the 3′ third of the control region), the human/marmoset homology dropped off considerably, with interspecific marmoset homology remaining high for another 225 bp, until the region known as CSB 1 (at approximately bp 765 of the marmoset control region), after which even the marmoset sequences were considerably more divergent. The two other conserved sequence blocks, known as CSBs 2 and 3, characteristic of several other mammalian control region sequences, were not clearly evident in these marmosets. There was also no evidence of any tandem repeat motifs similar to those that have been identified in a wide range of other mammalian taxa (reviewed in Rand 1993) and which have been associated with mtDNA heteroplasmy.

An "insertion" sequence of approximately 136 nt has been identified for some Old World primates (Saccone, Pesole, and Sbisá 1991) within the L domain, and in humans it is flanked by the following 11 nt direct repeat: 5′-TAGTACATAAA-3′. In marmosets, both these perfect repeats can be identified in roughly orthologous position to that in human (fig. 2), and the repeats had, for the vast majority of the 26 individuals, identical sequence to that for human. The marmoset "insertion" sequence is about 156 nt long, and shows no significant homology to human with the exception of the following identical stretch of 16 nt, occurring about 30 nt downstream of the 5′ direct repeat: 5′-CATGCTTACAAGCAAG-3′. The termination-associated sequence (TAS) in the L domain identified in humans as the putative signal for termination of D-loop synthesis was also present in the marmosets and had a high degree of homology to the human TAS (human: 5′-TACATAAAAACCCAAT-3′; marmoset consensus: 5′-TACATAAAATCCTAAC-3′; differing by only two transitions and one transversion). The R domain exhibited greater interspecies sequence divergence than did the L domain (mean sequence divergence for L and R, 0.207 and 0.244, respectively), particularly so for *Cebuella*; this R domain also had a greater number of indels, at least in the four individuals for which complete sequence was determined.

Table 2
Extended

13	14	15	16	17	18	19	20	21	22	23	24	25	26
0.054	0.113	0.112	0.132	0.134	0.136	0.143	0.143	0.131	0.143	0.132	0.133	0.172	0.237
0.053	0.114	0.114	0.134	0.135	0.138	0.144	0.144	0.132	0.145	0.134	0.132	0.171	0.237
0.063	0.105	0.103	0.131	0.131	0.135	0.138	0.138	0.134	0.147	0.134	0.142	0.164	0.227
0.070	0.102	0.101	0.135	0.136	0.138	0.140	0.140	0.134	0.146	0.130	0.136	0.170	0.228
0.064	0.104	0.103	0.132	0.132	0.136	0.136	0.136	0.134	0.148	0.135	0.143	0.165	0.229
0.069	0.088	0.086	0.122	0.123	0.128	0.128	0.128	0.119	0.131	0.120	0.128	0.164	0.220
0.063	0.105	0.103	0.131	0.131	0.135	0.137	0.137	0.134	0.146	0.134	0.142	0.165	0.228
0.083	0.105	0.105	0.124	0.125	0.126	0.128	0.128	0.118	0.135	0.124	0.133	0.169	0.233
0.084	0.108	0.106	0.123	0.123	0.126	0.131	0.131	0.119	0.133	0.125	0.134	0.170	0.230
0.085	0.106	0.106	0.125	0.126	0.127	0.129	0.129	0.119	0.135	0.124	0.134	0.170	0.230
0.086	0.104	0.105	0.125	0.126	0.127	0.130	0.130	0.119	0.136	0.125	0.133	0.168	0.229
0.070	0.099	0.098	0.125	0.126	0.128	0.120	0.120	0.120	0.135	0.123	0.135	0.164	0.218
	0.104	0.104	0.126	0.127	0.130	0.136	0.136	0.131	0.137	0.130	0.130	0.167	0.230
3.47		0.003	0.143	0.144	0.147	0.135	0.135	0.134	0.142	0.132	0.150	0.184	0.239
3.25	2.00		0.140	0.141	0.144	0.139	0.139	0.133	0.140	0.130	0.146	0.184	0.236
1.97	2.30	2.16		0.000	0.009	0.070	0.070	0.065	0.073	0.060	0.110	0.143	0.238
1.97	2.30	2.16	*		0.009	0.071	0.071	0.065	0.073	0.060	0.110	0.144	0.236
2.00	2.37	2.23	*	*		0.074	0.074	0.068	0.076	0.065	0.112	0.145	0.239
1.59	1.85	1.85	6.14	6.14	6.43		0.000	0.034	0.043	0.039	0.123	0.144	0.247
1.59	1.85	1.85	6.14	6.14	6.43	*		0.034	0.043	0.039	0.123	0.144	0.247
1.56	1.94	1.82	6.14	6.14	6.43	11.5	11.5		0.025	0.021	0.114	0.137	0.237
1.57	1.85	1.74	5.75	5.75	6.25	9.33	9.33	4.67		0.023	0.127	0.151	0.247
1.64	1.91	1.79	6.50	6.50	7.17	28.0	28.0	15.0	6.50		0.119	0.150	0.237
1.74	1.83	1.73	1.92	1.92	1.96	1.74	1.74	1.64	1.78	1.83		0.117	0.248
1.43	1.69	1.61	1.57	1.57	1.62	1.40	1.40	1.35	1.61	1.59	1.93		0.233
1.37	1.34	1.29	1.25	1.25	1.25	1.19	1.19	1.21	1.19	1.21	1.06	1.13	

In the resulting phylogenetic sequence alignment (all 26 individuals represented over 920 bp), a total of 359 positions were variable, 248 of these being phylogenetically informative. The majority (i.e., 66%) of the variable positions were located between base pairs 84 and 480 of this alignment, corresponding to the L domain of the control region (fig. 2). A hypervariable region between base pairs 237 and 406 of this alignment (corresponding to the primate insertion sequence) contained one third of the variable positions in only 18% of the total sequence, and, conversely, a highly conserved block of sequence between 484 and 620 (corresponding to most of the conserved central domain) contained only 2.5% of the variable positions in 15% of the sequence. A total of 49 indels of 1–5 nt in length (the vast majority being 1–3 nt in length) were apparent, with a single longer deletion of 15 bp in *Cebuella pygmaea* 105, in a surprisingly otherwise moderately conserved location (position 762).

Sequence divergence amongst the various *Callithrix* individuals ranged from 0.000 to 0.148 (table 2). Divergence figures between *Cebuella* and the Amazonian *Callithrix* species were less than those in many of the congeneric *Callithrix* comparisons. Sequence divergence between the two *Cebuella* individuals (0.117) was greater than that between many of the purported species of marmosets. Marmoset/lion tamarin divergence ranged from 0.218 to 0.248. Several individuals within purported taxa showed no sequence divergence. Transition/transversion ratios ranged from 1.11 to 28.0 (table 2). The highest transition/transversion ratios involved comparisons within the Amazonian *Callithrix* species. Several conspecific comparisons exhibited only transitions. Transitions accumulated much more rapidly than transversions, up to a sequence divergence of approximately 9%–10%, after which transversions continued to accumulate to about 15%, with little or no increase in transitions. At about 20% sequence divergence, there was a further increase in the number of transitions, reflecting several changes in conserved blocks of control region sequence in comparisons involving the outgroup. Using only those positions for which it could be unambiguously determined, there was a slight bias in the transition pathways, with A to G representing about 35% of the changes, G to A 16%, C to T 21%, and T to C 28%.

Phylogenetics

All three bootstrap analyses (parsimony, transversion parsimony, and neighbor joining) exhibited a high degree of congruence in their resulting topologies and clade strength. The principal features of the consensus topology (fig. 3) included a split between the Amazonian and Atlantic forest marmosets, with *Cebuella* joining convincingly at the base of the Amazonian clade. *Callithrix aurita* was the first to branch off the Atlantic group. A large number of clades were supported by 100% (or very nearly) in all three analyses. Several of the purported morphological species formed strongly supported monophyletic groups in all three analyses, including *C. aurita, C. geoffroyi, Cebuella, C. jacchus,* and *C. argentata.* The lowest clade strength occurred in the area of the tree depicting the relative relationships between *C. kuhli, C. jacchus,* and *C. penicillata.* Morphological species that did not form monophyletic groups included *C. mauesi, C. penicillata,* and *C. kuhli.* Although *C. mauesi* was not monophyletic it did form a strongly supported clade with *C. humeralifer* (bootstrap support of 100% for all three analyses). The five

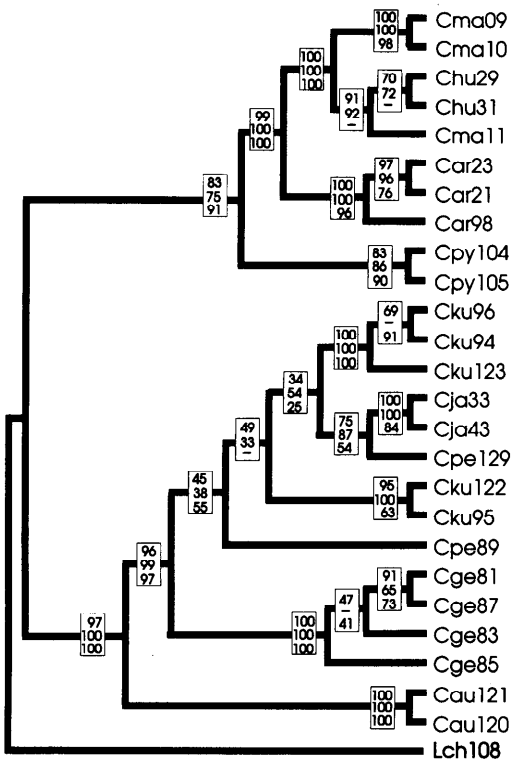

FIG. 3.—Majority consensus bootstrap trees of parsimony, neighbor-joining, and transversion parsimony analyses of control region sequences. Numbers indicate percentage bootstrap figures from 1,000 replications of the data. The top value corresponds to unweighted parsimony, the middle figure corresponds to neighbor joining, and the bottom figure corresponds to transversion parsimony. The neighbor-joining figures are based on a Kimura two-parameter divergence matrix with a 2.0 transition/transversion ratio, a figure which fairly closely approximates the mean for the data set as a whole. A dash in one of these three positions indicates that that particular analysis did not agree with the indicated majority topology for that node. See table 1 for species abbreviations.

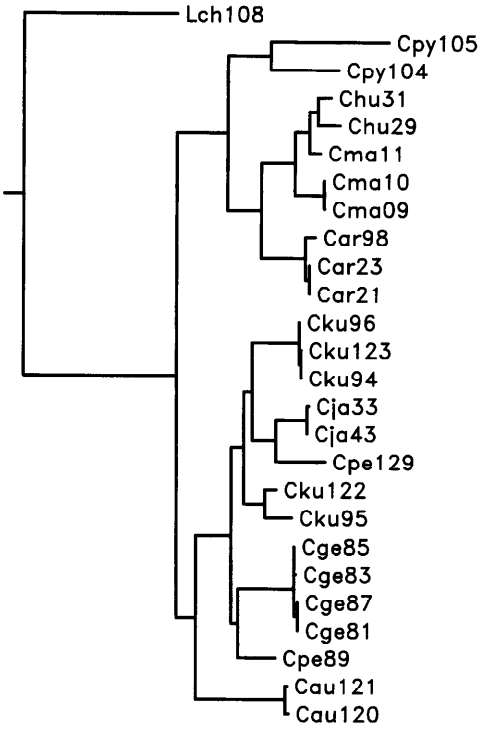

FIG. 4.—Maximum-likelihood tree resulting from analysis of the control region sequences. Branch lengths are drawn proportional to the amount of sequence change. See table 1 for species abbreviations.

C. kuhli individuals formed two strongly supported clades interrupted by a grouping of *C. jacchus* and *C. penicillata.* Different distance-based methods and transition/transverion ratios in the NJ analyses resulted in only very trivial differences in bootstrap values.

The maximum-likelihood analyses (fig. 4) agreed very closely with the bootstrap topologies; different transition/transversion ratios had only a minimal effect on branch length with no effect on topology. Several monophyletic species groupings including *C. aurita, C. geoffroyi, C. jacchus,* and *C. argentata* exhibited very short branch lengths separating individuals within the species, but had long branch lengths separating that clade from the adjacent taxa (fig. 4).

A large number of unique synapomorphies defining various groupings were evident in a site-by-site examination of the sequence alignment. For example, 19 such positions were identified in support of a *Cebuella*/Amazonian marmoset clade, in which all eight Amazonian marmosets and the two pygmy marmosets shared the same nucleotide, that was different from all 18 other sequences in the alignment. A further seven positions were identified in which only 1 of the 10 *Cebuella*/Amazonian marmoset taxa differed, with all others sharing a unique synapomorphy. Of these 26 synapomorphies, 12 were transversions, 6 were transitions, and 8 were insertions or deletions. A tree that had a monophyletic *Cebuella* on their own branch of the tree coming off directly after the outgroup, followed by a split defining the respective monophylies of the Amazonian and Atlantic marmosets, added 16 steps to the MP score and was judged significantly different from that MP tree.

Several other clades were similarly well supported by unique synapomorphies. The Atlantic forest clade was supported by 14 such positions (4 transitions, 2 transversions, 8 indels), *C. geoffroyi* by 10 (6 transitions and 4 transversions), *C. argentata* by 9 (8 transitions and 1 transversion), *C. mauesi*/*C. humeralifer* by 5 (2 transitions, 3 transversions), and *C. aurita* by 20 (11 transitions, 6 transversions, 3 indels).

Constraining the tree to support a monophyletic *C. mauesi* added six substitutions and was judged significantly different from the MP tree. A similar approach defining a monophyletic *C. penicillata* and *C. kuhli* added 12 and 1 steps respectively; the monophyletic *C. penicillata* tree was significantly different from the MP tree, but the *C. kuhli* arrangement was not.

Discussion

D-Loop Sequence Characteristics

The basic sequence features of the marmoset control region were roughly similar to those described for other mammals, with the central highly conserved domain flanked by the considerably more variable L and R domains (Saccone, Pesole, and Sbisá 1991). However,

not all of the CSBs characteristic of the R domain in several other mammals were identifiable in the complete marmoset sequences. CSB 1 was present in all marmosets, but the position and/or presence of CSBs 2 and 3 was not clearly recognizable. CSB 1 appears to be the more universally conserved sequence, present in a wide range of mammals (Saccone, Pesole, and Sbisá 1991); CSBs 2 and 3 are not clearly identifiable in cow or sheep (Saccone, Pesole, and Sbisá 1991; Wood and Phua 1996), with a hybrid CSB 2/3 suggested for these two artiodactyls (Wood and Phua 1996). CSB 3 appears to be absent from dolphin (Saccone, Pesole, and Sbisá 1991) and from platypus (Gemmell et al. 1996). All three CSBs have been suggested to act as processing signals for the enzymes involved in the generation of RNA primers for heavy-strand replication, and Bennet and Clayton (1990) have identified in human and mouse a site-specific endoribonuclease which recognizes CSBs 2 and 3. Sequence data of ours indicate that CSBs 2 and 3 are also not clearly recognizable in *Alouatta belzebul* (unpublished data), another New World monkey from a different subfamily. A relatively conserved sequence in the marmosets just upstream of CSB 1 (21 bp upstream) showed some similarity to the human CSB 2 (human: 5′-CCAAACCCCCCCTCCCCC-3′; marmoset: 5′-CCAR−−−−−CCCGCCCCC-3′). This sequence is also conserved in *A. belzebul* (unpublished data). If this is the same functional domain, it places the three CSBs in a different relative order than that for other mammals; this is not, however, without precedent in other vertebrates (see, e.g., Brown, Beckenbach, and Smith 1993). In *C. argentata* and *C. mauesi,* another region 136 bp downstream of CSB 1 showed some homology to CSB 2; however, this was not the case for *Cebuella* and *C. penicillata,* with at least part of this putative element deleted in both of these species (fig. 1). *Alouatta belzebul* for this same region also has a putative CSB 2 element, but again with part of it missing (i.e., *A. belzebul* sequence: CCCCCCTACC). Still another region, 187 bp downstream of CSB 1, showed quite close homology to CSB 2 in *C. argentata* and *C. mauesi* (Car: CCAAAACTCCCCACCC; Cma: CCAAAGCCCCCC-ACCC), but *C. penicillata, Cebuella,* and *A. belzebul* for this same region exhibited no such homology (fig. 1). Two regions downstream of CSB 1 (19 bp and 70 bp, respectively) showed weak homology to CSB 3, but neither was particularly conserved across marmoset species (fig. 1). We would suggest that whatever the role of CSBs 2 and 3, there are some salient features that differ in New World monkeys based on the extensive modification or, possibly, complete absence of these sequence features in the Callitrichinae and in howler monkey.

The TAS identified in humans as the putative element signaling the end of D-loop synthesis was very well conserved in marmosets and forms part of the 5′ direct repeat which flanks the "primate insertion" sequence. The TACAT pentanucleotide which forms part of this TAS element as well as those of other mammals (Gemmell et al. 1996) is repeated throughout the L domain of the control region in marmosets, is conspicuously absent from the middle conserved block, and is only rarely present in the R domain. This is similar to the situation in other mammals (Saccone, Pesole, and Sbisá 1991). These sequences have been shown to be associated with stable secondary structures in a range of mammals (Wilkinson and Chapman 1991; Saccone, Pesole, and Sbisá 1991; Wood and Phua 1996; Stewart and Baker 1994) and have been implicated as recognition sites for the termination of H-strand synthesis. It is not clear whether it is solely the primary sequence of the TAS element which acts as the signal to stop synthesis or whether its association with secondary structures may also play a role (Stewart and Baker 1994). The complete TAS element, as well as the TACAT pentanucleotide repeats within these marmosets, was generally (but not always) part of the stable secondary structures that are possible in this L domain (determined using the Zuker-Stiegler algorithm in DNASIS).

The insertion sequence in the L domain typical of some Old World primates was also evident in the marmosets, defined by the same conserved direct repeats present in human. The approximately 150-bp insertion sequence did not show any significant homology to human, except for a 16-bp region just inside the 5′ direct repeat. The presence of the direct repeats and of the conserved block of sequence inside the proposed primate insertion sequence confirms that this insertion took place prior to the evolutionary splitting event that gave rise to New and Old World monkeys. The conserved block of sequence inside the direct repeat suggests some possible functional role, perhaps unique to primates. This insertion sequence is otherwise a hypervariable region that, along with the $tRNA^{Phe}$ end of the D-loop, should serve as a target area for attempts at genetic discrimination of closely related New World primates.

Similar to results reported for other animal mtDNA control regions (e.g., rainbow fishes [Zhu et al. 1994] and *Drosophila* [Desalle et al. 1987]), marmoset transitions accumulate much more rapidly than transversions (up to about 4% sequence divergence virtually all changes are transitions) and appear to saturate at about 9%–10% total sequence divergence after which transversions accumulate. This overall pattern has been attributed to high A+T content, and the composition of these marmoset control regions would support this view. The A+T content of the marmoset control region, at 60%, is higher than that in hominid primates, but slightly lower than figures for various other mammals, which, in turn, are lower than those reported for most other vertebrates (Saccone, Attimonelli, and Sbisá 1987; Saccone, Pesole, and Sbisá 1991; Zhu et al. 1994).

Phylogenetics and Conservation Perspectives

The various methods of analysis were highly congruent in their overall topologies. The first feature, already widely recognized, is that there was an ancient evolutionary splitting event that gave rise to the Atlantic and Amazonian marmosets. An important distinctive feature of this event, suggested by these control region data, is that the pygmy marmoset lineage arose after the Amazonian/Atlantic split and not as the marmoset sister

group, which is the widely held traditional view based on immunological data (Cronin and Sarich 1978), dental ontogeny (Byrd 1981), cytotaxonomic criteria (Boer 1974), and features of the postcranium (Ford 1980). All of these different types of data have suggested a close evolutionary relationship of pygmy marmosets with marmosets. Rosenberger and Coimbra-Filho (1984), using craniodental features, suggested that pygmy marmosets are in fact so closely related to marmosets that they should be included in the same genus. Snowdon (1993) examined vocal characteristics of callitrichids and found pygmy marmosets and *C. argentata* to have remarkably similar long calls and contact trills. Their resulting phylogenetic arrangement based on these call characteristics was highly similar to that of Rosenberger and Coimbra-Filho. Sequence data from intron 1 of the IRBP gene, in agreement with our D-loop sequence, also supports *Cebuella* inside the *Callithrix* clade, although with these particular nuclear data it is not clear whether pygmy marmosets have a closer affinity with the Atlantic or the Amazonian clade (M. Goodman, personal communication). Our suggestion of a phylogenetic arrangement that has *Cebuella* at the base of the Amazonian clade concurs with the geographic distribution of this species, which is in the upper Amazon basin, west of the Rio Purus in Brazil (Rylands, Coimbra-Filho, and Mittermeier 1993). We would suggest that additional characteristics of the biology of pygmy marmosets be reevaluated in light of this phylogenetic proposal.

Because of the time-dependent accumulation of genetic differences in the absence of gene flow, species and subspecies will exhibit phylogenetic partitioning, which, in turn, dictates that most species and subspecies will be monophyletic, a view referred to as the "phylogenetic species concept" (Cracraft 1983). A partial solution to the problem of knowing where to draw the "monophyletic line" in any application of this concept is to have sequence data from a number of individuals from a number of different populations (ideally proximally and distally located) of each of the purported taxa, and to interpret the phylogenetic trees in light of the resulting branch lengths and bootstrap support.

In the case of the present marmoset data there are several purported taxa which do form strongly supported monophyletic clusters, which also have long branch lengths separating them from the adjacent group. These include *C. aurita, C. geoffroyi, Cebuella,* and *C. argentata. Callithrix mauesi* and *C. humeralifer* form a strongly supported clade, with a relatively long branch leading to that node. One of the *mauesi* (Cma11) is more closely related to the two *C. humeralifer* based both on sequence divergence and high bootstrap support, suggesting the existence of naturally occurring hybrids of *humeralifer* and *mauesi.* The distribution of *C. mauesi* is parapatric with *C. humeralifer* and *Callithrix chrysoleuca* (Mittermeier, Schwarz, and Ayres 1992) and occurs well within the geographic distribution for *C. humeralifer* described by Hershkovitz (1975). Mittermeier, Schwarz, and Ayres (1992) suggest that, among the Brazilian marmosets, *C. mauesi* is most closely related to *C. humeralifer* but that it is readily distinguishable by differences in the shape of the ear tufts and the overall dark color. Our D-loop sequence data do not corroborate this view of *C. mauesi* as a distinct taxon. Instead, our data suggest the possibility that *C. humeralifer* and *C. mauesi* may not be distinct evolutionary entities. We emphasize that this suggestion is a tentative one because of the still-limited sampling in terms of both loci and individuals. Also important to realize is the possibility that *C. humeralifer* and *C. mauesi* are valid species, but the gene tree does not support that view because of a polymorphism in the ancestral population.

It remains a possibility, of course, that there is a hybrid zone on the contiguous borders of the *mauesi* distribution and that individuals in the more internal regions of the proposed distribution may more clearly represent a distinct lineage. Our samples, however, come from the border between *C. chrysoleuca* and *C. mauesi* not from the border with *C. humeralifer.* The proposed distribution for *C. mauesi* is west of the Rio Maués, east of the Rio Urariá and north and east of the Rio Abacaxis (Mittermeier, Schwarz, and Ayres 1992). Our samples were collected along the east bank of the Rio Abacaxis, with *C. chrysoleuca* occurring on the west side of the river (see fig. 1). The closest *humeralifer,* according to Mittermeier, Schwarz, and Ayres' estimates of the distributions, is about 150 km east, across an area that they indicate is occupied by *C. mauesi.* The *humeralifer* we have sample for were collected around Santarem, which is about 400 km east of the Rio Abacaxis. Santarem is the point where the distributions of *C. argentata* and *C. humeralifer* become contiguous. One of our *C. argentata* samples (98) comes from Santarem, and it clearly groups with the other two *C. argentata* (21, 23) which were collected about 500 km east (see fig. 1). These sampling points concomitant with the tree topology suggest a clear genetic distinction between *C. argentata* and *C. humeralifer,* but not so for *C. mauesi/C. humeralifer.* We offer the hypothesis that the marmosets west of Santarem, immediately south of the Amazon River, to the River Abacaxis (and possibly further west to the inclusion of the proposed distribution for *C. chrysoleuca*) may in fact represent a relatively continuous genotype. Similarly, we suggest that the marmosets east of Santarem may all belong to a *C. argentata* genotype, in agreement with the already-proposed distribution for that species (e.g., Mittermeier, Schwarz, and Ayres 1992).

In regard to the Atlantic forest group, there are, as in the Amazonian clade, examples of purported taxa which are not monophyletic. One such example of this is *C. kuhli,* which forms a mixed group with *C. penicillata* and *C. jacchus. Callithrix kuhli* comes from the Atlantic forests of southern Bahia. Hershkovitz (1975) and Vivo (1991) regard the marmosets from this region as hybrids of *C. penicillata* and *C. geoffroyi.* Rylands, Coimbra-Filho, and Mittermeier (1993) disagree and regard them as a distinct species. Our trees certainly do not come down in strong support of a monophyletic *C. kuhli,* although their paraphyly is also only weakly supported (i.e., a monophyletic *C. kuhli* adds only one substitution to the MP tree). This absence of distinct mono-

phyly occurs despite the fact that all five animals come from the same relatively small area (table 1 and fig. 1); the greatest distance between our sample collection sites for this species was about 100 km. We interpret these results as not providing any convincing indication that *C. kuhli* should be regarded as a distinct taxon since its monophyly cannot be clearly demonstrated. If it were a hybrid form, then it would be closely related to one of the parental types, which it is; if it were clearly a distinct species, then the members would form a strongly supported monophyletic group, which they do not. Coimbra-Filho (1991) argued that the destruction of habitat over large areas of the Atlantic forest since the European discovery of Brazil in 1500, along with frequent and repeated introductions of *C. jacchus* and *C. penicillata,* has resulted in a confused picture of hybrids between these species and possibly also between *C. penicillata* and *C. kuhli.* Our trees are certainly consistent with this view regarding these three purported species.

In contrast to the confused picture regarding the *C. penicillata, C. kuhli, C. jacchus* group, *C. geoffroyi* form a very strongly supported clade, suggesting that they might more confidently be regarded as a distinct evolutionary entity; however, all animals did come from the same private breeding farm, which suggests the possibility that they were simply members of the same population. Similarly convincing support was apparent for *C. aurita*; however, only two individuals were represented (albeit from different places), and we did not have samples of the species for which there is some argument about hybridization (*C. flaviceps*). The data do confidently indicate that *C. aurita* represents an early branch of the Atlantic forest group.

Conclusions

The early parts of the next century are going to be a critical period for the conservation of biodiversity throughout the world, particularly in richly biodiverse countries such as Brazil. Approximately one quarter of the world's living primate species are found in Brazil, of which about 45% are endemic (Mittermeier, Schwarz, and Ayres 1992). An important feature of the conservation effort is going to be the preservation of species genetic diversity, and molecular genetic data should be of considerable assistance in this regard. As Avise (1989, 1994) has pointed out, one area in which molecular evolutionary genetics can be of some assistance is in the identification of "taxonomic mistakes" with their following two possible ramifications: (1) the recognition of groups which actually exhibit very little evolutionary differentiation and (2) the lack of recognition of forms which are phylogenetically distinct. In both such cases, conservation efforts can be misdirected with regard to the protection of biological diversity. In the present example, claims of *C. mauesi* and *C. humeralifer* as distinct taxa are not corroborated by our molecular genetic data. We emphasize, however, that we regard these data as a first tentative step toward an attempt at clarification of the phylogenetic diversity within these Brazilian primates and that a larger representation of individuals from various geographic locations is necessary before any more firm conclusions should be drawn. Similarly, our data do not support a clear taxonomic distinction between *C. kuhli, C. penicillata,* and possibly *C. jacchus,* which, again, we regard as a tentative proposal but one that needs to be further explored because of its possibly important ramifications in regard to the future conservation management of these endangered primates.

Sequence Availability

The nucleotide sequence data reported in this paper appear in GenBank under the following accession numbers: Cja33, U86526; Cja43, U88840; Cku94, U88841; Cku95, U88842; Cku96, U88843; Cku122, U88991; Cku123, U88992; Cge81, U88993; Cge83, U88994; Cge85, U88995; Cge87, U88996; Cpe129, U88997; Car23, U88998; Car98, U88999; Cau120, U89000; Cau121, U89001; Cma09, U89002; Cma11, U89003; Cpe89, U89004; Car21, U89005; Cma10, U89006; Chu29, U89007; Chu31, U89008; Cpy105, U89009; Cpy104, U89010; Lch108, U89011.

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