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A Reexamination of the Phylogenetic Position of *Callimico* (Primates) Incorporating New Mitochondrial DNA Sequence Data

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Abstract. The New World monkeys are divided into two main groups, Callitrichidae and Cebidae. *Callimico goeldii* shares traits with both the Cebidae and the Callitrichidae. Recent morphological phyletic studies generally place *Callimico* as the most basal member of the Callitrichidae. In contrast, genetic studies (immunological, restriction fragment, and sequence data) have consistently placed *Callimico* somewhere within the Callitrichidae, not basal to this clade. A DNA sequence data set from the terminal 236 codons of the mitochondrial ND4 gene and the $tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$ genes was generated to clarify the position of *Callimico.* The sequences of 887 base pairs were analyzed by maximum-parsimony, neighbor-joining, and maximum-likelihood methods. The results of these various methods are generally congruent and place *Callimico* within the Callitrichidae between the marmosets (*Callithrix* and *Cebuella*) and the tamarins (*Saguinus* and *Leontopithecus*). Combined analyses of all suitable nuclear and mitochondrial gene sequences confirm the position of *Callimico* between the marmosets and the tamarins. As available molecular evidence indicates that *Callimico* is more closely related to the marmosets than to the tamarins, a reconsideration of the morphological evidence in light of the consensus tree from DNA sequence analyses is warranted. The marmosets and tamarins share four morphological characters (loss of the third molar, loss of the hypocone, reduced body size, reproductive twinning). Dwarfism may have evolved repeatedly among the Callitrichidae. It is well-known that the loss of a character can occur many times independently. The reproduction of marmosets and tamarins is extremely specialized and it is difficult to imagine that this complex and unique twinning system evolved separately in marmosets and tamarins. However, it is possible that a secondary reversal to single offspring took place in *Callimico.*

Key words: *Callimico goeldii* — Goeldi's monkey — Callitrichidae — Primates — Phylogeny — Morphology — Sequencing — Mitochondrial DNA — ND4 gene — tRNA genes

Introduction

The New World monkeys (Platyrrhini) have traditionally been divided into two families, the Callitrichidae and the Cebidae. The marmosets (*Callithrix* and *Cebuella*) and tamarins (*Saguinus* and *Leontopithecus*) are distinguished from the Cebidae by their small size [range of species means: 110–560 g for marmosets and tamarins and 730 g–13 kg for Cebidae (Martin 1992)], having claws rather than nails on all digits except the big toe, and the presence of two molar teeth instead of three in each toothrow. Despite having a single-chambered (simplex) uterus and a single pair of teats (features usually found in mammals characterized by single births), mar-

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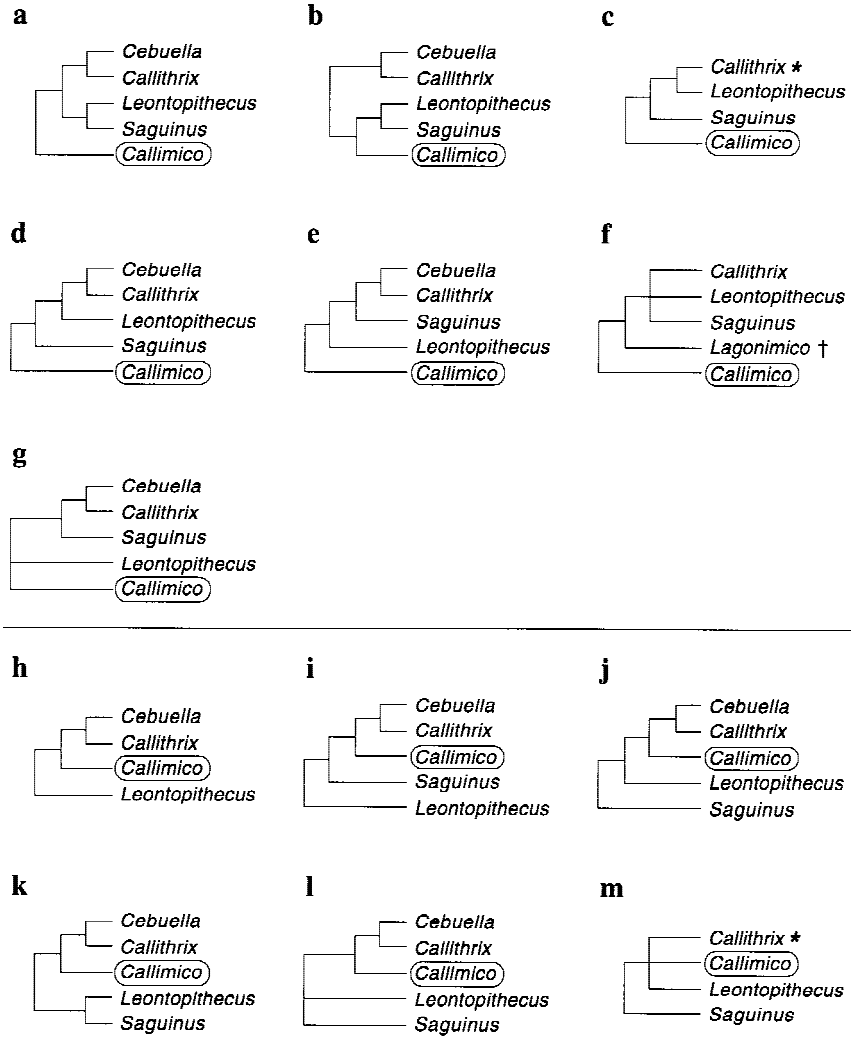

Fig. 1. Phylogenetic trees based on **(a, c)** various morphological features (Hill 1959; Rosenberger and Coimbra-Filho 1984; Ford 1986); **(b)** dental eruption (Byrd 1981); **(d)** vocalization (Snowdon 1993); **(e)** dental characteristics (Kay 1990); **(f)** morphological features including the fossil specimen *Lagonimico* (†) (Kay 1994); **(g)** urinary estrogen excretion (Pryce et al. 1995); **(h)** chromosomes (Canavez et al. 1996); **(i)** immunological data (Cronin and Sarich 1978); **(j)** LINE-1 restriction fragment data (Seuánez et al. 1988); **(k)** 1,928 bases of the ϵ-globin gene sequence (Schneider et al. 1993); **(l)** 542 bases of the mitochondrial 16S ribosomal gene sequence (Horovitz and Meyer 1995); **(m)** 1,843 bases of the IRBP gene and 1,928 bases of the ϵ-globin gene sequence (Barroso 1995) and additional sequences for the von Willebrand factor gene (Schneider et al. 1996). (*) Genus *Callithrix* includes *Cebuella pygmaea.*

mosets and tamarins typically give birth to twins. These features have led to the suggestion that marmosets and tamarins are specialized primates that have undergone secondary reduction in body size (dwarfing) during their evolution.

Callimico goeldii, the only species of its genus, is also believed to have undergone phyletic dwarfism but shares traits with both the Cebidae and the Callitrichidae. *Callimico* resembles marmosets and tamarins in having claws on all digits except the hallux and a relatively small body weight (360 g; Encarnación and Heymann 1998), but it resembles cebid monkeys in having third molars (albeit markedly reduced in size and lacking a distinct hypocone) and in producing only one infant at a time. As a result, *Callimico* has been variously placed in the family Cebidae (Thomas 1913; Weber 1928; Simpson 1945; Simons 1972; Martin 1990), in the family Callitrichidae, or even in its own family, Callimiconidae (Chiarelli 1972; Hershkovitz 1977). Its taxonomic status varies according to the relative importance given to these features and also to their interpretation as primitive or specialized. Most phylogenies, regardless of the use of external characters (Pocock 1920), dental and skeletal morphology (Hill 1959; Rosenberger 1977; Ford 1986; Kay 1990), or vocal characters of long calls (Snowdon 1993), have agreed in placing *Callimico* as the most basal member of the Callitrichidae (Figs. 1a, c–f). However, there have been a few exceptions, to this interpretation. Byrd (1981), using dental ontogeny, places *Callimico* between the marmosets and the tamarins (Fig. 1b). Similar phylogenies were supported by Cronin and Sarich (1975, 1978) and Seuánez et al. (1989), based on immunological data (albumin and transferrin) and on restriction mapping of LINE-1 repetitive elements, respectively (Figs. 1i, j). More recently, studies on chromosomes (Canavez et al. 1996), the nuclear ϵ-globin gene (Schneider et al. 1993), intron 1 of the IRBP gene (Barroso 1995), intron 11 of the von Willebrand factor gene (Schneider et al. 1996), and the mitochondrial 16S rRNA gene (Horovitz and Meyer 1995) have consistently linked *Callimico* more closely with the marmosets, excluding the tamarins as a separate clade (Fig. 1h, k–m).

All genetic and morphological studies agree that the two marmoset genera, *Callithrix* and *Cebuella*, are more

closely related to one another than either of them is to *Saguinus, Leontopithecus,* or *Callimico.* There are even indications from morphological (Rosenberger and Coimbra-Filho 1984) and genetic (Barroso 1995) studies that *Cebuella pygmaea* should be included in the genus *Callithrix*. The branching order among the marmosets, *Saguinus, Leontopithecus,* and *Callimico,* differs among the studies. In fact, consensus conclusions from morphological and molecular evidence are in direct conflict concerning the position of *Callimico* within the Callitrichidae. In addition, the detailed nodal relationships of the two tamarin genera, *Saguinus* and *Leontopithecus,* are unresolved. Both morphological and genetic studies produce conflicting results, either having *Saguinus* form a clade with *Leontopithecus* or having these genera branch away separately among callitrichids.

Different regions of the mitochondrial DNA genome have been shown to evolve at different rates (Brown 1983; Miyamoto and Boyle 1989), allowing a choice of temporal scale by selection of a suitable region of the molecule. Because ND4 has already been successfully used in vertebrates (Forstner et al. 1995) and, especially, in primate studies (Hayasaka et al. 1988; Wang et al. 1997), a DNA sequence data set from part of the mitochondrial protein-coding ND4 gene and three flanking tRNA genes (histidine, serine, leucine) has been generated for use in the examination of these competing hypotheses. Our results are compared to, and combined with, published sequence data and discussed in terms of general implications for the evolution of New World monkeys.

Materials and Methods

Samples

We used either hair or tissue samples from seven genera of New World monkeys, including *Callimico,* two marmosets, two tamarins, and two cebids, as our source of DNA. Specifically, samples were collected from three *Callimico goeldii* (Studbook Nos. 1236, 1243, 1419), three *Callithrix jacchus,* and one *Cebuella pygmaea* housed in the New World primate colony at the Anthropological Institute, University of Zürich. Samples for *Leontopithecus* were provided by L. Forman and T. Fanning. The tissue sample for *Ateles geoffroyi* was provided by the Fort Worth Zoo. Hair samples were also collected from one *Saguinus midas,* one *Cebus apella,* and one *Lemur catta* (prosimian, outgroup taxon) carcass kept deep-frozen (−20°C) at the Anthropological Institute. Additional sequences from outgroup taxa were available from GenBank.

Laboratory Methods

DNA was extracted from either hair or tissue samples with PCI (25:24:1 mix of phenol, chloroform, and isoamyl alcohol) and chloroform (Sambrook et al. 1989) or with QIAamp kits (QIAGEN 1995a). The segment of the mtDNA amplified and sequenced in this study includes the 3′ region of the NADH dehydrogenase subunit 4 (ND4) gene as well as the $tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$ genes. The template DNA was amplified in 100-μl reactions using *Thermophilus aquaticus* (*Taq*) polymerase in a Perkin Elmer Cetus 480 DNA thermal cycler. The amplification primers are ND4 (5′ TGA CTA CCA AAA GCT CAT GTA GAA GC 3′), ND4#2 (5′ TA, CGA CAA ACA GAC CTA AAA TC 3′) or ND4#2M (5′ ACA AGC TCA AYC TGC CTA CGA 3′), and Leu (5′ TA CTT TTA CTT GGA TTT GCA CCA 3′) or MLeu (5′ TA CTT TTA TTT GGA GTT GCA CCA 3′). Successful amplifications were obtained using the following protocol: 35 cycles of denaturing at 95°C for 30 s, primer annealing at 50°C for 60 s, and extension for 60 s at 72°C. The polymerase chain reaction (PCR) products were checked by electrophoresis in 1% agarose minigels against a λ-*Hin*dIII size standard marker, visualized using ethidium bromide and UV light, and verified using negative (no DNA) controls. The PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN 1995b). The sequencing reactions were carried out with the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems 1995). The following internal sequencing primers were utilized: LND4#2 (5′ CTA CAA CAA ACA GAC CTA AAA TCC CT 3′), MND4Rev (5′ (TAT TAA GYT GTT TTC TCG 3′), MonkAGram (5′ GC GTG GCT TGC AAG TAA TCA TCA 3′), Monkyrench (5′ TAT GAA TAT TAA TYT GTT TTC CCG 3′), N#2Alt (5′ ACA TCA TCC CTA CTA TTC TGC 3′), TRLeu (5′ ATA TTT ACC TCA ACA CAA CGA GG 3′), and TRNpri (5′ GCA GAA TAG TAA YGA TGA GGT 3′). Sequencing products were cleaned of excess dyes with CENTRI-SEP Columns (Princeton Separations, Inc.). The reactions were electrophoresed and the sequences were analyzed on Applied Biosystems Model 373A or 377 DNA sequencing systems.

Quantitative Analysis

The sequences obtained were entered into the Phylogenetic Analysis Using Parsimony (PAUP) 3.1.1 (Swofford 1990) or PAUP*4.0d59 beta test version (by permission, Swofford, personal communication) computer programs. Sequences were aligned by eye and using CLUSTAL (Higgins and Sharp 1988) against the homologous region of human mtDNA sequence (Anderson et al. 1981) and against other previously generated sequences (Accession Nos. M22650—M22651, M22653–M22657, M22681, L00015, V00658, V00659, V00672, V00675, D38116). Sequences for a lizard (*Sceloporus grammicus*) and cow (*Bos taurus*) were also included (Forstner et al. 1995). Gaps were considered as a fifth character state and deleted from all analyses. The aligned sequences were analyzed in different ways using maximum-parsimony, neighbor-joining, and maximum-likelihood methods. Examination of the sequences suggested additional analyses limited to those mutations resulting in a transversion or with down-weighting of mutations resulting in a transition. Bootstrap (BP) and jackknife (JK) analyses (Felsenstein 1985) of 2,500 replicates (10 random addition heuristic searches each for bootstraps) were performed to examine the relative support of each relationship in the resultant topologies. Neighbor-joining analyses were performed using MEGA (Kumar et al. 1993), with distances calculated using the Tamura and Nei (1993) correction for multiple substitutions and a relatively rapid rate of divergence. Maximum-likelihood analyses were performed using PHYLIP 3.5 (Felsenstein 1990). Previously published sequences for these taxa from other genes (see Introduction) were obtained from GenBank, tested for homogeneity, and combined where appropriate in a total genetic data set for analysis by the above methods.

Results

The new mtDNA sequences generated for the taxa we examined have been deposited in GenBank (Accession Nos. AF053684–AF053697). Each new nucleotide se-

Table 1. Summary of the variation for the sequences across the 13 New World monkey taxa examined

	Region					
Characters	All	ND4	tRNAs	His	Ser	Leu
Total	887	710	177	69	60	48
Invariant	562	429	133	50	42	41
Uninformative	95	76	19	8	7	4
Informative	230	205	25	11	11	3
Proportion of informative characters	0.26	0.29	0.14	0.15	0.18	0.06

quence was aligned against published homologous sequences from GenBank. Aligned sequences are available from the first author upon request. The nucleotide sequences span a total of 936 base positions (bp), of which 49 are primer sequences. The analyzed data set consists of about 50% of the ND4 gene (710 bp) along with the histidine (69 bp), the serine (59 bp; 1 gap character deleted from analyses), and part of the leucine (48 bp) tRNA genes.

A summary of the frequencies of invariant, uninformative, and informative characters along the segment sequenced is given in Table 1. The proportion of informative characters is 0.26 (Table 1).

We examined the overall substitution rates of the two types of genes (protein-coding ND4 and structural tRNAs) by plotting the relative substitution rates (data not shown). As expected, ND4 demonstrates a faster relative rate of evolution compared to that of the tRNAs and both types of genes demonstrate increases in sequence divergence with increasing phylogenetic distance. The distance table provides the Tamura and Nei (1993) distances and uncorrected distances for the compiled ND4 and tRNA genes (Table 2).

Parsimony analyses with all characters weighted equally result in three trees of 768 steps. The strict consensus of these trees groups *Callimico* with *Leontopithecus* as a sister group to *Callithrix*/*Cebuella* (data not shown). This arrangement is weakly supported. Bootstrap analyses by maximum-parsimony or jackknife iterations by the neighbor-joining method result in topologies which provide four unresolved clades within the Callitrichidae, represented by *Saguinus*, *Leontopithecus*, *Callimico*, and *Callithrix*/*Cebuella* (Fig. 2a).

Obviously, there is a phylogenetic signal in the data (g1 is −1.00 from 10 million random trees) (Hillis and Huelsenbeck 1992). Therefore, we examined the potential necessity for a posteriori weighting of the data to obtain better phylogenetic resolution. One commonly applied method is differential weighting of transversions (TV) over transitions (TI) (Miyamoto and Cracraft 1991; Hillis et al. 1996). Figure 3 plots the absolute number of TV and TI over eight taxonomic levels. While the TV show no saturation within the primates, the TI climb rapidly and appear to begin to plateau at the distances within the Callitrichidae (Fig. 3). This indicates that the TI will give only limited information for questions within the Callitrichidae, and the resulting homoplasy may account for the lack of resolution in the unweighted analyses. One method of correcting for this is to weight TV and TI compensatorily according to the TI:TV ratio of the data set (Hillis et al. 1996). Following compensatory weighting of the TV by 3 and the T1 by 1, we obtain three trees 1,209 steps in length, the strict consensus of which is identical to the well-supported bootstrap topology (Fig. 2b). The *Callithrix* group and *Cebuella* form a subclade within the Callitrichidae (BP = 99%). However, the JK analyses fail to resolve these two taxa as separate clades. The analyses unambiguously linked *Callimico* to the marmosets (i.e., *Callithrix* and *Cebuella*), with BP = 80% and JK = 99%. *Leontopithecus* and *Saguinus* together form a clade, with BP = 62% and JK = 79%. Finally, the monophyly of the family Callitrichidae is supported, with BP = 93% and JK = 87%.

Another potential weighting takes into account positional substitution bias among the codons of ND4. If the substitutions by position for the eight taxonomic levels of comparison used before are considered, the first and second positions remain linear (nonsaturated) over the entire taxonomic evaluation. Third-position substitutions show a linear substitution rate for the relationships within primates but begin to saturate upon reaching the simian/prosimian split (data not shown). Thus, third-position changes should be informative for the portion of the graph specifically of concern here, namely, that reflecting relationships within the Callitrichidae.

The results of the maximum-likelihood analysis of the complete data set for all taxa are presented in Fig. 2c. The phylogram presented maintains branch lengths proportional to the number of changes. The phylogenetic relationships of the terminal taxa are identical to those from the analyses presented in Fig. 2b. The position of *Callimico* as sister group to the marmosets is maintained.

Our aligned mitochondrial sequences were combined in tandem with 1,928 bp of the ϵ-globin gene (Schneider et al. 1993), 1,843 bp of the IRBP gene (Barroso 1995; Harada et al. 1995), and 543 bp of the mitochondrial 16S ribosomal gene (Horovitz and Meyer 1995). The seven genes to be included in the combined analyses were analyzed using the combinability test in PAUP*4.0d59 (Farris et al. 1994; Swofford 1991). The IRBP gene (Barroso 1995; Harada et al. 1995) was found to be significantly ($P < 0.01$) incongruent with the remaining six

Table 2. Tamura–Nei distance (above the diagonal) and uncorrected distance (under the diagonal) matrix derived from the sequence data set

	H.s.	*A.g.*	*C.a.*	*L.r.* 1	*L.r.* 2	*L.c.*	*S.m.*	*C.g.* 1	*C.g.* 2	*C.g.* 3	*C.p.*	*C.j.* 1	*C.j.* 2	*C.j.* 3
Homo sapiens	—	0.324	0.320	0.341	0.341	0.343	0.315	0.342	0.346	0.342	0.313	0.296	0.303	0.305
Ateles geoffroyi	0.254	—	0.211	0.225	0.225	0.234	0.236	0.211	0.216	0.211	0.216	0.213	0.207	0.209
Cebus apella	0.252	0.176	—	0.220	0.220	0.225	0.222	0.211	0.212	0.211	0.193	0.195	0.188	0.192
L. rosalia 1	0.266	0.187	0.183	—	0	0.023	0.214	0.192	0.190	0.192	0.182	0.206	0.208	0.208
L. rosalia 2	0.266	0.187	0.183	0	—	0.023	0.214	0.192	0.190	0.192	0.182	0.206	0.208	0.208
L. chrysomelas	0.267	0.193	0.186	0.023	0.023	—	0.221	0.187	0.185	0.187	0.179	0.202	0.201	0.200
Saguinus midas	0.252	0.195	0.184	0.178	0.178	0.183	—	0.198	0.195	0.198	0.193	0.188	0.181	0.180
Callimico goeldii 1	0.264	0.177	0.174	0.164	0.164	0.160	0.168	—	0.008	0	0.164	0.166	0.159	0.160
Callimico goeldii 2	0.266	0.181	0.175	0.163	0.163	0.159	0.166	0.008	—	0.008	0.166	0.164	0.157	0.159
Callimico goeldii 3	0.264	0.177	0.174	0.164	0.164	0.160	0.168	0	0.008	—	0.164	0.166	0.159	0.160
Cebuella pygmaea	0.248	0.181	0.164	0.157	0.157	0.155	0.164	0.140	0.141	0.140	—	0.109	0.104	0.103
Callithrix jacchus 1	0.238	0.178	0.164	0.173	0.173	0.170	0.160	0.140	0.139	0.140	0.098	—	0.014	0.013
Callithrix jacchus 2	0.243	0.175	0.159	0.174	0.174	0.169	0.156	0.135	0.134	0.135	0.094	0.014	—	0.006
Callithrix jacchus 3	0.244	0.176	0.163	0.174	0.174	0.169	0.155	0.137	0.135	0.137	0.093	0.012	0.006	—

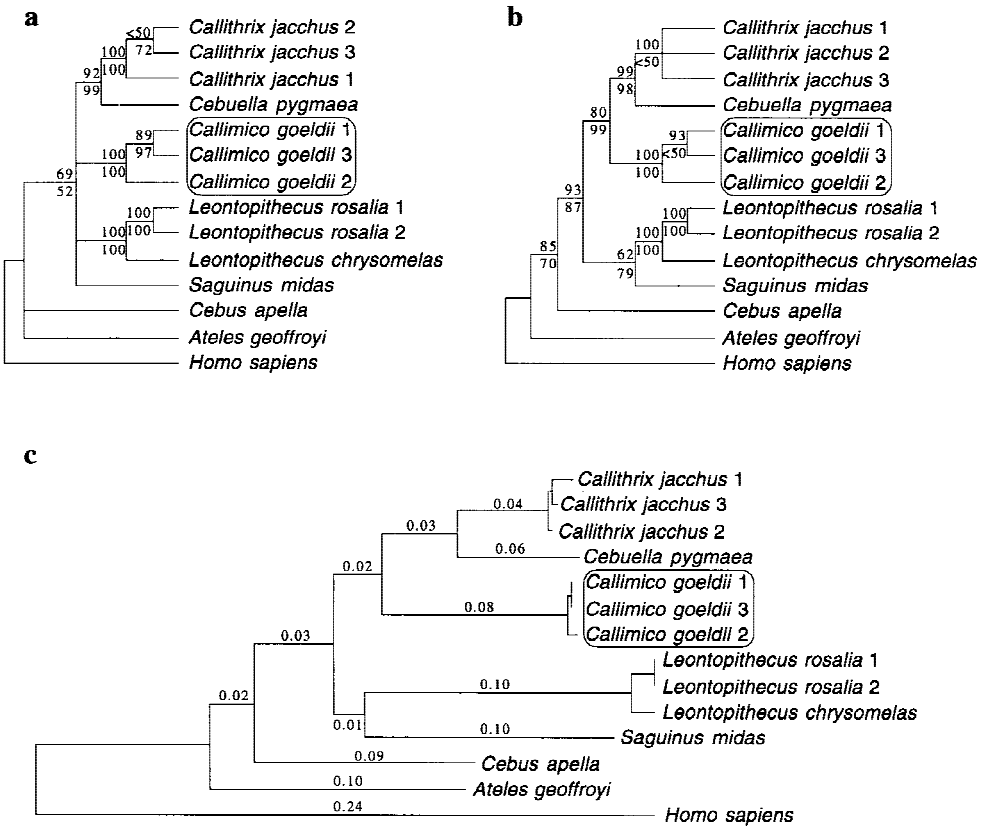

Fig. 2. Phylogenetic trees based on sequences of part of the mitochondrial ND4 gene and the $tRNA^{His}$, $tRNA^{Ser}$, and $tRNA^{Leu}$ genes for 13 New World monkeys and *Homo.* **a** Maximum-parsimony tree with bootstrap values (as percentages, above nodes) obtained in 2,500 replicates with 10 random additions each and neighbor-joining jackknife tree with jackknife values (below nodes) from 2,500 iterations with 50% deletion. **b** Maximum-parsimony tree weighting transversions over transitions (3:1) with bootstrap values (above nodes) obtained as in **a** and neighbor-joining jackknife tree resulting from analysis of only those mutations resulting in a transversion (below nodes) obtained as in **a. c** Maximum-likelihood phylogram with proportional branch lengths (values provided on each branch).

genes based upon 10,000 replicates. The remaining genes were not found to be significantly incongruent ($P < 0.21$) and were combined for total genetic analyses. Analyses which combined IRBP in spite of the significant incongruence were also performed and compared to assess the effect of incongruency upon the final trees.

In all maximum-parsimony analyses of the combined data set, whether IRBP is included or not and whether weighted or not, *Callithrix* and *Cebuella* form a subclade within the Callitrichidae, with *Callimico* as the next sister group. However, the equal-weights analyses (Fig. 4a) provide no resolution for *Saguinus* and *Leontopithecus.*

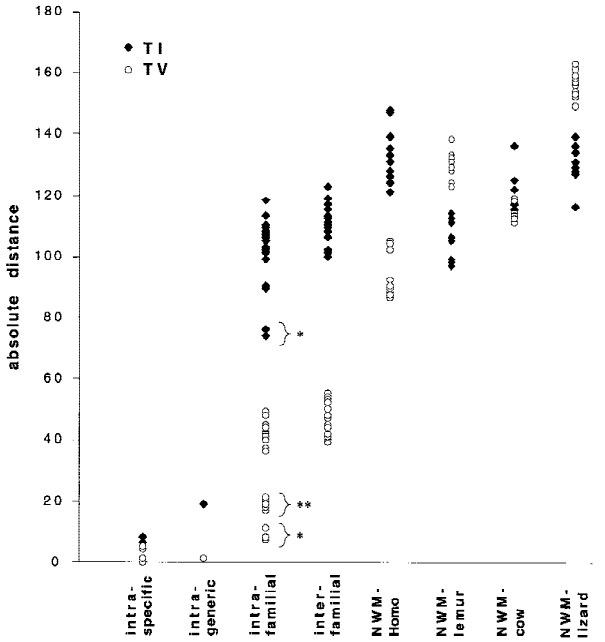

Fig. 3. Absolute numbers of transversions (TV) and transitions (TI) over eight defined taxonomic levels. (*) The distance values between *Cebuella* and *Callithrix*. (**) The distance values between *Callimico* and *Cebuella* or *Callithrix*.

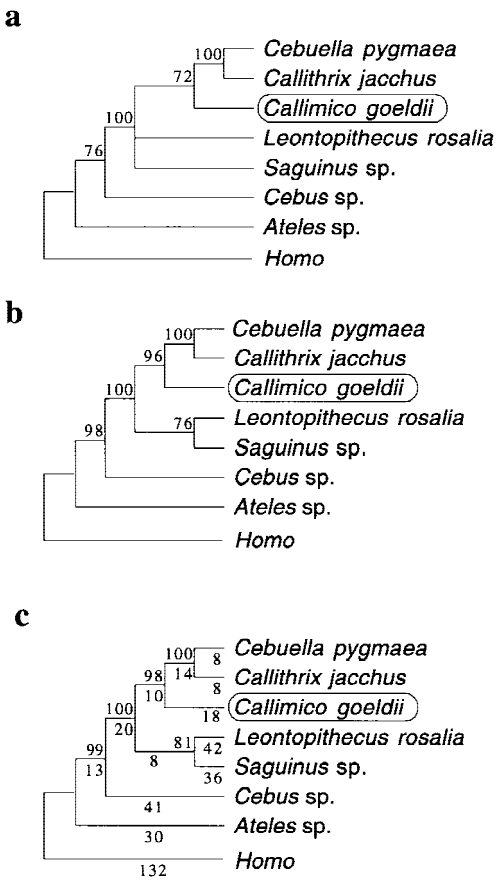

Fig. 4. Phylogenetic trees for a tandemly combined sequence of the ϵ-globin gene (Schneider et al. 1993), mitochondrial 16S ribosomal gene (Horovitz and Meyer 1995), and ND4 and tRNA genes from this study. The combined *Callithrix jacchus* sequence contains the ND4 sequence from ''*Callithrix jacchus* 3.'' The sequence from *Saguinus* sp. consists of sequences from both *S. midas* and *S. geoffroyi* sequences: likewise, that from *Cebus* sp. is a species mixture from *C. apella* and *C. kaapori*, and *Ateles* sp. is compiled from *A. geoffroyi* and *A. belzebuth.* **a** Maximum-parsimony tree with bootstrap values (as percentages) obtained in 10,000 replicates of 10 random additions each. **b** Maximum-parsimony tree weighting transversions over transitions (3:1) with bootstrap values obtained in 10,000 replications of 10 random additions each. **c** Maximum-parsimony tree from analysis of only those mutations resulting in a transversions. Branch lengths are provided for each branch below the lines. Bootstrap values obtained in 10,000 replications with 10 random additions each are presented above each line for those nodes supported at 50% or greater.

In weighted (3:1) TV parsimony *Leontopithecus* and *Saguinus* form a sister-group relationship (Fig. 4b). However, if IRBP is included in the weighted TV parsimony analyses, *Saguinus* branches off before *Leontopithecus*, with weak bootstrap (BP = 60%) support. If only TV are considered, *Saguinus* and *Leontopithecus* together form the most basal clade of the Callitrichidae, with strong bootstrap (BP = 81%) support (Fig. 4c). If IRBP is included in the TV-only analyses, the relationships remain but support of this basal clade is weakened (BP = 59%; data now shown). Despite support in most analyses, it remains possible that the sister-group relationship indicated for *Saguinus* and *Leontopithecus* is an artifact of long branch attraction in these two taxa (Fig. 4c). Inclusion of additional taxa, in a much broader examination of the New World primates, may be able to assist in resolving this tissue.

The use of *Homo* as the outgroup specified in all the analyses presented is potentially problematic due to the large phylogenetic distance between Old and New World primates. However, the results of the entire analytical suite are not significantly different with regard to relationships within the Callitrichidae if *Cebus* or *Ateles* is chosen as the outgroup, with *Homo* deleted from the data set. Rooting with *Homo* does decrease the bootstrap support of each branch by a few percentage points from the values obtained when rooted with *Ateles.* Alternative rootings do not affect the sister relationship of *Callimico* and *Cebuella*/*Callithrix.*

Finally, we compared the morphological tree topology (Figs. 1c–f) with the tree that results from analyses of molecular data (Figs. 2b, c and 4b, c) using the Templeton (1983) test. The molecular tree provides a significantly shorter topology ($P < 0.05$; $T_s = 25$; $n = 16$) using the two-tailed probability of the Wilcoxon rank sum test (Sokal and Rohlf 1987). Constraining the molecular data to the topology suggested by morphological characters increases the tree length significantly.

Discussion

Molecular Evidence

Overall, the results of the various methodologies are consistent: *Callithrix* and *Cebuella* are grouped together,

Callimico is the sister group to these marmosets, and *Saguinus* and *Leontopithecus* form the other subclade among callitrichids. In analyses which combine all available DNA sequence data, the topologies of the trees obtained with unweighted and weighted parsimony methods are identical concerning the position of *Callimico* (Fig. 4).

Comparing the trees obtained for the Callitrichidae with immunological data (Cronin and Sarich 1978), LINE-1 restriction fragment data (Seuánez et al. 1988), ϵ-globin gene sequences (Schneider et al. 1993), IRBP gene sequences (Barroso 1995) von Willebrand factor gene sequences (Schneider et al. 1996), and mitochondrial 16S rRNA sequences (Horovitz and Meyer 1995), there are conspicuous differences in branching orders among the three clades represented by *Saguinus, Leontopithecus,* and *Callimico*/*Callithrix*/*Cebuella* (Figs. 1i–m). However, all of these genetic studies agree in placing *Callimico* between the marmosets and the tamarins. In our own analyses we have chosen to be conservative in combining different data sets. Combining data sets whether they represent different genes or morphological versus molecular evidence is a contentious topic in systematic biology (de Quieroz et al. 1995). The results we obtained indicate that intron 1 of the IRBP gene is statistically incongruent with other genetic data. However, the only changes seen in the phylogenetic reconstruction that included the IRBP gene occurred in weakly supported branches (i.e., the relationship between *Saguinus* and *Leontopithecus*).

The use of a partial gene sequence for phylogenetic reconstruction may be questioned (Arnason and Gullberg 1994). Cummings et al. (1995) inferred phylogenetic trees from individual genes and random samples of nucleotides from the mitochondrial genomes of 10 vertebrates and compared the results to those obtained by analyzing the whole mitochondrial genomes. They found that blocks of contiguous sites were less likely to lead to the whole-genome tree than samples composed of sites drawn individually from different places in the genomes (Cummings et al. 1995). However, Cracraft and Helm-Bychowski (1991) examined the same region and genes as presented here to investigate the effects of sample size of characters on the cladistic structure of the data. The results of their subset analyses of this region demonstrate that, although potential resolution decreases with smaller fragments of a gene, partial analyses do resolve the correct topology. An analysis of the three tRNAs suggests that their contribution to instability is minimal. Further, the inclusion of different genes—as in this case—actually assists in the reconstruction of more robust nodes on the final topology (Cracraft and Helm-Bychowski 1991). Douzery and Catzeflis (1995) investigated the complete 12S rRNA gene sequences of 43 mammals and determined that different relative weightings of loops versus stems did not alter the phylogenetic conclusions. We feel confident that sequencing only part of the ND4 gene and the three tRNAs represents a suitable data set for addressing phylogenetic questions within the Callitrichidae and our inclusion of genetic data from other genes in the combined analyses increases the likelihood of obtaining the best estimate of the true phylogeny (Fig. 4).

The genetic divergence between *Cebuella* and *Callithrix* is markedly lower than all other values of the same level of intrafamilial comparison (Table 2, Fig. 3). This supports the proposed close relationship between *Callithrix* and *Cebuella* (Rosenberger and Coimbra-Filho 1984; Barroso 1995). While the relative genetic distinction is not as low as comparisons between *L. rosalia* and *L. chrysomelas,* we cannot exclude the possibility that *Cebuella* and *Callithrix* are congeneric. A congeneric classification would support the conclusion of Barroso (1995) from IRBP gene sequences that *Cebuella* should be included within the genus *Callithrix.*

Morphological Evidence

There are two major alternative hypotheses of callitrichid relationships that can be derived from the morphological evidence. One is that the marmosets and tamarins form a monophyletic group and that the four characters which they share (loss of the third molar, loss of the hypocone, reduced body size, reproductive twinning) are homologous and retained from a common ancestor that underwent dwarfing at some point in the past. The other is that these characters are not all homologous and that there have been two or more independent dwarfing events within the Callitrichidae (Ford 1980). If *Callimico* really is more closely related to the marmosets than to the tamarins, as suggested by the molecular evidence, there are two possible interpretations. The first is that the development of reproductive twinning, loss of the third molar, loss of the hypocone, and reduction in body size must have taken place independently at least twice in the Callitrichidae. The second is that there has been a reversal in one or more of these four characters in *Callimico.*

It is well-known that the loss of a character can occur many times independently. *Callimico* has a small third molar, whereas the marmosets and tamarins have only two molars. The third molar is small in several Cebidae and is occasionally absent in some specimens (Rosenberger 1977). Since reduced third molars are widespread among New World monkeys, it seems clear either that relatively small third molars are primitive for Cebidae or that the trend to their reduction is almost universal within the group (Ford 1980). Rosenberger et al. (1977), for example, are convinced that the loss of the third molar in the fossil *Xenothrix* and in the marmosets and tamarins was achieved convergently. Therefore, the loss of the third molar cannot be considered as conclusive evidence that marmosets and tamarins form a clade excluding *Callimico.*

In the Cebidae, the upper molars usually possess four cusps, although the third molar frequently lacks a hypocone (Swindler 1976). *Callimico* normally has quadritubercular first and second upper molars with a small hypocone (Kinzey 1973), but individuals lacking a hypocone have also been found (Swindler 1976). The hypocone is usually lacking in the marmosets and tamarins (Swindler 1976), but *Callithrix* and *Saguinus* specimens with four cusps sporadically occur (Kinzey 1973; Swindler 1976). The absence or presence of the hypocone therefore does not seem to be a reliable feature for resolution of phylogenetic relationships within the Callitrichidae.

All four characters that unite marmosets and tamarins may be interpreted as being the direct result of phyletic dwarfing (Ford 1980). A nearly complete but badly crushed skull and mandible of *Lagonimico conclucatus* from the middle Miocene has been interpreted as intermediate between *Callimico* and the other Callitrichidae (Kay 1994) (Fig. 1f). Estimates from *Lagonimico*'s jaw size suggest a body weight of about 1,200 g, which is greater than that of *Callimico* or any other living callitrichid (Kay 1994). Nevertheless, *Lagonimico* shows some callitrichine features (e.g., loss of the hypocone), although small third molars are still present (Kay 1994). Some anatomical features shared by marmosets and tamarins may therefore not be causally connected to phyletic dwarfing. If size reduction itself did not cause the evolution of all these features, it could have occurred several times independently within the Callitrichidae.

There is, however, a major problem involved in proposing that certain similarities in reproductive biology shared by marmosets and tamarins but lacking in *Callimico* arose through convergent evolution. Marmosets and tamarins are unique among simian primates in typically giving birth to twins, rather than to single offspring. The reproduction of marmosets and tamarins is extremely specialized in several respects and this supports the inference that twinning is derived rather than primitive (Martin 1992). In particular, marmosets and tamarins are apparently unique among mammals in that the twins (which are dizygotic) share a common placental circulation within a single chorionic membrane. This highly unusual system is associated with mutual exchange of cells (chimerism), such that (for example) blood-forming cells of both twins are combined in each individual. As one outcome of this, multilocus fingerprints derived from blood samples are identical between littermates (Dixson et al. 1988). It is difficult to imagine that this complex and unique system evolved separately in marmosets and tamarins. There is, however, an alternative possibility. It has already been shown that *Callimico* attains a relatively high reproductive output through a reduced age of sexual maturity rather than through twinning (Martin 1992). It is therefore possible that, if *Callimico* is indeed really more closely related to marmosets than to tamarins, secondary reversal to single offspring took place after *Callimico* diverged from the common ancestor of marmosets. Such secondary loss of the complex character of twinning would be a relatively simple transformation. If this is, in fact, what happened, there is a remote possibility that detailed studies of the reproductive system of *Callimico* might reveal relict features reflecting an original adaptation for twinning.

Our molecular analyses of new mitochondrial sequences as well as the combined analyses of available molecular data confirm the marmoset clade containing *Callithrix* and *Cebuella* and provide supplementary support for a tamarin clade grouping *Saguinus* with *Leontopithecus.* On the basis of our mitochondrial data, *Callimico* is placed between the marmosets and the tamarins, agreeing with all previously published DNA sequence analyses (Schneider et al. 1993; Barroso 1995; Horovitz and Meyer 1995; Schneider et al. 1996). In contrast, most morphological studies have led to the conclusion that *Callimico* is the most basal member of the Callitrichidae. Obviously, there is a direct conflict (Templeton test $P < 0.05$) between inferences based on morphology and molecules concerning the position of *Callimico* within the Callitrichidae.

Dwarfism may have occurred repeatedly among the Callitrichidae. Reduced body size, loss of the third molar, and loss of the hypocone may be explained by homoplasy. It is important to notice that characters add additional support to a phylogenetic hypothesis only to the extent that they are independent of the other characters in the analysis. If loss of the hypocone, loss of the third molar, and twinning are all direct consequences of the dwarfing event, these factors are not independent. A data set with many dependent factors can be positively misleading (Shaffer et al. 1991). Another bias in the morphological data set may be introduced by selecting apparently significant characters at the outset of the analysis.

Although this conflict of evidence cannot be resolved conclusively at the moment, we believe that the phylogenetic link between marmosets and *Callimico* is now overwhelmingly supported by genetic studies and that a reconsideration of the morphological evidence in the light of the consensus tree from DNA sequence analyses (Figs. 2 and 4) is needed. It would be advantageous to increase taxonomic coverage for the dual purposes of addressing both the question of paraphyly in Cebidae and potential artifacts of subset taxon sampling. Moreover, sequencing additional callitrichid taxa and individuals within the taxa already analyzed should improve our insight into the branching order of *Saguinus*, *Leontopithecus*, and the *Callimico*/*Callithrix*/*Cebuella* clade as well as permitting assessment of the questionable generic status for *Cebuella pygmaea.*

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