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Phylogenetic Relationships of the New World Monkeys (Primates, Platyrrhini) Based on Nuclear G6PD DNA Sequences

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In order to test hypotheses about the phylogenetic relationships among living genera of New World monkeys, 1.3 kb of DNA sequence information was collected for two introns of the glucose-6-phosphate dehydrogenase (G6PD) locus, encoded on the X chromosome, for 24 species of New World monkeys. These data were analyzed using a maximum parsimony algorithm. The strict consensus of the three most-parsimonious gene trees that result shows support for the following clades: a pitheciine clade including *Callicebus* within which *Chiropotes* and *Cacajao* are sister taxa, an *Alouatta*–atelin clade within which *Brachyteles* is the sister taxon of *Lagothrix* and which is sister to another clade containing the callitrichines, and a callitrichine/*Aotus*/*Cebus*/*Saimiri* clade. Within the callitrichines, *Callimico* is the sister taxon of *Callithrix*. *Cebus* and *Saimiri* form a clade. These results are broadly consistent with previously published DNA sequence analyses of platyrrhine phylogeny and provide additional support for groupings provisionally proposed in those earlier studies. Nevertheless, questions remain as to the relative phylogenetic placement of *Leontopithecus* and *Saguinus*, the branching order within the *Aotus*/*Cebus*/*Saimiri*/callitrichine clade, and the placement of the pitheciine clade relative to the atelines and the callitrichines.

INTRODUCTION

There are sixteen extant genera of New World monkeys, or platyrrhines. Table 1 lists these genera and their common names. Three recent morphological studies on the systematics of these genera are shown in Fig. 1 (Rosenberger, 1984; Ford, 1986; Kay, 1990). In addition, three DNA sequence datasets for platyrrhines have been analyzed phylogenetically: mitochondrial 16S rRNA (Horovitz, 1995), epsilon-globin (Schneider *et al.*, 1993; Harada *et al.*, 1995; Porter *et al.*, 1995, 1997a,b), and the interphotoreceptor-retinoid binding protein (IRBP) (Harada *et al.*, 1995; Schneider *et al.*, 1996; Barroso *et al.*, 1997) (Figs. 2–4).

In broad terms, all of these analyses agree on the following clades for platyrrhines: an *Alouatta*–Atelini (*Ateles, Brachyteles, Lagothrix*) or Atelinae clade, a Pitheciini (*Pithecia, Chiropotes, Cacajao*) clade, and a Callithrichinae (*Callimico, Callithrix, Cebuella, Saguinus, Leontopithecus*) clade. They disagree on the branching order between these three major clades and that within the ateline and callitrichine clades and on the placement of the four genera *Saimiri, Cebus, Callicebus,* and *Aotus.*

Looking in more detail at the DNA sequence gene trees, the epsilon-globin (Fig. 3) and IRBP (Fig. 4) topologies are largely in agreement. Both link *Cebus* and *Saimiri* in a clade and link that clade with the callitrichines and *Aotus* to yield the family Cebidae. Both link *Callicebus* with the pitheciins to yield the Pitheciinae or pitheciine clade. Both also link *Brachyteles* with *Lagothrix* within the ateline clade. Finally, both show *Callimico* not as the most basal member of a callitrichine clade, but as the sister taxon to either *Callithrix* (epsilon-globin) or *Leontopithecus* (IRBP). The two gene trees disagree on the branching order within the callitrichines and on the placement of the pitheciine clade: IRBP places the pitheciines as a sister group to the new family Cebidae, while epsilon-globin places the pitheciines sister to the atelines.

The 16S rRNA topology (Fig. 2) also links *Aotus, Cebus,* and *Saimiri* with the callitrichines, and it shows *Callimico* as the sister taxon to *Callithrix.* It disagrees with the other two DNA sequence topologies in linking *Pithecia* with *Cebus* and linking *Callicebus* with *Saimiri.* The analyses conducted by Horovitz and Meyer (1995) demonstrate that the topology for 16S is highly sensitive to the method of analysis used, particularly in terms of weighting. In addition, the 16S dataset includes representatives of only 12 of the 16 genera of platyrrhines. Thus, the gene tree presented in Horovitz and Meyer (1995) and reproduced in Fig. 2 should be considered provisional, and the majority of compari-

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TABLE 1

Genera and Common Names of New World Monkeys

Genus	Common name
Alouatta	Howler monkey
Aotus	Night monkey, owl monkey, douroucouli
Ateles	Spider monkey
Brachyteles	Woolly spider monkey
Cacajao	Uakari
Callicebus	Titi
Callimico	Goeldi's marmoset
Callithrix	Marmoset
Cebuella	Pygmy marmoset
Cebus	Capuchin
Chiropotes	Bearded saki
Lagothrix	Woolly monkey
Leontopithecus	Golden lion tamarin
Pithecia	Saki
Saguinus	Tamarin
Saimiri	Squirrel monkey

sons made in this paper will be to the gene trees for epsilon-globin and IRBP.

In order to test the placements hypothesized on the basis of these existing DNA sequence datasets, 1.3 kb of DNA sequence information from introns of the glucose-6-phosphate dehydrogenase (G6PD) locus were collected for 24 platyrrhine taxa. As an outgroup, a sequence was included for *Homo sapiens* (Chen *et al.*, 1991). G6PD is a single-copy gene located on the X-chromosome in mammals. It is 18 kb long in humans (Martini *et al.*, 1986) and codes for an enzyme that is important in glucose metabolism. This is one of the first uses of G6PD DNA sequences in a phylogenetic study.

MATERIALS AND METHODS

Sample Provenance

Table 2 lists the species studied and source of the sample for each. In addition to the species newly sequenced in this study, sequence information for *Homo sapiens* was also included (DDBJ/EMBL/GenBank Accession No. X55448; Chen *et al.*, 1991).

DNA Extraction

Extraction of DNA from all of the non-DNA samples above was performed using the IsoQuick DNA Extraction Kit (ORCA Research, Bothell, WA), which is a guanidine thiocyanate-based method. The long protocol for the "Total Nucleic Acid (DNA/RNA) Extraction" was followed, with the alteration that the second cycle of lysis with Reagent 4 was omitted, as it was found to be unnecessary. DNA was resuspended in water and kept at −20°C.

PCR Amplification and Visualization

DNA sequence data were obtained for two introns of the X-encoded glucose-6-phosphate dehydrogenase locus (G6PD). These two introns, referred to here as introns D and E, correspond to introns 4 and 5 of Martini *et al.* (1986); D is 550 bp long in humans, and E is 573 bp long in humans. The polymerase chain reaction (PCR) was used to amplify target DNA in

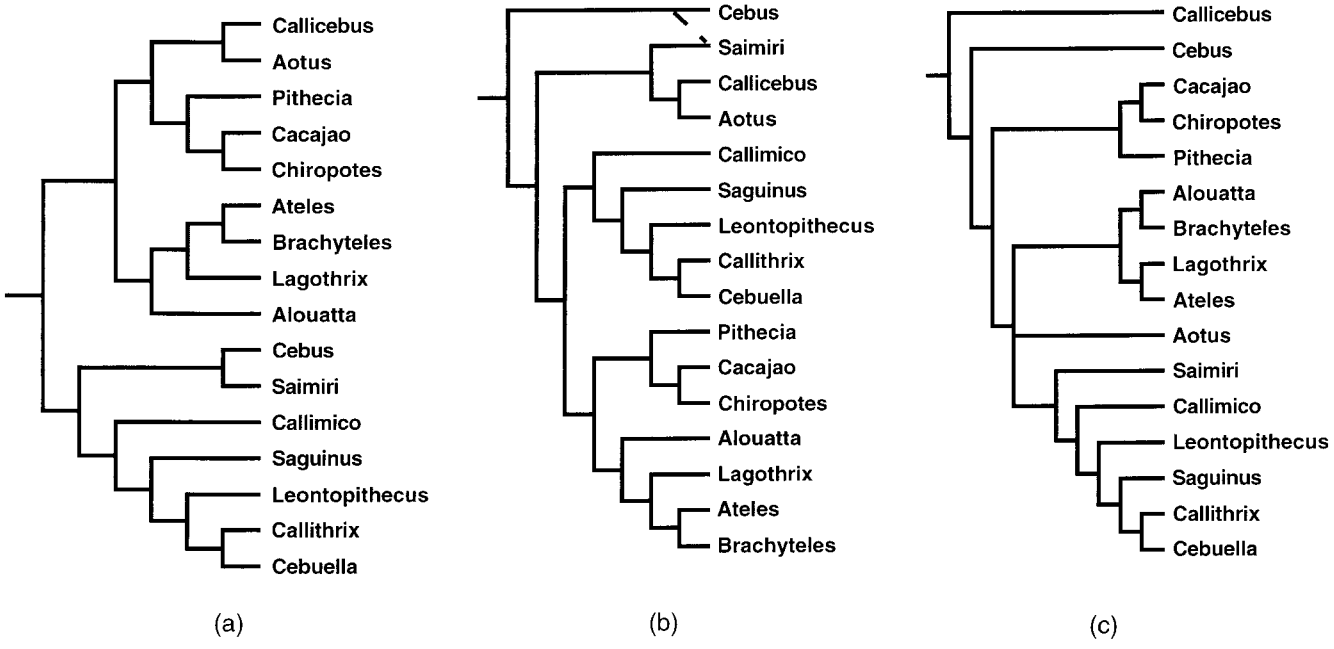


FIG. 1. (a) New World monkey phylogeny (Rosenberger, 1984) based on a variety of morphological characters. (b) New World monkey phylogeny (Ford, 1986) based on dental, cranial, and postcranial characters, analyzed cladistically. The dashed line indicates uncertainty as to the placement of *Saimiri*. (c) New World monkey phylogeny (Kay, 1990) based on dental characters, analyzed cladistically.

preparation for sequencing. Because G6PD is located on the X chromosome, there is only one copy of the locus in male mammals. The majority of individuals sampled in this study were male and thus there was no question of heterozygosity for the sequences obtained from these samples. Three female individuals were included in the study (*Cacajao calvus*, *Cacajao rubicundus*, and *Lagothrix lagothricha*) but no heterozygosity was detected, as discussed below in the section on DNA sequence verification.

There is a processed pseudogene of G6PD in New World monkeys, which because it is smaller in size (due to lack of intron sequence) amplifies preferentially (Ruvolo, Yoder, and Heider, unpublished data). Thus, for many taxa each intron was amplified in halves using one external (exon) and one internal (intron) primer, to avoid amplification of the processed pseudogene. Table 3 shows the primer sequences for G6PD. For Intron D, the primers used were G4P26-GDM376/OWM and GDP288-G5M32; for intron E, the primers used were G5P160-GEM401 and GEP222-G6M97. There were some exceptions to this sequencing strategy. The entire intron was amplified in sufficient quantities (the intron is easy to distinguish from the pseudogene on an agarose gel due to its much larger size) and sequenced in the following cases: for Intron D, G4P26-G5M32 was used for *Alouatta caraya*, *Brachyteles arachnoides*, *Cebus capucinus*, *Callimico goeldii*, and *Leontopithecus rosalia;* for Intron E, G5P160-G6M97 was used for *Saimiri boliviensis* and *Callimico goeldii*. These sequences were then used to design the primers internal to the introns.

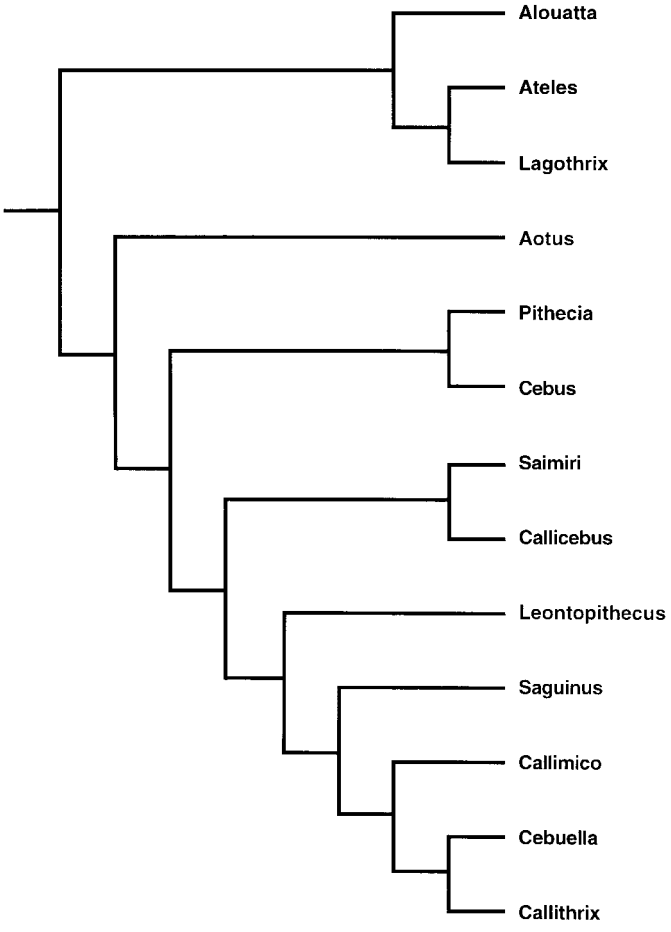


FIG. 2. New World monkey gene tree (Horovitz and Meyer, 1995) based on mitochondrial 16S rRNA sequence data, analyzed using maximum parsimony and successive weighting.

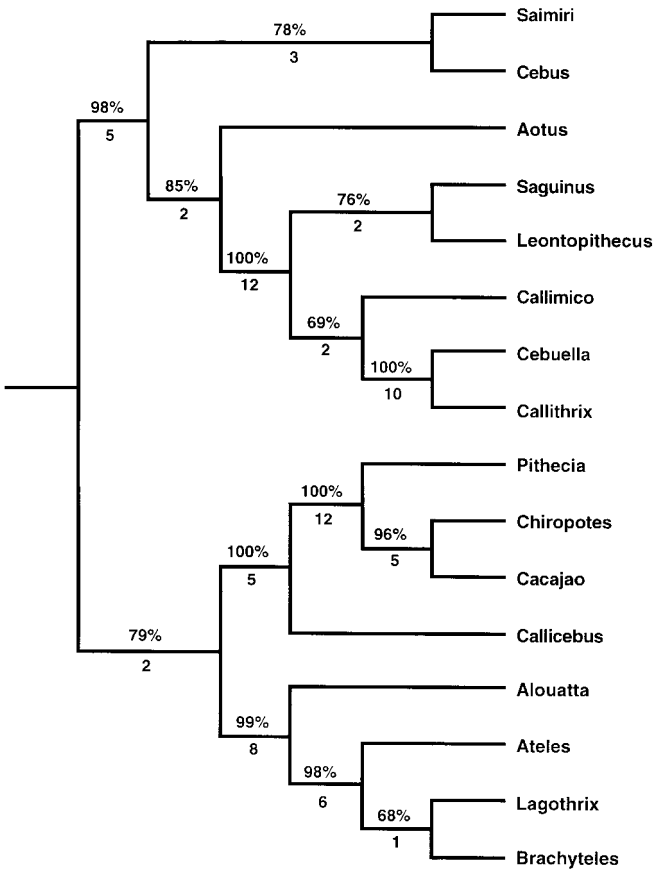


FIG. 3. New World monkey gene tree (Harada *et al.*, 1995) based on epsilon-globin DNA sequence data, analyzed using maximum parsimony. Strict consensus of two most parsimonious trees is shown with bootstrap values above branches and "strength of grouping" (Harada *et al.*, 1995) numbers below branches.

PCR amplification conditions for G6PD were as follows (all reactions were 25 µL reactions): 1.5–3 mM $MgCl_2$, 200 µM each dNTP, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 25 pM each primer, 1.2 µL TaqStart Antibody preparation containing 0.22 µg TaqStart Antibody (Clontech, Palo Alto, CA) and 1 unit *Taq* Polymerase diluted in TaqStart Antibody dilution buffer in a ratio of 1:4:1 (Antibody:Dilution Buffer:*Taq*) added to 3 µL template DNA (concentration unquantified, from 100 µL starting material, IsoQuick extracted); cycle parameters were 3′ at 95°C followed by 35–40 cycles of 30″ at 94°C, 30″ at 66–68°C, and 45″ at 72°C. Magnesium concentration

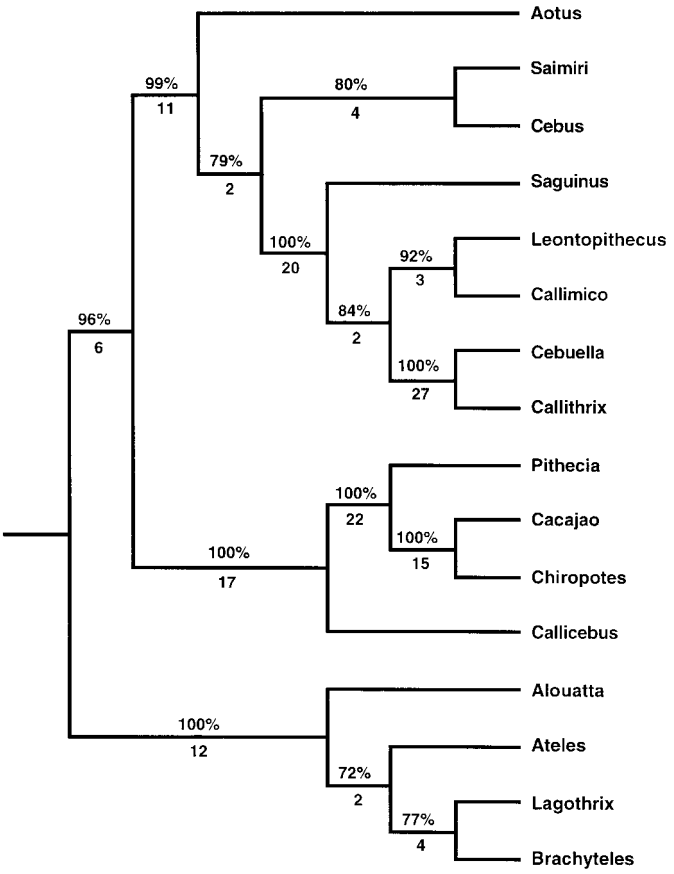


FIG. 4. New World monkey gene tree (Schneider *et al.*, 1996) based on IRBP DNA sequence data, analyzed using maximum parsimony. Bootstrap values are above branches, and "strength of grouping" (Harada *et al.*, 1995) numbers are below branches.

and annealing temperature varied with the primer combination used: for primers G4P26-GDM376/OWM, optimum annealing temperature was 68°C and optimum magnesium concentration was 3 mM except in the case of *Aotus*, for which the optimum magnesium concentration was 1.5 mM (Aot2) or 2 mM (Aot3); for primers GDP288-G5M32, optimum annealing temperature was 62°C and optimum magnesium concentration was 3 mM, except in the case of *Chiropotes*, for which the optimum annealing temperature was 65°C; for primers G5P160-GEM401, optimum annealing temperature was 68°C and optimum magnesium concentration was 3 mM; for primers GEP222-G6M97, optimum annealing temperature was 66°C and optimum magnesium concentration was 3 mM. In all cases a negative PCR control using a water blank instead of template DNA was run alongside the reactions to verify that no contaminating DNA was present in the reagents.

Double-stranded PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide for UV visualization. A DNA size marker of known concentration (ϕX176/*Hae*III digest, GibcoBRL, Gaithersburg, MD) was used to verify that a band of the expected size was present, and the amount of DNA was roughly quantified by comparing the brightness of the band with that of the size marker.

The sample was then electrophoresed on a 1% low-melt agarose gel (SeaPlaque GTG, FMC, Rockland, ME/UltraPure Low-Melt Agarose, Gibco-BRL), stained with ethidium bromide, and excised from the gel under long-wave UV illumination. The excised band was placed in a 1.5-mL tube and kept at 65°C for 10 min. The melted sample was equilibrated to 37°C, agarase was added (7.5 units/100 μL), and the sample was incubated at 37°C for 30 min or overnight. The resulting sample was used directly in sequencing reactions. This protocol for band-excised DNA purification was developed by Conroy (1995).

Sequencing

Sequencing of double-stranded PCR product was performed using the ABI Prism Dye-Deoxy Reaction Kit (Applied Biosystems, Foster City, CA), which uses fluorescent rather than radioactive labeling as a modification of the standard dideoxynucleotide DNA sequencing method. Sequencing was carried out following the ABI protocol, with 3.2 pM of primer per reaction as specified. Sequencing was carried out in a Perkin–Elmer 9600 PCR machine, using the following reaction parameters: tubes were placed in the machine after it had reached 96°C, and then 25 cycles were performed of 15″ at 96°C, 10″ at 50°C, and 4′ at 60°C.

Sequencing reactions were purified before loading onto the gel to remove unincorporated nucleotides and primers using one of three methods. Centri-Sep columns (Princeton Separations, Adelphia, NJ) and Centri-Flex columns (AGTC, Gaithersburg, MD) were both used according to the manufacturer's directions. Alternatively, empty columns were filled with 0.8 mL suspension of Sephadex G-50 Fine (Sigma Chemical, St. Louis, MO), centrifuged for 2 min at 3000*g* to remove excess water, and then centrifuged again in the same way as before after pipetting the sample into the column. Purified sequence reactions were dried down in a SpeedVac concentrator without heat and stored at −20°C until use. Before use the samples were resuspended in loading buffer containing formamide and EDTA (according to instructions from Applied Biosystems, Inc.) with 30 mg/mL Blue Dextran dye.

Electrophoretic gels for sequencing were made using either the SequaGel kit from National Diagnostics to a 4.75% acrylamide concentration or the Long Ranger kit from FMC to a 6% acrylamide concentration. Resuspended samples were loaded onto the gel and run for 12 h. Data were collected on an Apple Macintosh Quadra 650.

Data Analysis

DNA sequence verification. All electropherogram files generated by the automated DNA sequencer were

TABLE 2

Complete Sample Information for All Samples Obtained during This Study

Taxon name	Code, this study	Name/zoo ID	ISIS no.	Sex	Source	Origin of animal	Sample type
Alouatta caraya	Alc1	Dagwood/2033	n/a	M	Lincoln Park Zoo (C.D.)[a]	Asuncion, Par.	RBC lysate
Alouatta seniculus	Als1	OR449	n/a	M	San Diego Zoo	n/a	DNA
Aotus trivirgatus KI	Aot2	102.85	n/a	M	New England RPRC (C.D.)	captive born	RBC lysate
Aotus trivirgatus KVI	Aot3	330.83	n/a	M	New England RPRC (C.D.)	captive born	RBC lysate
Ateles fusciceps	Atf1	n/a	102570	M	National Zoological Park (C.D.)	n/a	RBC lysate
Brachyteles arachnoides	Bra1	FE2	n/a	M	Theresa Pope, Duke Univ.	wild caught	Whole blood
Cacajao rubicundus	Cjr1	n/a	2193	F	Los Angeles Zoo (C.D.)	n/a	RBC lysate
Cacajao calvus	Cjc2	BK1B	n/a	F	San Diego Zoo	n/a	DNA
Callicebus moloch	Ccm1	CMO-17947	n/a	M	Davis RPRC (C.D.)	captive born	RBC lysate
Callimico goeldii	Cmg3	n/a	106661	M	National Zoological Park (C.D.)	captive born?	RBC lysate
Callithrix jacchus	Cxj6	MJ-4185	n/a	M	Marmoset Research Center (C.D.)	captive born	RBC lysate
Cebus albifrons	Cba5	Quigley/15512	n/a	M	Oregon RPRC (C.D.)	captive born	RBC lysate
Cebus apella	Cbp8	Octavius/542	n/a	M	Brookfield Zoo (C.D.)	captive born	RBC lysate
Cebus capucinus	Cbc1	Heime/2516	n/a	M	Sedgwick Zoo (C.D.)	wild caught	RBC lysate
Cebus nigrivittatus	Cbn1	Houdini/786	n/a	M	Sedgwick Zoo (C.D.)	captive born?	RBC lysate
Chiropotes satanas	Csa1	n/a	n/a	M	Paula Schneider, Univ. Fed. do Para	wild caught	DNA
Lagothrix lagothricha	Lgl1	Accession no. 83	95283	F	Los Angeles Zoo (C.D.)	n/a	RBC lysate
Leontopithecus rosalia	Lnr1	n/a	105951	M	National Zoological Park (C.D.)	captive born?	RBC lysate
Pithecia pithecia	Pip1	Mr. M	911009	M	Roger Williams Park Zoo	captive born	RBC lysate
Pithecia pithecia	Pip2	SJM1060	n/a	M	Roger Williams Park Zoo (C.D.)	n/a	Whole blood
Saguinus fuscicollis	Sgf1	MH-4861	n/a	M	Marmoset Research Center (C.D.)	captive born	RBC lysate
Saimiri boliviensis	Smb1	24	n/a	M	Univ. of South Alabama (C.D.)	wild caught	RBC lysate
Saimiri oerstedii	Smo1	Stash/0203	n/a	M	Santa Ana Zoo (C.D.)	wild caught	RBC lysate
Saimiri sciureus	Sms2	228	n/a	M	Univ. of South Alabama (C.D.)	wild caught	RBC lysate

[a] (C.D.), Charlene Dickinson donated these samples; the institutions indicated are those from which she originally obtained the sample.

examined by eye to verify the sequence assignments made by the computer. Both strands were sequenced and for each primer pair at least two amplification reactions were performed and sequenced. Sequences generated from different primer pairs were compared using AutoAssembler (Applied Biosystems Inc., 1993) and then rechecked by eye.

For the three female individuals included in this study, multiple sequencing reactions were performed and heterozygosity was tested by examining the electropherograms for double peaks and comparing sequences from different primers. No evidence of heterozygosity was detected.

TABLE 3

Primer Sequences for G6PD

Primer	Sequence (5′ to 3′)
G4-P26	GTGGCTGTTCCGGGATGGCCTTCTGC
G5-P160	AACCGCCTCTTCTACCTGGCCTTGC
G5-M32	AGTTGCGGGCAAAGAAGTC
G6-M97	CAGGTAGTGGTCGATGCGGTAGATCTG
GD-P288	CCAGTGTACTCCCAGGTCAAAGTG
GD-M376/OWM	CCATCTCAGCAGCTCTGCACATCCAG
GE-P222	GCCAGGCCGCTAGGTGGTAGCC
GE-M401	GCTGGGTCCCCAGTGGATTGGA
GE-P358	CAAACCAATGAGGAAGCTATGCC

Note. Primers were used for both PCR amplification and DNA sequencing.

Alignment. Alignment and insertion of gaps were performed by eye. Although the G6PD data are intron data and thus not constrained in the same way as protein-coding data, the amount of sequence divergence existing between taxa in this study is small enough that alignment for the most part is straightforward. Intron D proved to be more variable than Intron E, and in the former case alignments were also submitted to the computer alignment algorithm CLUSTAL V (Higgins *et al.*, 1992) and then reexamined by eye.

Phylogenetic analysis. Maximum parsimony analyses were performed using test version 4.0d56 of PAUP*, written by David L. Swofford. Analyses were performed on PowerMac 7100 and 7200 computers.

In order to find the most parsimonious tree for each dataset, a branch-and-bound search with MULPARS was conducted. Transversions were weighted twice transitions to reflect empirically derived models of molecular evolutionary change (Wakeley, 1996). Gaps were treated as missing data.

Nonparametric bootstrap analyses were also performed. In this case 100 bootstrap replicates with 100 heuristic replicates per bootstrap replicate were carried out. Transition:transversion weighting was 1:2 as in the branch-and-bound tree searches. The resulting

bootstrap values are presented as measures of the relative strength of support in the data for particular groupings on the maximum parsimony tree. In a test of the accuracy of bootstrap values using both numerical simulations and experimentally produced phylogenies (Hillis and Bull, 1993), it was shown that bootstrap proportions are biased estimators of the true clades in such a way that for any bootstrap over 50%, the bootstrap is an underestimate of the true probability of being correct, and bootstrap proportions over 70% represent true clades over 95% of the time. Bootstrap values below 50% indicate that a clade is not well supported by the data.

The "list of apomorphies" output of PAUP* was used to find the putative nucleotide sequence changes reconstructed along each branch of the phylogenetic trees. Synapomorphies were divided into those that changed unambiguously along a particular branch and those that are ambiguously assigned, and the number of unambiguous synapomorphies for each branch is shown in the figures to indicate which clades have relatively larger numbers of supporting synapomorphies.

MacClade (Maddison and Maddison, 1993) was used to map the G6PD data onto the topologies generated by epsilon-globin (Harada *et al.*, 1995) and IRBP (Schneider *et al.*, 1996). The "tree lengths" output of PAUP* was used to perform a Kishino–Hasegawa (Kishino and Hasegawa, 1989) test of the difference between these alternative gene trees and the G6PD gene trees.

Estimates of divergence times. To estimate divergence times for representative nodes on the G6PD gene tree, branch lengths were estimated by applying a Jukes–Cantor correction using the minimum evolution optimality criterion in PAUP*, with the tree topologies obtained in the maximum parsimony search used as constraint trees. The branch lengths obtained using this procedure were used in a "local clock" calculation (Bailey *et al.*, 1991, 1992a).

Because there is only one outgroup included, the calibration point for the tree had to be within the platyrrhine clade. The earliest known fossil platyrrhine is *Branisella boliviana,* a generalized platyrrhine dated to approximately 26 million years ago (myr) (MacFadden, 1990); by 19–20 myr specimens are found that show affinities to modern forms, suggesting that the radiation of modern platyrrhines had begun. These specimens include *Dolichocebus gaimanensis,* linked with *Cebus* or *Saimiri,* and *Tremacebus,* linked with *Aotus* (Fleagle, 1988, p. 346). Thus a calibration date of 20 myr for the split between the pitheciine clade and the remaining platyrrhines was used as a conservative, minimum estimate. Because of the uncertainty in this estimate, the dates given here are only rough estimates of the divergence times for the various nodes in the tree.

RESULTS

DNA Sequences

Aligned sequences for the 25 G6PD DNA sequences collected in this study are shown in Fig. 5. These sequences include 1286 aligned base positions, consisting of 571 aligned positions for Intron D and 715 aligned positions for Intron E.

Maximum Parsimony Analyses

Using a 1:2 transition:transversion weighting, three most-parsimonious trees, length 756, were found (Fig. 6). The number of unambiguous changes reconstructed for each branch is shown below the lines as generated in PAUP*. The arrangements that differ in the three trees involve the relationship between *Aotus,* a clade containing *Cebus* and *Saimiri,* and a clade containing *Callithrix, Callimico, Leontopithecus,* and *Saguinus* (the Callitrichinae). The alternative arrangements are: (((*Aotus*) (*Cebus, Saimiri*)) (Callitrichinae)); ((*Aotus,* Callitrichinae) (*Cebus, Saimiri*)); and ((*Aotus*) ((*Cebus, Saimiri*) (Callitrichinae))).

The strict consensus of the three most-parsimonious trees is shown in Fig. 7. Nonparametric bootstrap percentages are shown above the branches, and unambiguous changes are shown below the branches. As discussed above, there is a trichotomy between *Aotus,* a *Cebus–Saimiri* clade, and the callitrichines. Except for this trichotomy the consensus tree in Fig. 7 has fully resolved dichotomous branchings. All three most-parsimonious trees for G6PD agree in showing a basal split between a *Callicebus*–pitheciin clade and all other platyrrhines and an *Alouatta*–atelin clade sister to an *Aotus/Cebus–Saimiri*/callitrichine clade.

The branch separating the pitheciines and *Callicebus* from the remaining platyrrhines is poorly supported, with a bootstrap percentage of 40% and one of the lowest values on the tree for unambiguous changes (2). Within the pitheciine clade, however, the branches are all very strongly supported, showing *Cacajao* and *Chiropotes* as sister taxa with 100% bootstrap support and nine unambiguous changes; *Pithecia* appears as the sister taxon to the *Cacajao–Chiropotes* clade with 100% bootstrap support and nine unambiguous changes. *Callicebus* is the most basal member of the pitheciine clade, with 78% bootstrap support and five unambiguous changes.

The *Alouatta*–atelini clade is supported at 70% (with four unambiguous changes). The Atelini or atelins (*Ateles, Brachyteles,* and *Lagothrix*) appear as a clade, with 89% bootstrap support and five unambiguous changes; within that group, *Brachyteles* and *Lagothrix* emerge as sister taxa, although this relationship is less strongly supported, with a bootstrap percentage of 72% and only one unambiguous change along the branch linking those two taxa.

The *Aotus/Cebus–Saimiri*/callitrichine group ap-

peared in 83% of the bootstrap trees, and there are either four or six unambiguous changes supporting the node, depending on which of the underlying topologies is used to reconstruct changes. The relationships within this group are the locus of disagreement between the three most parsimonious trees; there are no unambiguous changes to support any alternative topology, and the bootstrap values for the three alternative topologies range from 24% (for an *Aotus*–callitrichine clade) to 29% (for a (*Cebus*–*Saimiri*)–callitrichine clade) to 33% (for an *Aotus*–(*Cebus*–*Saimiri*) clade). Support for the *Cebus*–*Saimiri* clade is high, with a bootstrap value of 93% and eight or nine unambiguous changes, again depending on which underlying topology is used to reconstruct changes.

Support for the callitrichine clade is also high, with a bootstrap percentage of 100% and 13 unambiguous changes. Within the callitrichine clade, *Callimico* and *Callithrix* appear as sister taxa; this clade has an 80% bootstrap percentage and 4 unambiguous changes. The *Callimico*–*Callithrix* clade is linked to *Leontopithecus* (92% bootstrap support, 3 unambiguous changes) and then to *Saguinus*.

Synapomorphies

The total putative synapomorphies supporting each proposed clade in the tree are shown in Table 4. Unambiguous changes are marked with an asterisk. In cases where the reconstructions differ for the three most-parsimonious trees, that is indicated by placing the synapomorphic position in parentheses and describing which tree supports it (a, b, or c of Fig. 6).

Length Comparisons with Epsilon-Globin and IRBP

Mapping the G6PD data onto the topologies found using epsilon-globin (see Fig. 3) and IRBP (see Fig. 4) yields trees with lengths of 760, four steps longer than for the G6PD parsimony trees. Using the Kishino–Hasegawa test (Kishino and Hasegawa, 1989) as implemented in PAUP* 4.0d56, these trees are not significantly different in length (IRBP: difference = 4, s.d. (diff) = 2.82733, $t = 1.4148$, $P = 0.1574$; epsilon-globin: difference = 4, s.d. (diff) = 3.16154, $t = 1.2652$, $P = 0.2060$) from the trees derived from the G6PD data, suggesting that the gene trees are not significantly different.

Estimates of Divergence Times

Starting with a calibration date of 20 myr for the basal split between the pitheciine clade and the remaining platyrrhines, and using a "local clock" calculation method (Bailey *et al.*, 1991, 1992a), divergence date estimates were made for representative nodes of the G6PD gene tree. These calculations were performed using a Jukes–Cantor correction and the minimum evolution optimality criterion as implemented in PAUP* to fit branch lengths to all three most-parsimonious trees; the resulting dates, though not substantially different, were averaged.

If the basal split in the G6PD tree (Fig. 7), between the pitheciines and the cebids, is set at 20 myr, then the subsequent split between the atelines and the cebines falls at 17.5 myr. The split between *Alouatta* and the Atelini falls at 15.1 myr and the basal split in the Atelini falls at 12 myr. The unresolved split between *Aotus*, *Cebus*, and *Saimiri*, and the callitrichines falls at 15.8 myr; the split between *Cebus* and *Saimiri* falls at 12.6 myr; and the basal split in the callitrichine clade occurs at 11.3 myr.

Returning to the basal node and examining the pitheciine clade, the branch separating the basal node from the split between *Callicebus* and the pitheciins is too short to meaningfully differentiate the divergence time from the basal 20 myr; the split between *Pithecia* and the *Chiropotes*–*Cacajao* clade falls at 12 myr and the split between *Chiropotes* and *Cacajao* occurs at 6.3 myr.

DISCUSSION

The G6PD gene tree recovers the ateline, pitheciine, and callitrichine clades upon which all studies have agreed. In its details, the G6PD gene tree is largely in agreement with those reconstructed from sequence information for epsilon-globin and IRBP, and as mentioned above, the G6PD data are not significantly incompatible with the topologies generated by epsilon-globin and IRBP. They are less concordant with the 16S rRNA results, but as mentioned in the introduction, those results should be considered with caution. The implications in terms of morphological features of the placements of a number of taxa are intriguing and will be discussed in detail below.

Cebus and Saimiri

Cebus and *Saimiri* form a clade in the G6PD analysis, in line with both the epsilon-globin and the IRBP results from published studies. A *Cebus*–*Saimiri* clade is not a new suggestion, and there is evidence from morphological and other non-sequence-based molecular data to support it. Using immunodiffusion distance data, Baba *et al.* (1979) found a sister group relationship between these two taxa. Using morphological data, Rosenberger (1977, 1980, 1981, 1984, 1992) has made a series of strong arguments for this grouping.

What are the implications in terms of the biology of these two species? Both have a number of unique features; as stated by Ford (1986, p. 119), "each has developed a host of autapomorphies." For *Saimiri*, these include small size and an interorbital fenestra; *Cebus*, on the other hand, is relatively large, apparently quite intelligent, and has a semiprehensile tail

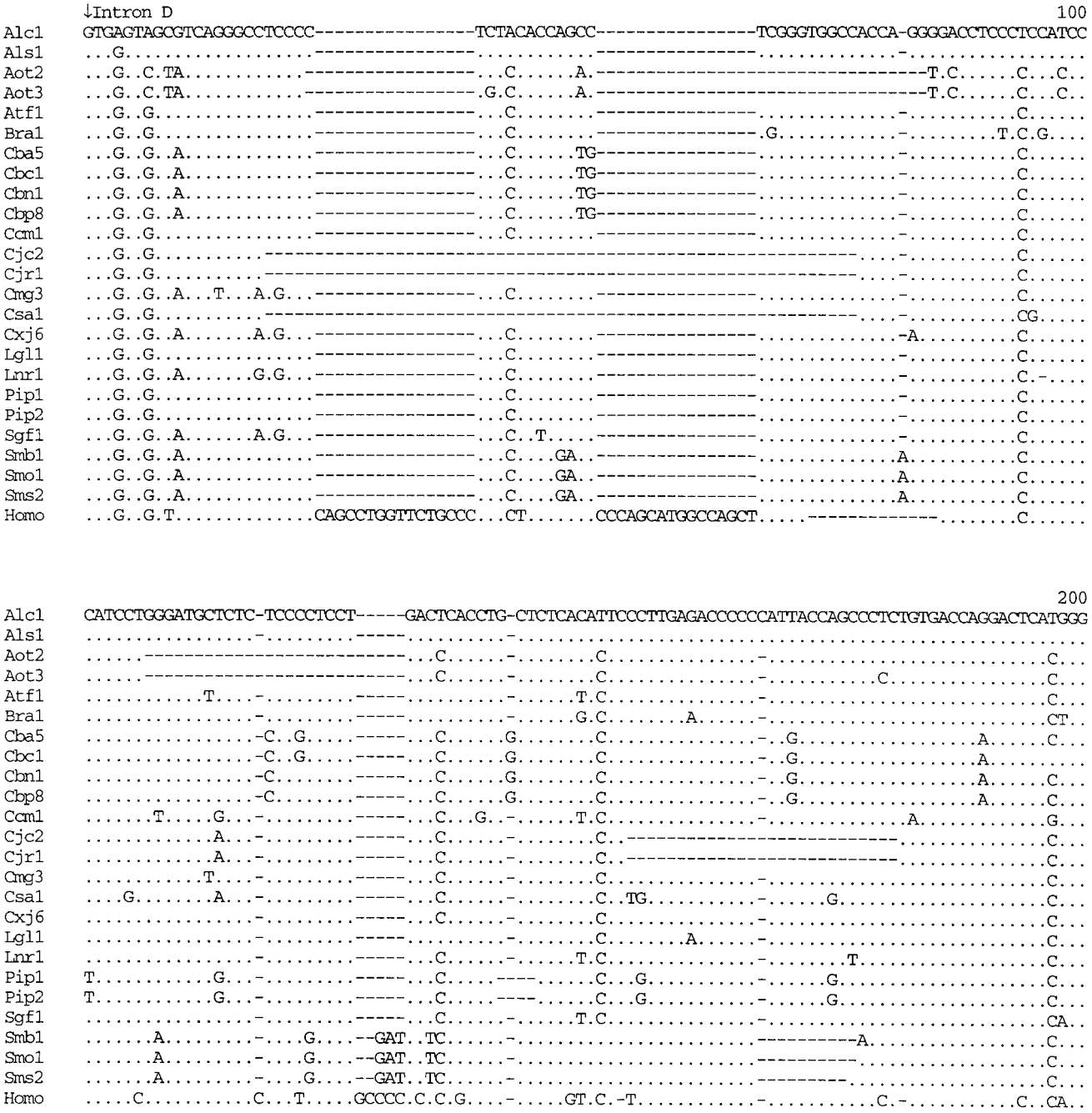
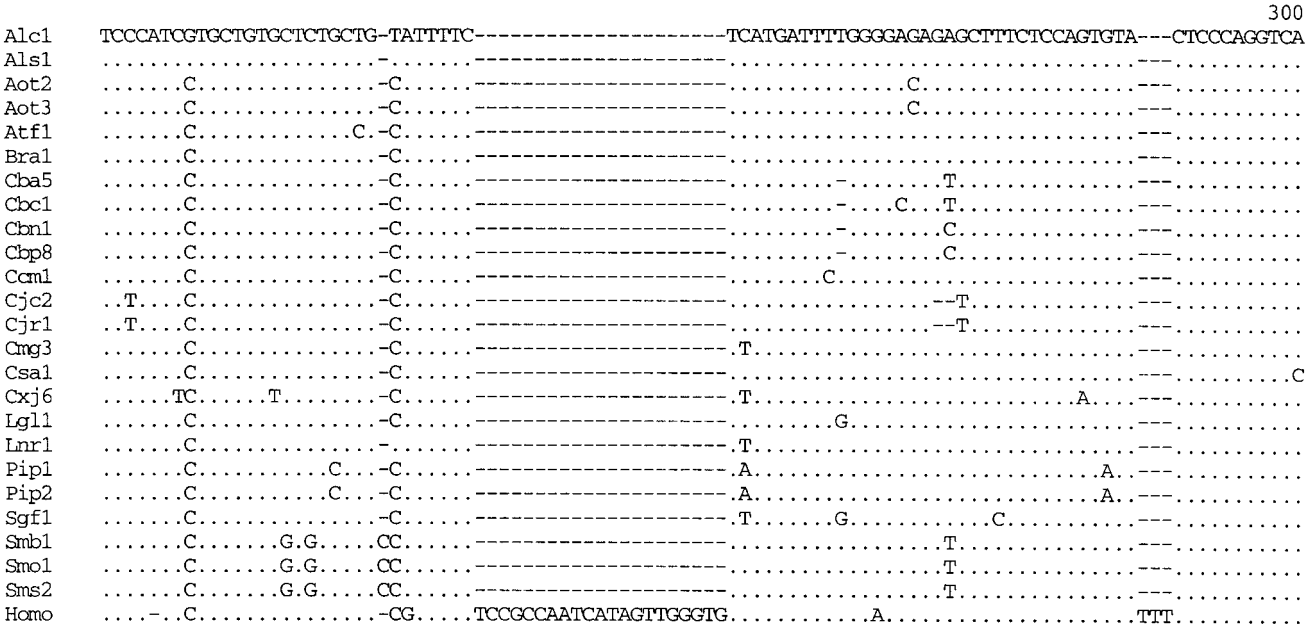


FIG. 5. Aligned sequences for glucose-6-phosphate dehydrogenase (G6PD) introns D and E, corresponding to introns 4 and 5 of Martini *et al.* (1986). A dot indicates that the nucleotide is the same as that shown in the top row. A dash indicates a deletion. Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under Accession Nos. AF028473–AF028496 (Intron D) and AF028497–AF028520 (Intron E).

(this last trait was previously used to group *Cebus* with the atelines, but the trait is currently interpreted as a parallelism; Ford, 1986). In terms of the morphological data used in Ford (1986), *Saimiri* and *Cebus* share two or three synapomorphies: enlarged second premolar, sharp posterior border of tibial trochlear facet, and, possibly, according to Ford (1986), relatively broad second through fourth premolars. Rosenberger (1977, 1980, 1981, 1984, 1992) argues that they also share a narrow interorbitum, long narrow nasals, a convex glabella, and vomer exposure in orbits, as well as several dental features.

Cebus, Saimiri, and Aotus with the Callitrichines

As in all of the other DNA sequence analyses, *Cebus*, *Saimiri*, and *Aotus* appear on the G6PD gene tree as



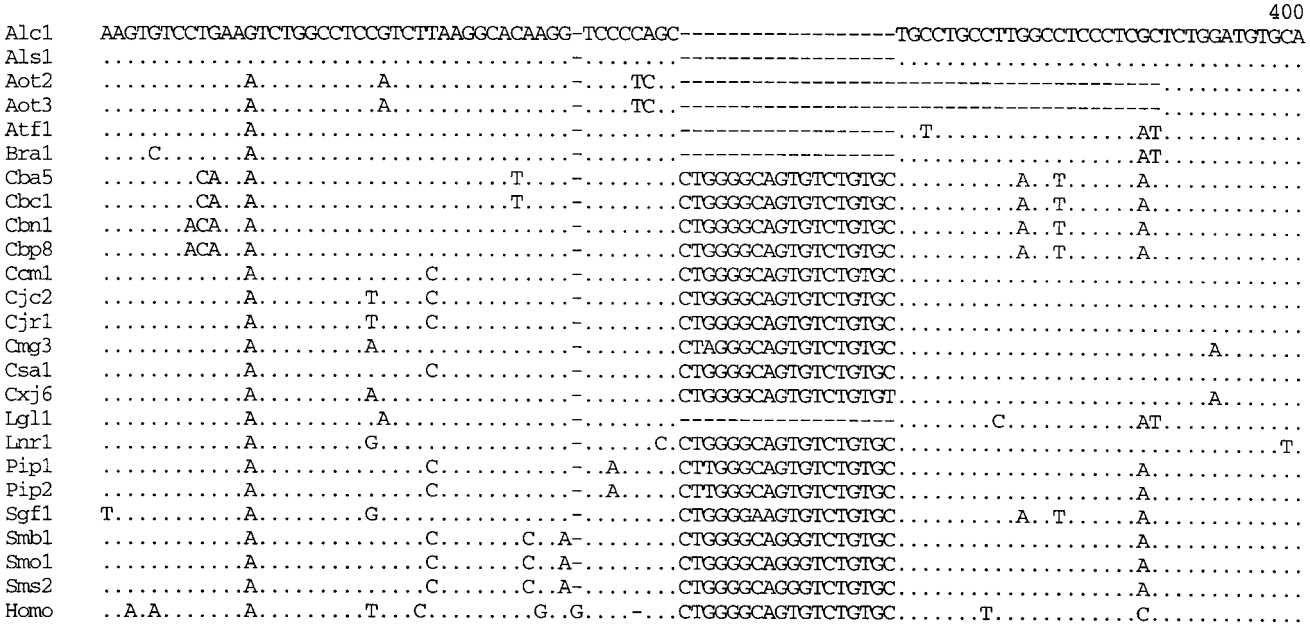


FIG. 5—*Continued*

members of a clade with the callitrichines. Like the *Cebus–Saimiri* clade, the placement of *Saimiri* and *Cebus* with the callitrichines rather than with the atelids is also not new, although it has not received the same degree of support. Again, Rosenberger (1981) has used morphological data to support this placement, arguing that *Saimiri* and *Cebus* share with the callitrichines enlarged canines and second premolar, a reduced third molar, gracile zygoma, and an open glenoid fossa. Other studies using morphological features (e.g., Ford, 1986; Kay, 1990) did not come to the same conclusion. However, these studies rely on different suites of morphological characters, with Kay (1990) using only dental characters. In particular, the placement of *Cebus* with the callitrichines might seem implausible on morphological grounds. However, both *Cebus* and *Saimiri* share a reduced third molar, as seen in *Callimico* (Martin, 1992).

No morphological analyses have supported the placement of *Aotus* with the callitrichines, although Kay's

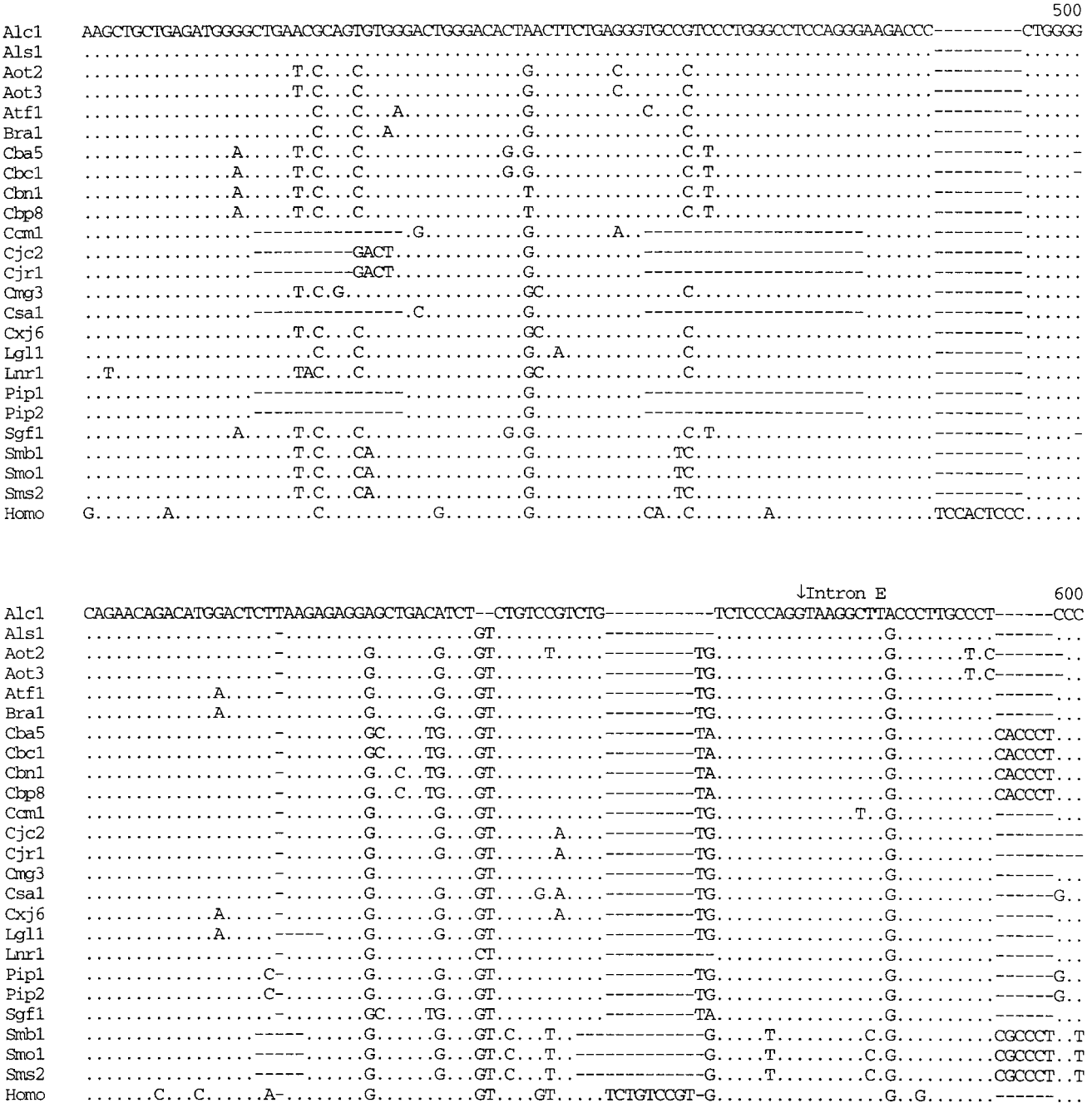


FIG. 5—*Continued*

(1990) tree topology shows the placement of *Aotus* as unresolved between the callitrichine and ateline clades. The most common placement of *Aotus* in the published morphological studies has been as a sister taxon to *Callicebus* and the pitheciines (Rosenberger, 1977, 1981); Ford (1986) has an *Aotus–Callicebus* clade more distantly related to the other platyrrhines. However, Dunlap *et al.* (1985), in a study of forelimb anatomy, found that there were large differences between *Aotus* and *Callicebus*. The placement of *Aotus* found using the DNA sequence data requires a reevaluation of the traditional morphological view of its affinities.

Branching Order within the Aotus/Cebus–Saimiri/Callitrichine Clade

Although the placement of *Aotus* with the callitrichids and the *Cebus–Saimiri* clade is strongly supported by the molecular data, there is less strong support for the exact branching order within that larger clade. The G6PD analysis here does not resolve this branching

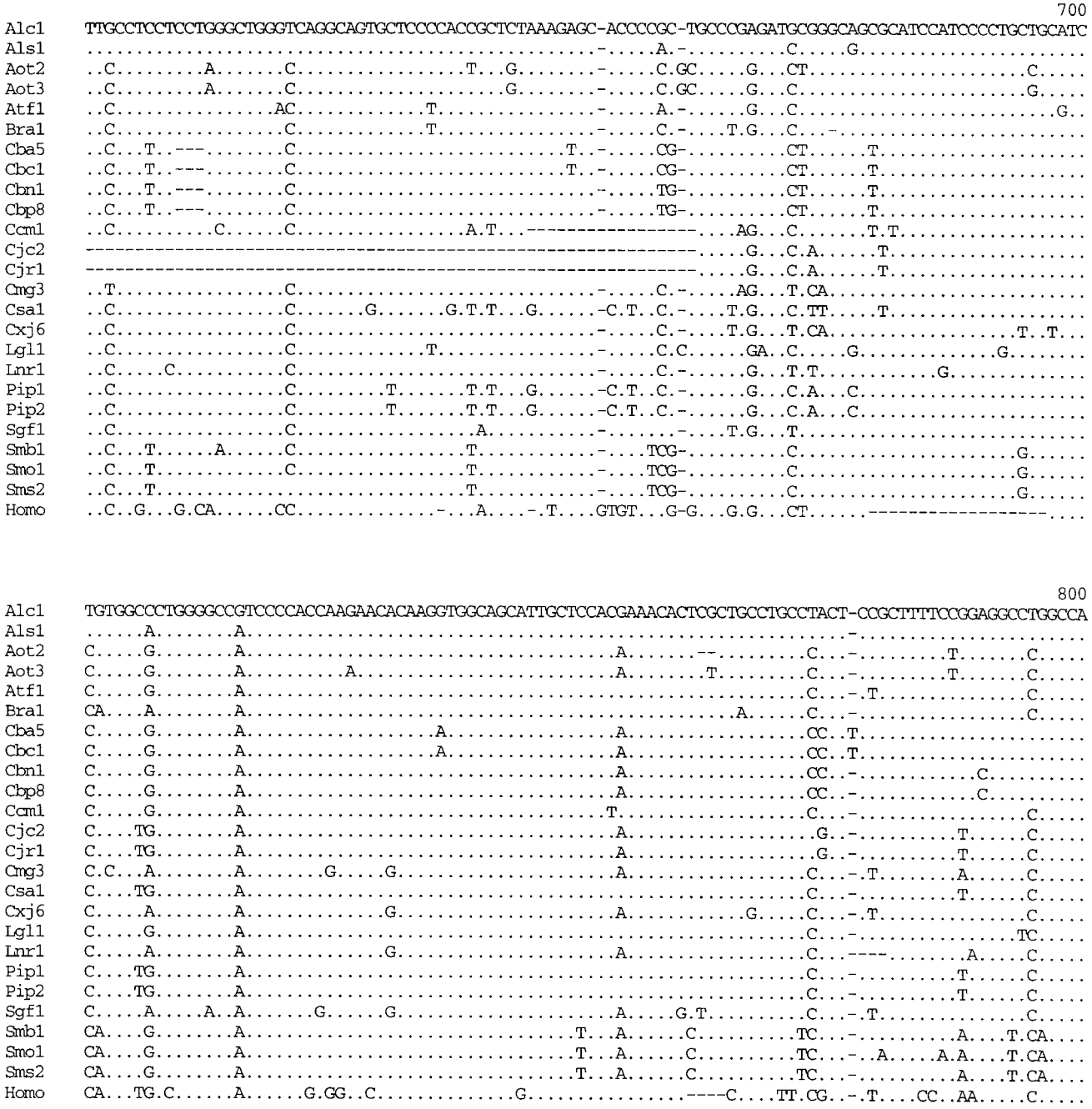


FIG. 5—*Continued*

order. An examination of the synapomorphies reconstructed for each of the three alternative topologies (Table 4) shows that a *Cebus/Saimiri*/callitrichine clade is supported by two synapomorphies (neither unambiguous), while an *Aotus/Cebus/Saimiri* clade is supported by one (ambiguous) synapomorphy and an *Aotus*/callitrichine clade is not supported by any putative synapomorphies. In the epsilon-globin analysis of Harada *et al.* (1995) *Aotus* appeared as the sister group of the callitrichines at 85% bootstrap support. In the IRBP analysis of Schneider *et al.* (1996) on the other hand, it is a *Cebus–Saimiri* clade that appears as the sister group to the callitrichines, at 79%. Thus, these datasets disagree with each other and this question requires further investigation.

Callicebus

Support is strong from the molecular data for a placement of *Callicebus* as the sister group to the pitheciines. This placement appears in all of the molecu-

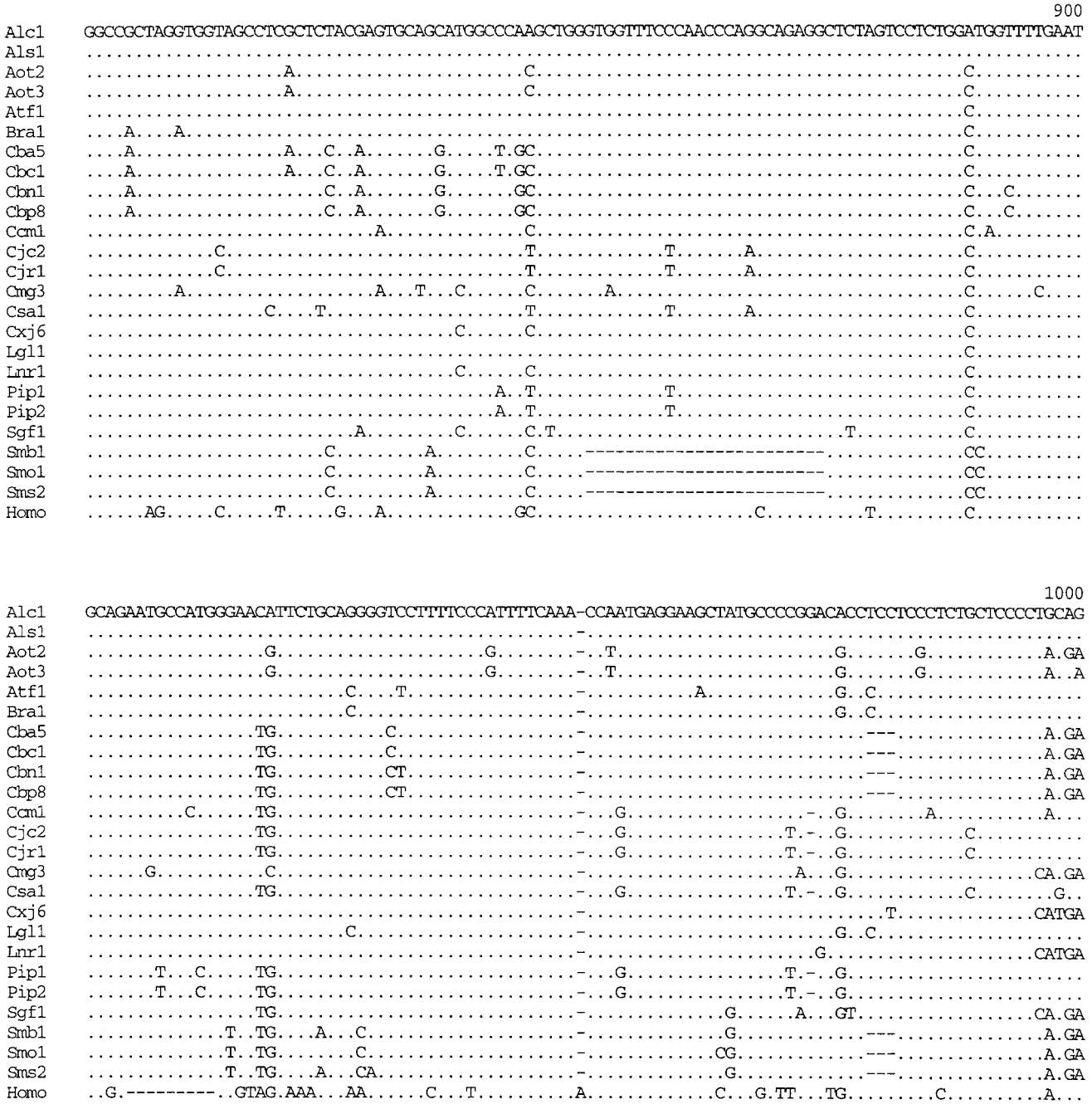


FIG. 5—*Continued*

lar datasets except the 16S analysis. In terms of morphological data, Rosenberger (1977, 1981) links *Callicebus* with the pitheciins based on morphological characters, arguing that they share two synapomorphies: an enlarged protocone and a distal fovea on the first and second lower molars. However, this placement does not appear in Ford (1986) or Kay (1990). The molecular evidence and the presence of morphological synapomorphies suggest that *Callicebus* is likely a member of the pitheciine clade.

Callimico

One of the most intriguing results to emerge from analysis of the molecular data sets is a placement of *Callimico* as a sister taxon to *Callithrix* or *Leontopithecus* rather than as the sister group to all of the marmosets and tamarins, as has been inferred from the morphological data. Conflict as to which genus is the sister group to *Callimico* is present among the existing gene trees. The G6PD analysis here supports a *Callimico–Callithrix* clade, as does the epsilon-globin lo-

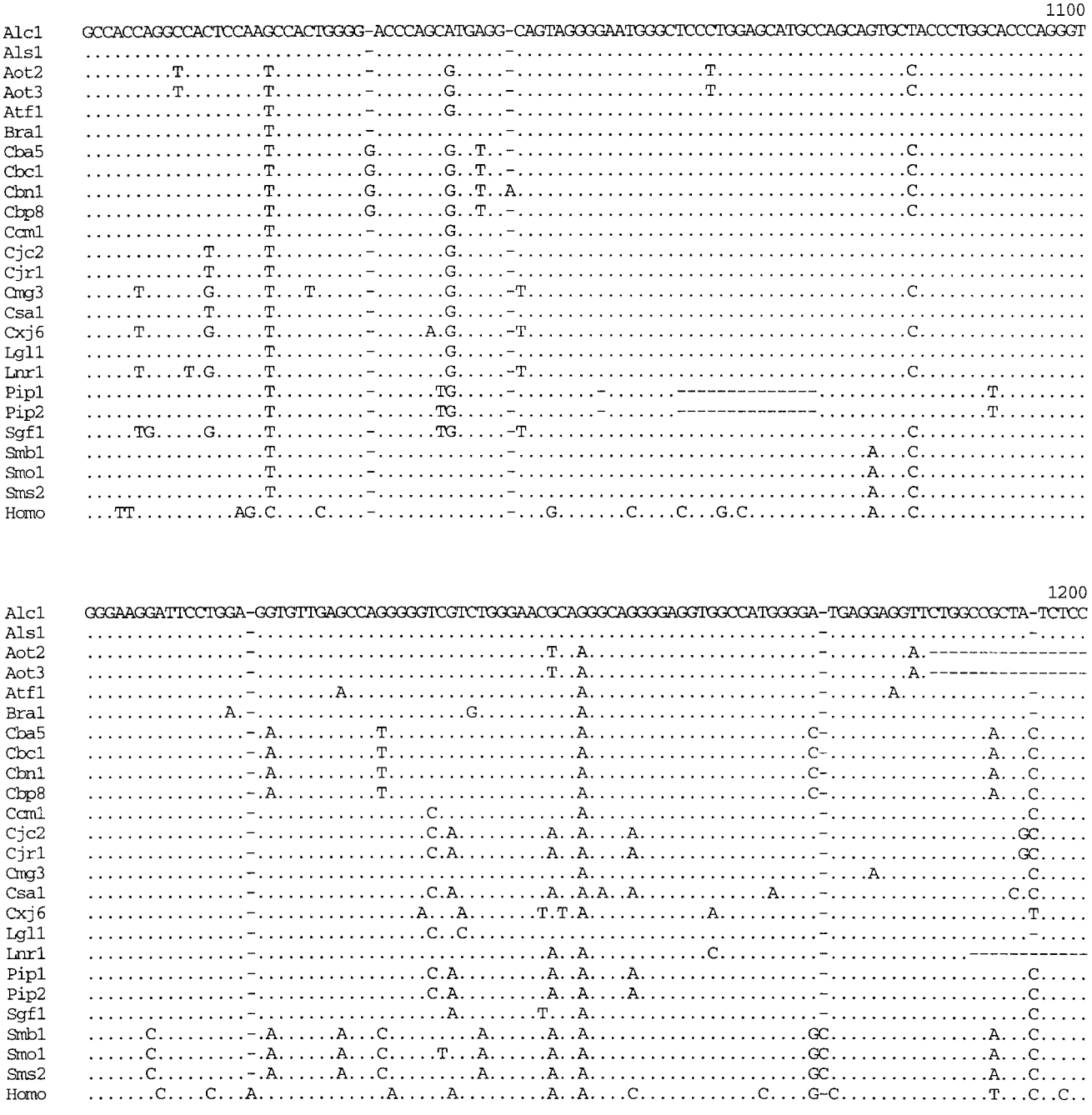


FIG. 5—*Continued*

cus. However, the IRBP locus supports a link between *Callimico* and *Leontopithecus*, rather than between *Callimico* and *Callithrix*. But what is clear from the DNA sequence evidence is that *Callimico* may not be simply the sister taxon to all the other marmosets and tamarins. Indeed, no DNA sequence data set supports the traditional view that holds *Callimico* as essentially an "intermediate" between the small-bodied marmosets and tamarins and the remaining, larger-bodied, platyrrhines.

Non-DNA-sequence molecular evidence has also supported this conclusion. Cronin and Sarich (1975), using albumin and transferrin immunological data, found a callitrichine clade showing *Leontopithecus* as the first lineage to branch, then *Saguinus*, and then *Callimico* and *Callithrix–Cebuella*. Seuánez *et al.* (1989), in a study of karyology and LINE elements, found the following branching order for the callitrichines: (*Saguinus*, (*Leontopithecus*, (*Callimico* and *Callithrix*))). It should be noted that one study of dental ontogeny (Byrd, 1981) placed *Callimico* as the sister group to the tamarins to the exclusion of the marmosets, a finding in accord with the IRBP results.

```
                                                                                                                                1286
Alc1    AGGGAGGGCGTT----------------------------------------------------------------------------------AGGGTGACGCAGCTCGGGGCTCCCAGCAG
Als1    ............----------------------------------------------------------------------------------............................
Aot2    ---------...----------------------------------------------------------------------------------............................
Aot3    ---------...----------------------------------------------------------------------------------............................
Atf1    ............----------------------------------------------------------------------------------............................
Bra1    ............----------------------------------------------------------------------------------........A.G................
Cba5    ........T...----------------------------------------------------------------------------------............................
Cbc1    ........T...----------------------------------------------------------------------------------............................
Cbn1    ........T...----------------------------------------------------------------------------------.......T....................
Cbp8    ........T...----------------------------------------------------------------------------------.......T....................
Ccm1    ..A.........----------------------------------------------------------------------------------........A....T.............
Cjc2    ........T...----------------------------------------------------------------------------------.A..................T.........
Cjr1    ........T...----------------------------------------------------------------------------------.A..................T.........
Cmg3    ........TA..----------------------------------------------------------------------------------.A............T....T.........
Csa1    ........T...----------------------------------------------------------------------------------.A..................T.........
Cxj6    ........T...----------------------------------------------------------------------------------.............T..............
Lgl1    ............----------------------------------------------------------------------------------.......T....................
Lnr1    --------T...----------------------------------------------------------------------------------............................
Pip1    ............----------------------------------------------------------------------------------........................G...
Pip2    ............----------------------------------------------------------------------------------........................G...
Sgf1    ........T...----------------------------------------------------------------------------------........A.......T............
Smb1    ........T...----------------------------------------------------------------------------------....................T....C....
Smo1    ........T...----------------------------------------------------------------------------------.......T................C....
Sms2    ........T...----------------------------------------------------------------------------------....................T....C....
Homo    T..........CTGAATGATGCAGCTGTGATCCTCACTCCCCGAAGAGGGGTTCAAGG....A.........C.............
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FIG. 5—*Continued*

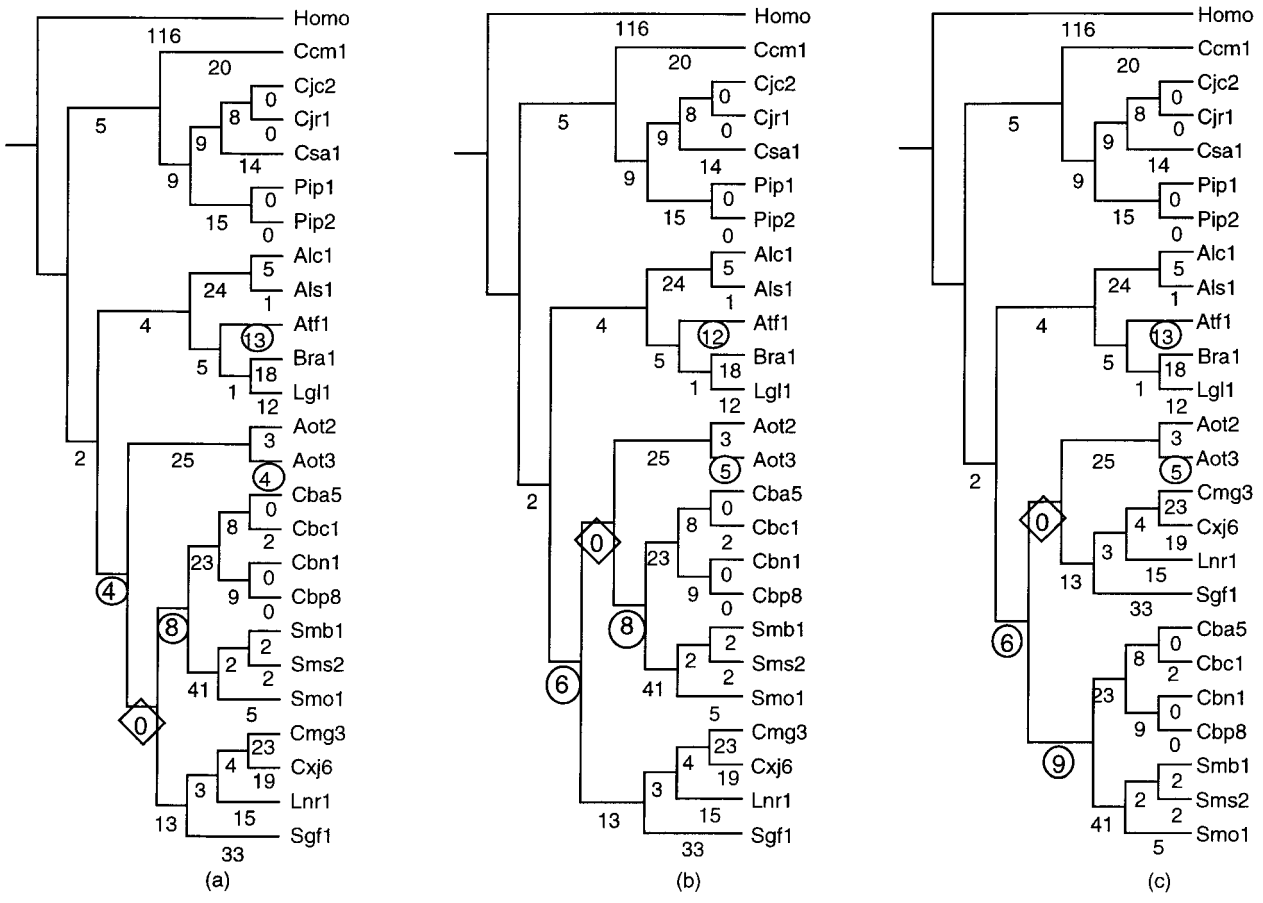

FIG. 6. Three most-parsimonious trees (length 756) for 25 aligned G6PD intron D and E sequences, analyzed using maximum parsimony. Transversions were weighted twice transitions. Numbers of unambiguous changes are shown below branches as generated in PAUP*. The three maximum parsimony tree topologies show (a) *Cebus* and *Saimiri* in a clade with the callitrichines; (b) *Aotus* in a clade with *Cebus* and *Saimiri;* (c) *Aotus* in a clade with the callitrichines. Circles indicate branches for which the number of unambiguous changes differs depending on reconstruction (a, b, or c); diamonds indicate branches with zero unambiguous changes, which support the three alternative topologies.

The placement is intriguing because it contradicts the clear implications of the majority of the morphological evidence. All recent morphological studies of the platyrrhines show *Callimico* as the most basal member of the callitrichine clade. Ford (1986) argues that there are no synapomorphies that would support a closer relationship between *Callimico* and particular callitrichids. The alternative placement proposed here would have ramifications for the evolution of both dental traits and reproductive parameters.

One of the major features distinguishing callitrichines from other platyrrhines is the lack of a third molar. Thus, if *Callimico* is not basal to the marmosets and tamarins but instead forms the sister group to *Callithrix,* independent complete loss of the third molar in tamarins and marmosets would seem to be required. However, Byrd (1981) notes that third molars are occasionally seen in tamarins and marmosets. Also, as noted above in the discussion of the affinities of *Cebus* and *Saimiri,* those taxa show a reduced third molar.

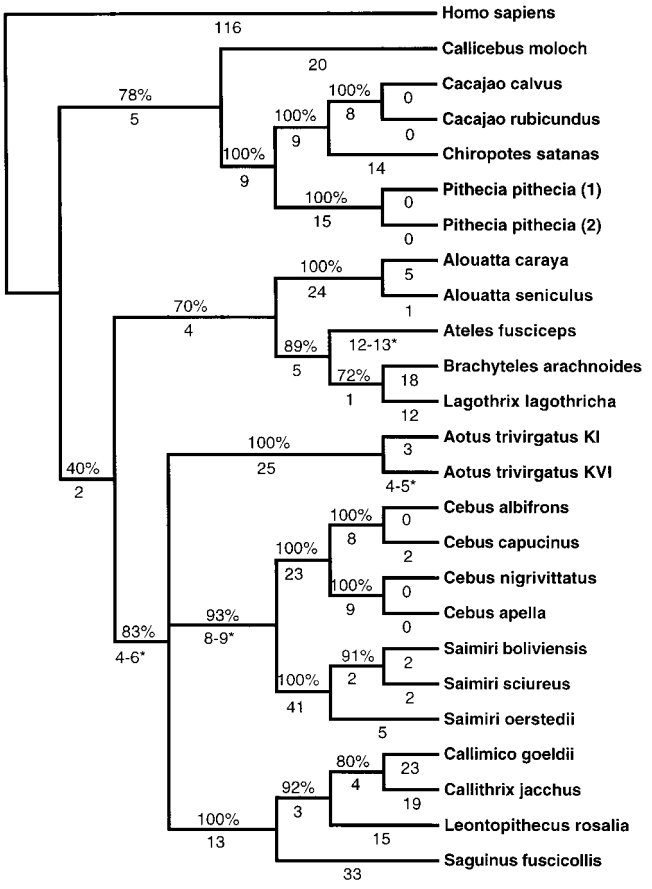

FIG. 7. New World monkey gene tree based on G6PD data; strict consensus of three most-parsimonious trees for aligned G6PD intron D and E sequences for 25 taxa. Nonparametric bootstrap values above branches; reconstructed unambiguous changes below branches, to indicate relative measure of support for each branch. *Number of unambiguous changes differs among three most-parsimonious trees (see Fig. 6).

TABLE 4

G6PD Nucleotide Sequence Positions with Synapomorphies[a,b] for Proposed Platyrrhine Clades

Alouatta/atelin/callitrichine/*Aotus/Cebus/Saimiri:* 130, 132, 457, 458, 469, 653*, 655*, 830, 1137, 1148
Callicebus/pitheciins: 114*, 328*, 387, 428, 429, 430, 431, 639, 641*, 645, 918, 954*, 1083, 1135*
Pitheciins: 156*, 175*, 598*, 673*, 706, 788*, 830, 845, 859*, 971, 997*, 1155*
Cacajao/Chiropotes: 114*, 155, 548*, 629, 637, 680*, 867*, 989*, 1013*, 1209*, 1259*, 1276*
Alouatta/atelins: 136*, 658, 845*, 919*, 997*, 1083
Atelins: 388*, 514*, 635*, 927*, 979*
Brachyteles/Lagothrix: 161*, 658
Aotus/Cebus/Saimiri/callitrichines: 10*, 422*, 660, 754*, 999 (* for trees b and c), 1000*, 1209 (* for trees b and c)
Callitrichines: 18*, 20*, 150, 254*, 323*, 671*, 707*, 731*, 779*, 838*, 996*, 1006*, 1013*, 1044*
Callimico/Callithrix/Leontopithecus: 387, 446*, 536, 673*, (918 for tree a), 919*, (976 for trees b and c), 998
Callimico/Callithrix: 150, 323*, 393*, 674*, 1272*
Cebus/Saimiri: 171, 271*, 607*, 659*, 667*, 825*, (918 for trees b and c), (976* for trees b and c), 1119*, 1130*, 1173, (1191* for trees a and c)
Cebus/Saimiri/callitrichines: (918, 976 for tree a)
Aotus/Cebus/Saimiri: (1191 for tree b)

[a] Asterisks indicate positions where changes are unambiguous.
[b] Parentheses indicate positions whose placement or status as unambiguous differs depending on which MP tree is used to reconstruct changes.

Thus, it is likely that this trait is quite labile, and at the very least it is polymorphic for presence/absence in some of the species in question. Dentally, the marmosets and tamarins are also characterized by the loss of the hypocone. Maier (1977) and Rosenberger (1977) argue that the *Callimico* pattern is intermediate between that seen in *Saimiri* and that found in the other callitrichines. If so, this would be another character evolving in parallel in the marmosets and tamarins if the nonbasal placement of *Callimico* is correct.

There are implications for the reproductive biology of these animals as well. Marmosets and tamarins are unique among the anthropoids in typically producing twins. Martin (1992, p. 379) notes: "the association of regular dizygotic twinning with a simplex uterus is apparently confined to marmosets, tamarins and hairy armadillos." While uterine morphology is the same in all of the callitrichines including *Callimico,* the marmosets and tamarins do have specialized placentation in which the dizygotic twins share a single placenta. Numerous vascular anastomoses exist, and bone marrow chimerism results (Benirschke *et al.,* 1962). Clearly, this could potentially be problematic if the twins were of different sexes, so there must exist special mechanisms to prevent, for example, deleterious hormonal effects. The DNA sequence evidence suggests that such a system may have evolved twice within the callitrichines.

Brachyteles and Lagothrix

The placement of *Lagothrix* and *Brachyteles* as sister groups emerges in the G6PD, epsilon-globin, and IRBP analyses, albeit with moderate bootstrap support. *Brachyteles* is not included in the 16S analysis, and thus there are only three molecular datasets available to address this question. In this case, all three datasets agree on the topology but the internal support in each data set is not as strong as that seen for other portions of the tree.

The morphological data generally support instead an *Ateles–Brachyteles* grouping; indeed, Ford (1986, p. 110) remarks: "there is little to no evidence that would argue for a closer relationship between *Lagothrix* and *Brachyteles*." Both *Ateles* and *Brachyteles* feature a highly reduced pollex as part of a complex of traits related to semibrachiation; both are highly agile and gracile, while *Lagothrix* is more robust (Rosenberger and Strier, 1989). If they are not sister taxa either these features have evolved twice, in *Ateles* and *Brachyteles,* or the characters in *Lagothrix* have undergone reversal. The placement supported by the molecular data, if it can be further confirmed, requires a fresh look at ateline evolution.

Leontopithecus and Saguinus

The G6PD results show *Leontopithecus* as the sister group to a *Callimico–Callithrix* clade and *Saguinus* as the sister group to the *Leontopithecus/Callimico/Callithrix* clade. Harada *et al.* (1995)'s epsilon-globin phylogeny instead links *Leontopithecus* and *Saguinus* into a clade that is sister to a *Callimico–Callithrix* clade. In a third variation, Schneider *et al.* (1996)'s IRBP analysis shows *Leontopithecus* and *Callimico* in a clade sister to *Callithrix*. *Saguinus* is again sister to the *Leontopithecus/Callimico/Callithrix* clade. Thus, there is some disagreement about the placement of *Saguinus.*

In terms of morphological studies, Ford (1986) and Kay (1990) place *Leontopithecus* as the sister group to *Callithrix* and *Cebuella* and *Saguinus* as the sister group to those three taxa. Rosenberger (1977) shows *Saguinus* as the sister group to *Callithrix–Cebuella,* with *Leontopithecus* being the sister group to the rest of the marmosets and tamarins, but indicates some uncertainty in that placement. Rosenberger and Coimbra-Filho (1984), in a study of the affinities of *Leontopithecus* based on craniodental traits, present strong arguments that the "tamarin" dentition is primitive for callitrichines and that *Leontopithecus* is indeed more closely related to *Callithrix* than to *Saguinus.* Thus, the majority of morphological and molecular analyses support a placement of *Saguinus* as the most basal member of the marmosets and tamarins.

Position of the Pitheciines Relative to Other Platyrrhine Clades

From the results of DNA studies, there are three alternative placements of the pitheciines. The G6PD data presented here support a placement of the pitheciines as the sister group to all other platyrrhines. However, bootstrap support for this placement is very low (40%). The epsilon-globin data set supports a pitheciine–ateline clade, with bootstrap support of 79% (Harada *et al.*, 1995). The IRBP data support a pitheciine–cebid clade, in which the cebids consist of *Aotus, Saimiri–Cebus,* and the callitrichines, with bootstrap support of 96% (Schneider *et al.*, 1996). The 16S topology published by Horovitz and Meyer (1995) also supports a closer link between the pitheciines and cebids than between the pitheciines and atelines (no bootstrap values given). Thus, there is conflict among the available molecular data sets on this question.

Published morphological analyses for platyrrhines largely support a pitheciine–ateline clade (Hershkovitz, 1977; Ford, 1986; Rosenberger, 1992). An exception is Kay (1990), who found a basal position for the pitheciines to be most parsimonious (in line with the G6PD results). Ford (1986) mentions the following synapomorphies for an ateline–pitheciine clade: enlarged hypocones, medially buttressed temporomandibular joining, ventrally sloping lower border of mandibular corpus, a reduced cristid obliqua, and a rounded deltopectoral crest on the humerus. This question of the basal phylogenetic relationships of the New World monkeys is clearly one that deserves further investigation.

Divergence Dates

Although the divergence date estimates based on the G6PD evidence are only rough estimates, they are generally concordant with palaeontological evidence. For example, the estimated date of 15.8 myr for the split between *Aotus, Cebus,* and *Saimiri* and the callitrichines is reasonable in light of fossil finds showing specimens clearly allied with these three clades from a time period between 12 and 16 myr: *Aotus dindensis,* only slightly different from the living *Aotus; Neosaimiri fieldsi,* very similar to modern *Saimiri;* and *Lagonimico conclucatus,* related to the living marmosets and tamarins (Fleagle, 1988, pp. 347–349; MacFadden, 1990; Rosenberger *et al.*, 1991; Kay, 1994). Similarly, the estimate for the split between *Alouatta* and the Atelini falls out at 15.1 myr, and *Stirtonia tatacoensis,* a form allied with *Alouatta,* has been dated to between 12 and 16 myr (Fleagle, 1988, p. 349; MacFadden, 1990).

CONCLUSIONS

The newly reported G6PD sequence data have added support to the results of previous DNA sequence studies on platyrrhine phylogeny in a number of areas. Gene trees for G6PD, epsilon-globin, and IRBP clearly support a placement of *Cebus, Saimiri,* and *Aotus* with the callitrichines and *Callicebus* with the pitheciines.

They also support a *Cebus–Saimiri* clade. The data sets support a placement of *Lagothrix* as the sister taxon to *Brachyteles* and *Callimico* as the sister taxon of either *Leontopithecus* or *Saguinus*, rather than the sister taxon of all other marmosets and tamarins.

There is disagreement among the topologies supported by the DNA sequence data sets in the following areas: the relative placement of *Saguinus* and *Leontopithecus*, the branching order within the cebid (*Aotus*/*Cebus*/*Saimiri*/callitrichine) clade, and the placement of the pitheciines relative to the atelines and cebids. These questions merit further research.

ACKNOWLEDGMENTS

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