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Systematics of New World Monkeys (Platyrrhini, Primates) Based on 16S Mitochondrial DNA Sequences: A Comparative Analysis of Different Weighting Methods in Cladistic Analysis

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In order to investigate the effects of different weighting methods on a phylogeny reconstruction based on DNA sequences and to evaluate the phylogenetic information content of various secondary structures, a fragment of the large ribosomal mitochondrial gene (16S) was sequenced from 13 species of New World monkeys, three species of catarrhines, and *Tarsius*. The data were analyzed cladistically without weighting characters or changes, and with different weighting methods: *a priori* differential weights for transitions and transversions, two variants of dynamic weighting for each kind and direction of change, and successive approximations, using both the character consistency index (CI) and the rescaled consistency index (RC). The results were compared with published trees constructed from nuclear sequences of ε-globins and morphological characters by different authors. The result of the analysis of the mtDNA data set with successive approximations, using the RC as weighting function, was the closest to the topology on which all molecular and morphological trees concur. Other relationships were unique to this tree. "Loops" were the type of secondary structure that showed maximum variation in sequence length and sites with the lowest character CI and RC. A large number of sites within loops showed high values for these indices, however, which suggests that uniform downweighting of these regions represents a large loss of phylogenetic information. Successive weighting, which assigns a weight for each particular character, seems to be a desirable alternative to this practice. We propose a new variant of dynamic weighting, which we call homoplasy-correcting dynamic weighting, that like dynamic weighting, is applicable to any kind of sequence, coding or noncoding.

INTRODUCTION

New World monkeys (Platyrrhini) inhabit the tropical areas of South and Central America. Their evolutionary history extends back over 30 million years. They constitute a wide radiation, both in morphology and behavior: some species are prosimian-like in habits, some are ape-like, and many exhibit no close analogy to other groups of primates (Fleagle, 1988). Their extensive morphological and ecological diversity has been attributed to the fact that they evolved in the absence of other competing groups of primates. Fleagle (1988, p. 148) pointed out that ". . . the extent of their adaptive diversity is indicated by the presence of six or more sympatric species throughout most of South America and up to thirteen species at some Amazonian sites."

New World monkey systematic relationships have been contentious for many decades. The morphological distinctiveness of most genera suggests that they underwent an early evolutionary radiation, with subsequent modifications, which renders identification of homologous characters difficult (Fleagle, 1988). Different authors have produced conflicting phylogenetic hypotheses using partially different data sets of morphological characters (Rosenberger, 1981, 1984; Ford 1986; Kay, 1990) (Figs. 1a–1c). All authors agree on the monophyly of the atelines (*Ateles, Lagothrix, Alouatta,* and *Brachyteles*), pitheciines (*Pithecia, Chiropotes,* and *Cacajao*), and callithrichids (*Callithrix, Cebuella, Leontopithecus, Saguinus,* and *Callimico*), but they disagree on the relationships within some of these groups, and also disagree in how these groups relate to each other and to *Cebus, Aotus, Callicebus,* and *Saimiri.* Immunological studies conducted by Baba *et al.* (1980) and Sarich and Cronin (1980) have not been able to confidently resolve the relationships among the seven clades mentioned above.

Schneider *et al.* (1993) recently published a study based on ε-globin sequences and obtained six most parsimonious trees. Three of these trees show alternative relationships among New World monkeys (Fig. 2) which differ in many aspects from all previous morphological ones, and the other three show different relationships among the outgroups, which were *Macaca mulatta, Pongo pygmaeus, Hylobates lar, Homo sapiens, Gorilla gorilla, Pan paniscus, Pan troglodytes,* and *Tarsius syrichta.* All nuclear sequence trees agree with

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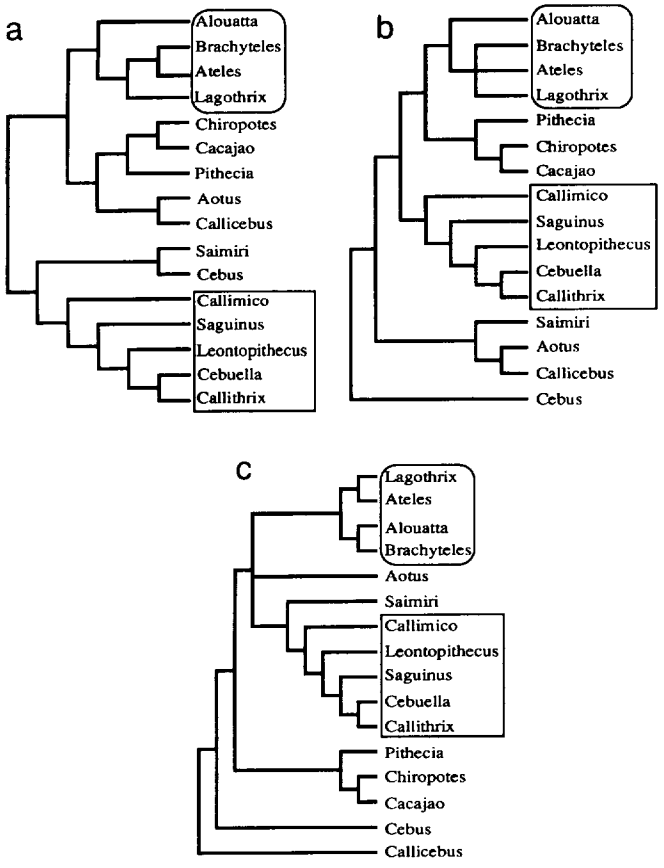

FIG. 1. Phylogenetic trees obtained with morphological data by (a) Rosenberger (1981, 1984); (b) Ford (1986) also suggested that *Saimri* may be related to *Cebus* instead of *Aotus-Callicebus* and that *Ateles* may be related to either *Brachyteles* or *Lagothrix*; and (c) Kay (1990). Callithrichids are enclosed in a squared outline, whereas atelines are enclosed in a rounded outline. The same scheme is used in subsequent figures.

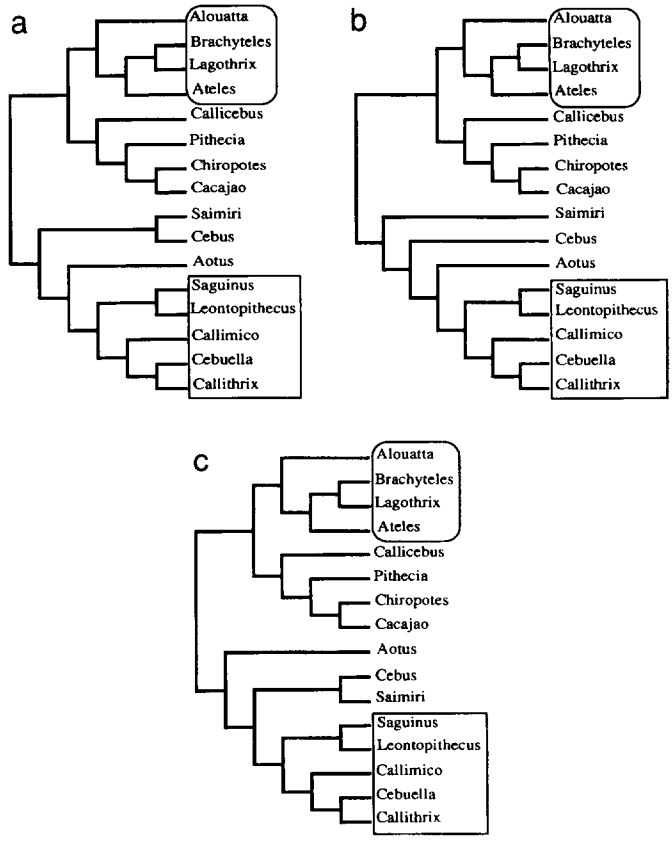

FIG. 2. Three most parsimonious trees for NewWorld monkeys relationships obtained by Schneider *et al.* (1993), using ε-globin nuclear gene sequences; the outgroups employed were *Macaca, Pongo, Hylobates, Homo, Gorilla, Pan* (*P. paniscus* and *P. troglodytes*), and *Tarsius,* which were related to each other in three most parsimonious additional rearrangements.

morphological ones on the monophyly of callithrichids, atelines, and pitheciines. In the present study, we sequenced portions of a mitochondrial gene, the 16S ribosomal gene, in order to present another data set to compare to nuclear or morphological ones, and explore its phylogenetic utility for the question of the evolutionary relationships among New World primates.

MATERIALS AND METHODS

Data Collection

DNA was extracted from frozen or ethanol-preserved muscle (Kocher *et al.*, 1989) or from frozen or Tris–SDS–EDTA buffer-preserved blood from the following species: *Cebus apella, Saimiri sciureus, Aotus trivirgatus, Callithrix jacchus, Cebuella pygmaea, Saguinus geoffroyi, Callimico goeldii, Leontopithecus rosalia, Ateles* sp., *Lagothrix lagothricha, Alouatta palliata, Pithecia pithecia,* and *Callicebus moloch,* and the outgroups *Hylobates lar, Nasalis larvatus,* and *Tarsius syrichta.* The sequence for *Homo sapiens* was obtained from Anderson *et al.* (1981).

A 542-bp fragment of the mitochondrial 16S ribosomal gene was sequenced for all species. This length is that obtained after alignment. Amplifications *via* the polymerase chain reaction (PCR) were conducted in 25 ml of Tris buffer (67 mM, pH 8.8) containing 2 mM $MgCl_2$, 1 mM of each dNTP, 1 mM of each primer, 1–1000 ng template DNA, and *Taq* polymerase, (0.5 units; Cetus). The primers used were 16Sar-L (5′-CGCCTGTTTTCAAAAACAT-3′) (Palumbi *et al.*, 1991) and 16Sbr-H (5′-CCGTCTGAACTCAGATCACGT-3′) (Palumbi *et al.*, 1991). PCR was performed for 27 cycles for the double-stranded PCR (45 s at 93°C, 60 s at 60°C, and 90 s at 72°C). An aliquot of 5 μl of the double-stranded PCR products was gel-purified on a minigel containing 2.5% NuSieve agarose in Tris–borate–EDTA buffer (0.1 M, pH 7.2), stained with ethidium bromide. The target product was excised and dissolved in 200–1000 ml H_2O. Asymmetric PCR was conducted for

35 cycles (Gyllensten and Erlich, 1988), with the same primers, in limiting concentration (0.01 mM; 40 s at 92°C, 60 s at 60°C, and 90 s at 72°C). The single-stranded amplification products were ultracentrifuged three times with 300 ml H_2O in spin columns (Millipore 30,000) before direct sequencing (Sequenase version 2.0; U.S. Biochemical; Sanger *et al.*, 1977). The sequences were electrophoresed in two complementary runs using 6% acrylamide–urea gels in TBE buffer (45 mM, pH 8.0) on a wedge and a straight gel.

Sequences have been deposited in GenBank under Accession Nos. U38997–U39012.

Data Analysis

Alignments were performed with Malign 1.85 (Wheeler and Gladstein, 1993) and the phylogenetic analyses were done with the heuristic algorithm in PAUP 3.1.1 (Swofford, 1993), with 50 replications. Aligned sequences are available from the authors upon request. Gaps were considered as a fifth character state. The following *a priori* weighting schemes were applied to the mtDNA sequences: TS/TV (TS are transitions, TV are transversions) from 1/2 to 1/20, and 0/1 (excluding transitions). We used two *a posteriori* methods to analyze the same data set: (i) dynamic weighting (Williams and Fitch, 1989, 1990; Fitch and Ye, 1991) in two variants, one by Maddison and Maddison (1992) and the other we propose in this paper, and (ii) two variants of successive approximations to character weighting (Farris, 1969, 1988; Carpenter, 1988). These methods are based on the idea that changes which have many steps are unreliable indicators of relationships and therefore one should weight changes inversely to the frequency of their occurrence.

Phylogenetic quality of different secondary structural regions of the 16S mtDNA molecule were investigated based on the model by Gutell and Fox (1988) and a classification of structures based on Vawter and Brown (1993).

Dynamic Weighting and Homoplasy-Correcting Dynamic Weighting

As addressed by dynamic weighting, some changes are rarer than others, so what dynamic weighting does is to examine ". . . the frequency with which the various nucleotide changes [for example] occurred in the best tree, weight the changes inversely to their frequency of occurrence and then redo the analysis . . ." (Williams and Fitch, 1989). The method utilizes *a posteriori* transformation weighting because it involves obtaining an initial tree and tallying numbers of changes on this tree, applying the weighting scheme to the data, and recalculating the tree, and then repeating the process until some stable solution is obtained. The data were weighted simultaneously in two ways with this method: with a transformation–cost matrix T for changes and with a vector C of weights for each site (the latter being equivalent to a successive weighting). The first run was performed without any weights and this first pool of (nine) trees was used as the starting point for the weighting. We built the vector C with the maximum value of the rescaled consistency index for each character from the trees resulting from the previous iteration. The matrix T was built weighting different directions of changes separately (i.e., A → C and C → A have different costs). Frequencies of changes were calculated from the tree/s resulting from the previous iteration, and the minimum number of steps for all possible optimizations and trees, as calculated by MacClade 3.0 (Maddison and Maddison, 1992), was used. The two variants of the method we employed differed in the function we used to weight changes in the T matrix. The first one was suggested by Maddison and Maddison (1992) and is

$$K_{ij} = -\ln (X_{ij}/X), \quad (1)$$

where K_{ij} is the cost of changing from state i to state j, X_{ij} is the number of i → j changes calculated from the tree/s, and X is the total number of changes on the tree. The second is a new variant that we suggest in this paper, which we call homoplasy-correcting dynamic weighting:

$$L_{ij} = -\ln (X_{ij}/2N_{ij}), \quad (2)$$

where L_{ij} is the new function for the cost of changing from state i to state j, X_{ij} is the same as defined above, and N_{ij} is the number of positions that show presence of character states i and j. The reason we suggest dividing the number of changes between character states by the number of sites where these character states appear in combination is because in this way we introduce a correction for degree of homoplasy. Simple abundance of a kind of change gives no indication of its degree of homoplasy, whereas if we consider the number of sites where this kind of change may potentially occur, we average for its degree of homoplasy across sites, a correction missing from K_{ij}. At the same time, the vector C that weights each site also takes overall site homoplasy into consideration. N_{ij} is multiplied by 2 to ensure that the denominator is always larger than the numerator, so the function always has the same sign. Gaps were included in the computations, since they were considered a fifth character state.

Successive Approximations to Character Weighting

The successive weighting of characters (Farris, 1969, 1988; Carpenter, 1988) was done using the maximum value of the RC and the CI for each character (Farris, 1989). Successive weighting also is an *a posteriori* method of weighting because it requires an initial tree or pool of trees to calculate a weight for each character

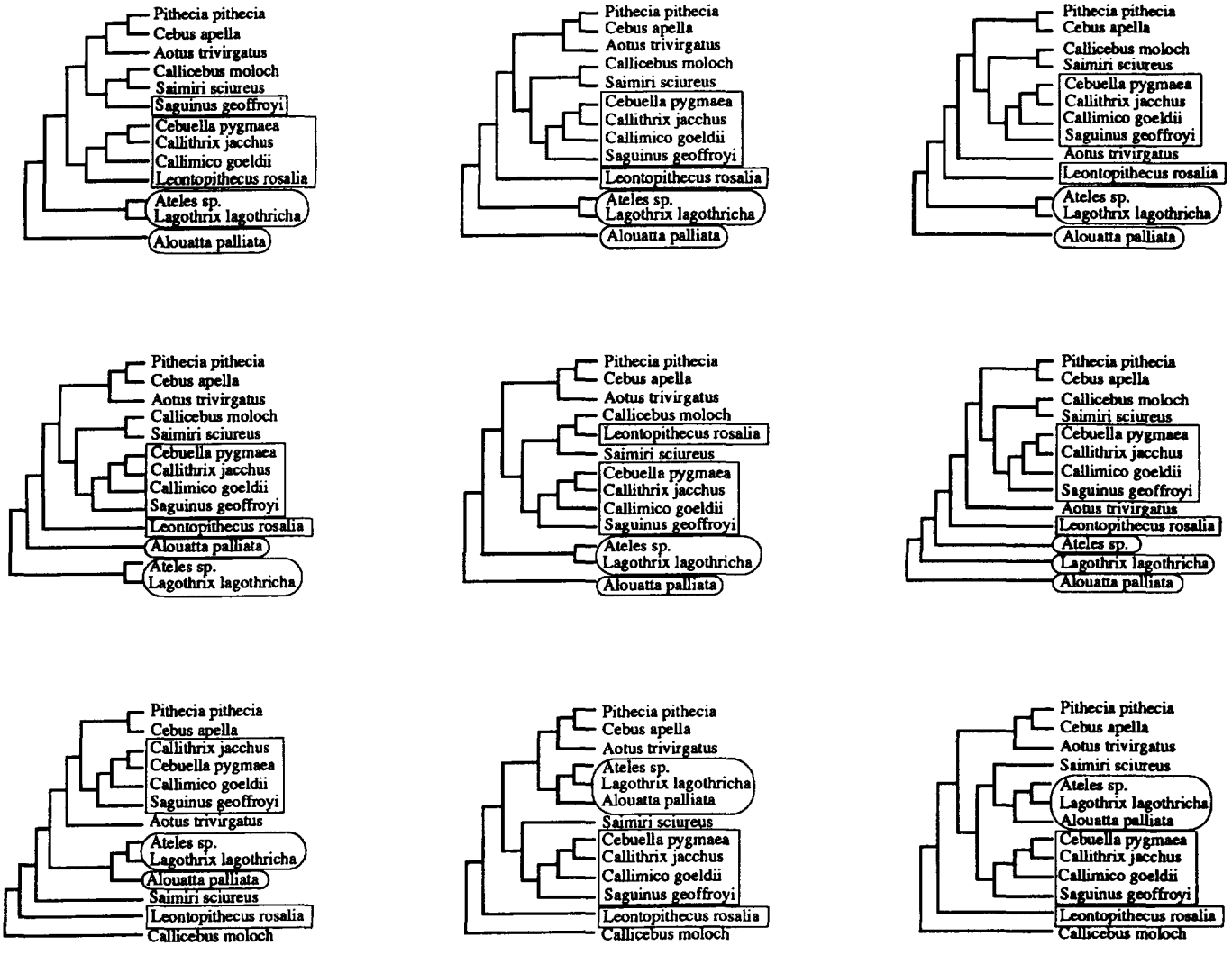

FIG. 3. Nine most parsimonious trees obtained from the phylogenetic analyses of partial 16S mtDNA sequences employing no weights (CI = 0.47, RC = 0.24). See Fig. 4 for relationships among outgroups.

based on their performance in the initial trees. The weighting function recommended by Farris (1988) is the RC. The weights are applied to the characters and a second round of trees (or a single tree) is obtained. The process is repeated until the topology does not change with new iterations.

RESULTS

Weighting Analysis

An initial analysis with all sites and types of changes weighted equally yielded nine most parsimonious trees (Fig. 3) which, when combined in a strict consensus tree, provided very poor resolution (Fig. 4). The trees were rooted with *Tarsius.* None of these trees showed callithrichids as monophyletic and in only three of them were atelins grouped as a clade. Trees in the initial unweighted pool showed low CI (0.47) and RC (0.24). Transitions showed a high abundance and number of steps in many sites. Though many sites showed transitions with a single step, the traditional scheme using different *a priori* weighting ratios of changes, with a single weight for all transitions, and another weight for all transversions, was tried on the data set to see how they affected the topology of the trees. The analysis yielded a series of topologies (Fig. 5). All of these show a common pattern: *Callimico* as sister group of *Callithrix* and *Cebuella,* and the next branch bears *Leontopithecus.* Other relationships varied from tree to tree. We obtained several trees when the TS/TV ratio varied from 1/2 to 1/5 (see consensus of trees using two ratios in Figs. 5a and 5b), whereas from 1/6 to 1/20, a single and the same tree was obtained in each run (Fig. 5c). In this tree, *Saguinus* was added to the core clade of callithrichids mentioned above, and the sister group of callithrichids was (*Aotus* (*Pithecia* (*Cebus, Saimiri*))).

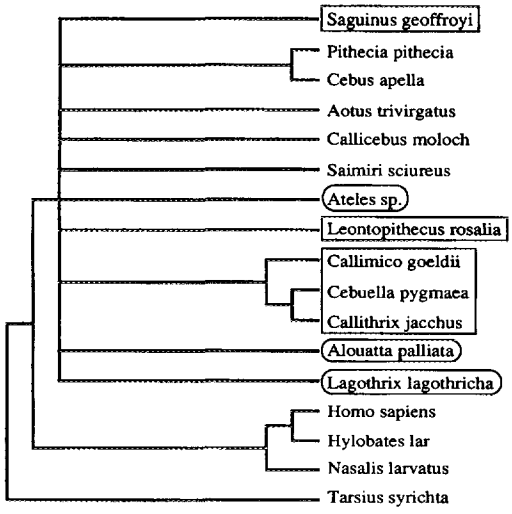

FIG. 4. Strict consensus of nine trees from Fig. 3.

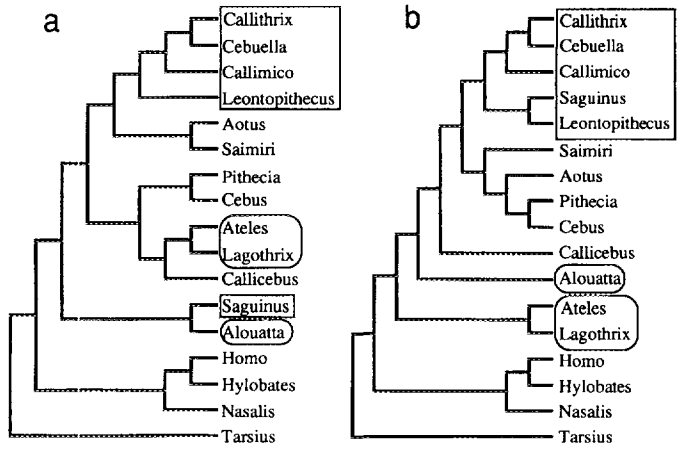

FIG. 6. Most parsimonious trees obtained with dynamic weighting using two different weighting functions for the transformation matrix T, and in both cases, C vector weights calculated with successive weighting using the maximum value of the rescaled consistency index as weighting function: (a) tree obtained after three iterations applying weighting function $K_{ij} = -\ln (X_{ij}/X)$ (Maddison and Maddison, 1992) for matrix T, with the minimum number of steps for X_{ij} for all optimizations and uninformative characters excluded, and (b) tree obtained after two iterations applying the new weighting function, the homoplasy-correcting dynamic weighting, $L_{ij} = -\ln (X_{ij}/2N_{ij})$, for matrix T, also with minimum number of steps for all optimizations and uninformative characters excluded.

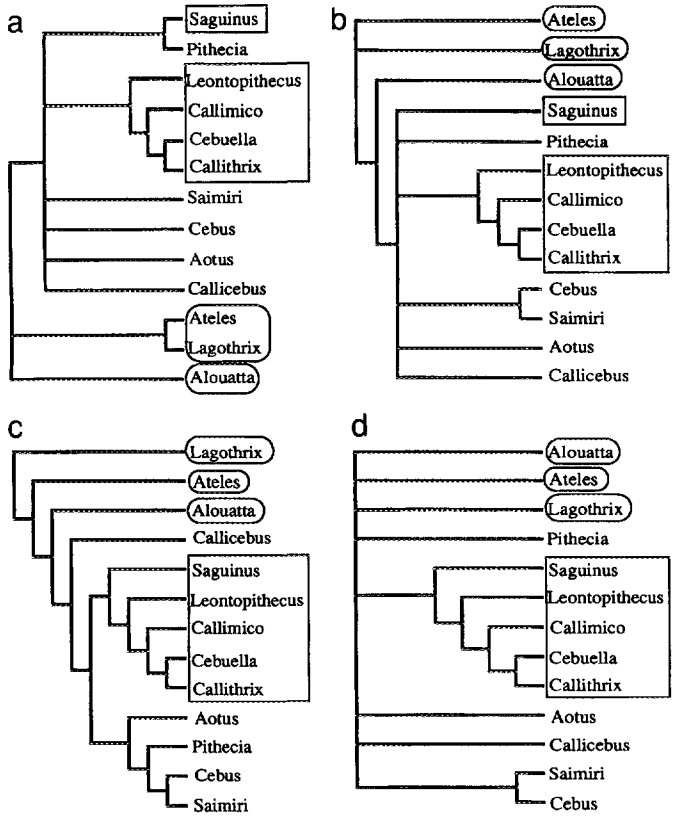

FIG. 5. Trees obtained with a series of weight ratios for transitions and transversions: (a) consensus of 5 trees obtained with a TS1/TV3 ratio, (b) consensus of 7 trees obtained with TS1/TV4 ratio, (c) tree obtained with ratios TS1/TV6 to TS1/TV20, (d) consensus of 15 trees obtained excluding TS.

Callicebus was on the next branch and successively outer groups were the three atelids used in this study, which therefore appeared as paraphyletic. Weighting transitions by "0", 15 trees were found, the consensus of which is shown in Fig. 5d. Callithrichids were monophyletic but the atelines still did not form a clade.

Dynamic weighting (Williams and Fitch, 1989) was applied to the data set in two different variants. These two variants yielded two different topologies. The first variant (Maddison and Maddison, 1992), using K_{ij} as weighting function, yielded a stable topology after three iterations (Fig. 6a). Neither callithrichids nor atelines formed clades. Variant L_{ij}, the homoplasy-correcting dynamic weighting, yielded a stable tree after two iterations (Fig. 6b), where callithrichids but not atelines form a clade, the latter being paraphyletic. It may be of interest to point out that after the first iteration, atelines formed a clade, but the clade was lost thereafter.

When the initial pool of unweighted trees was subjected to successive weighting using either the maximum values of the CI or the RC, a single tree was obtained in each case after the first iteration, which remained stable with further iterations (Figs. 7a and 7b). Whether the CI or the RC better represent character homoplasy is under debate (Goloboff, 1991); in this analysis the RC yielded the topology which is closest to that on which nuclear and morphological data agree (Fig. 7b). Using the CI as weighting function, atelines form a group but callithrichids fail to do so, with *Leontopithecus* left out, whereas using the RC, both groups

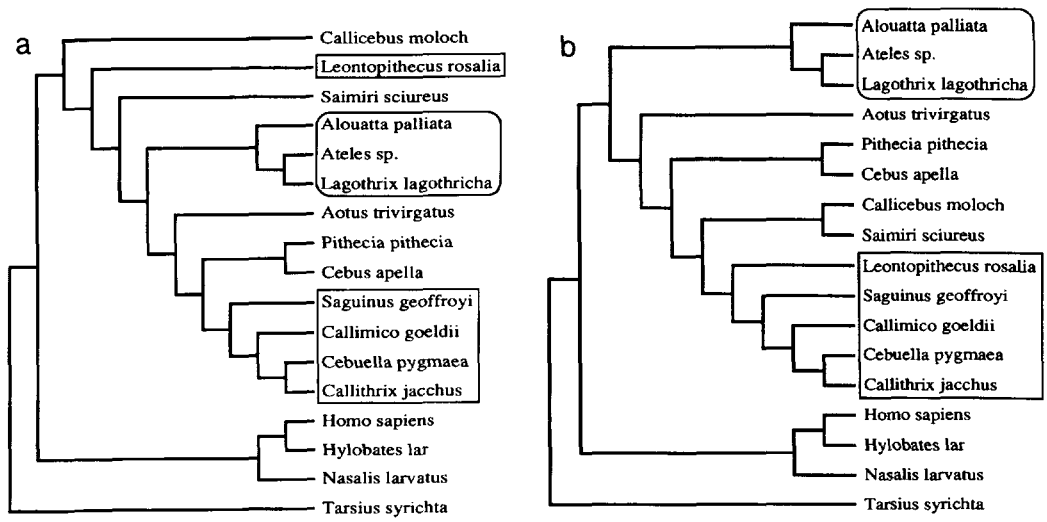

FIG. 7. Trees obtained with successive approximations to weighting, performed on the nine initial trees, employing the maximum value of (a) the CI of each character and (b) the RC of each character, as weighting function. Topology stability was obtained after one iteration in both cases.

appear. The relationships among these and other taxa differ from all other studies based on morphological or nuclear data.

Secondary Structure Analysis

Although all the trees mentioned above differ greatly in topology, they show several consistent features. Fourteen of the trees were examined for number of changes in individual positions to determine whether different segments of the sequence deserved to be weighted differentially according to their position in the secondary structure of the molecule and if these properties were consistent in several different topologies. These trees were: the 9 unweighted trees, the trees resulting from CI and RC successive approximations weighting, and the mitochondrial data plotted onto the 3 most parsimonious trees obtained from ε-globin sequences by Schneider *et al.* (1993). The number of steps of each character represents the relative rate of mutation of that character relative to other characters. Some positions showed very high rates across the 12 trees considered. Secondary structure was determined in the sequences according to Gutell and Fox (1988) (Fig. 8) and grouped into classes (stems, bulges, loops, and "other") as defined by Vawter and Brown (1993). Paired bases are classified as stems, unpaired regions within stems as bulges, unpaired bases at the end of stems as loops, and "other" includes all remaining cases of unpaired bases (Vawter and Brown, 1993). Different taxa were found to contain different numbers of nucleotides in each of the classes because point mutations cause some positions to shift from one kind of secondary structure to another, and also because of insertion/deletion events. Three loops were found to contain several very "fast evolving" positions (10, 9, and 8 steps), with the highest rates of all sites of the sequenced fragment of the molecule. These sites were found in loop 7 (Fig. 8: L7 positions 242–287) in our sequenced fragment after alignment, equivalent to positions 1084–1124 in the human mitochondrial genome), loop 9 (L9, positions 322–332, or 1159–1169 in the human mitochondrial genome) and loop 10 (L10, positions 345–384, or positions 1180–1218 in the human mitochondrial genome). Two of them had high variation in length across the sampled primates: loop 7 varied from 44 bp in *Tarsius* to 41 in *Pithecia* and loop 10 from 34 bp in *Tarsius* to 38 in *Cebuella*. These fast evolving positions were also highly homoplastic (RC = 0). Though loops were the regions that contained the positions with highest degree of homoplasy, they also contained conservative sites, ranging from those with no changes at all (43% of the loop sites, using the human 16S gene secondary structure as parameter) to those with RC of 1 (see Fig. 9 for frequencies). In fact, loops displayed most of the informative sites of the sequence, both because of their large total length and because they showed variability, low in some cases, higher in many others, with a wide range of RCs.

The next category in maximum rate of change at a site after loops was "other," with seven steps in most trees and eight steps in only one tree. Finally, stems and bulges displayed some sites with a maximum of six steps in all trees, and in one tree with a length of seven steps, belonging to a stem in some taxa, to a bulge in others. RCs for each structural class (after the human pattern) are shown in Fig. 9.

DISCUSSION

Differential weighting of characters in a cladistic phylogenetic analysis is a much debated subject

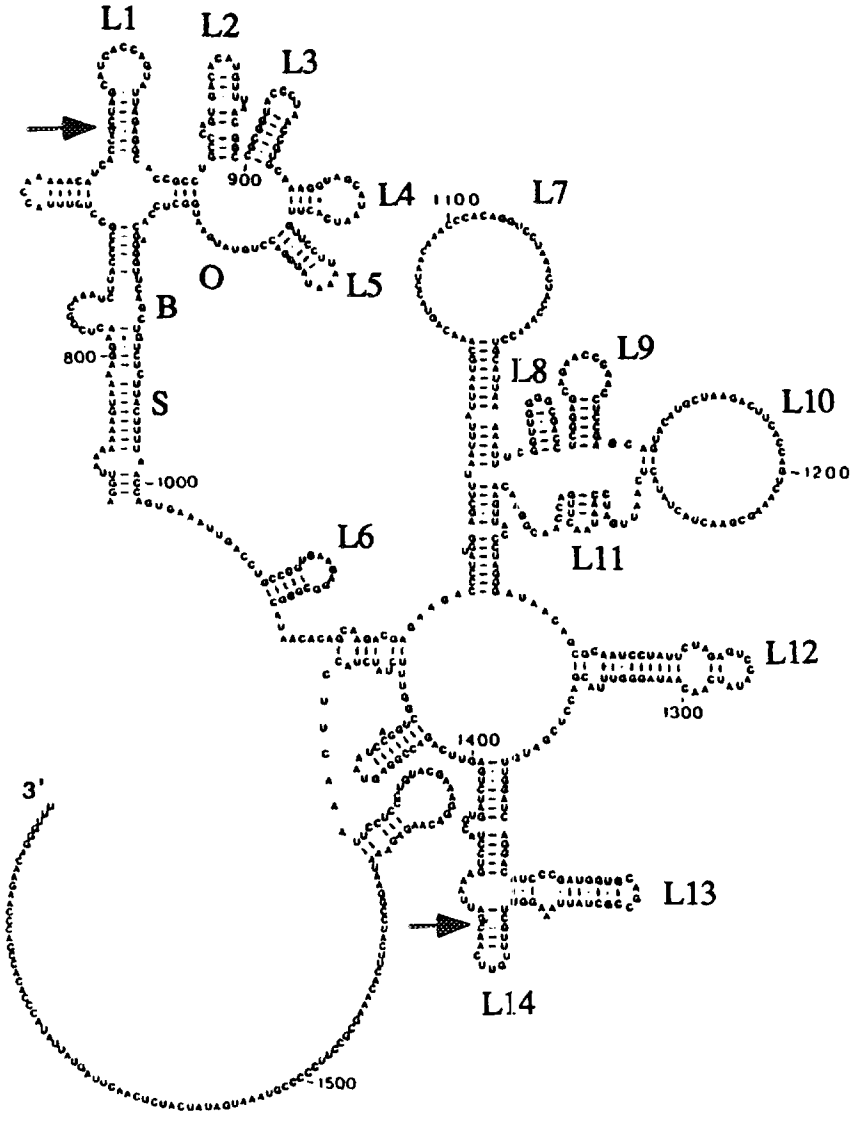

FIG. 8. Secondary structure of the 16S ribosomal DNA molecule in humans, 3′ half (Gutell and Fox, 1988). Arrows indicate beginning and ending of the sequenced region. L, S, B, and O indicate loops, stems, bulges, and "other," based on the classification scheme by Vawter and Brown (1993). Numbers preceded by Ls indicate sequence of loops. Figure modified from Gutell and Fox (1988).

(Farris, 1969; Carpenter, 1988; DeSalle *et al.*, 1987; Miyamoto *et al.*, 1994). Given the faster rate of change of mitochondrial sites compared to nuclear ones on the average, and the higher incidence of transitions (C ↔ T and A ↔ G) over transversions (all other substitutions), the former kind of change has a higher probability of producing homoplasy than the latter, and therefore the former has been proposed to be downweighted in phylogenetic analyses (Brown *et al.*, 1982; DeSalle *et al.*, 1987; Meyer and Wilson, 1990) because the quality of phylogenetic information provided by each kind of change will tend to be different. We applied different weighting methods to our data set and we compared our results with those published on morphological characters and nuclear sequence data (see Introduction). In all morphological and nuclear phylogenies, callithrichids, atelines, and pitheciines appear as monophyletic; therefore we consider this result a reliable one. Since we only included one pitheciine in our study, we only evaluated monophyly of callithrichids and atelines as obtained using the different weighting schemes.

As discussed by Fitch and Markowitz (1970) and Vawter and Brown (1993), changes should be tallied on a phylogeny and not on pairwise comparisons of taxa, to estimate both the total number of changes required by the phylogeny and to account for the specific nature of those changes. In our analyses, these tallies were used to weight the different kinds of changes with dynamic weighting (Williams and Fitch, 1989, 1990) and with successive approximations to character weighting (Farris, 1969, 1988; Carpenter, 1988). The two weighting functions K_{ij} and the new L_{ij} we employed to

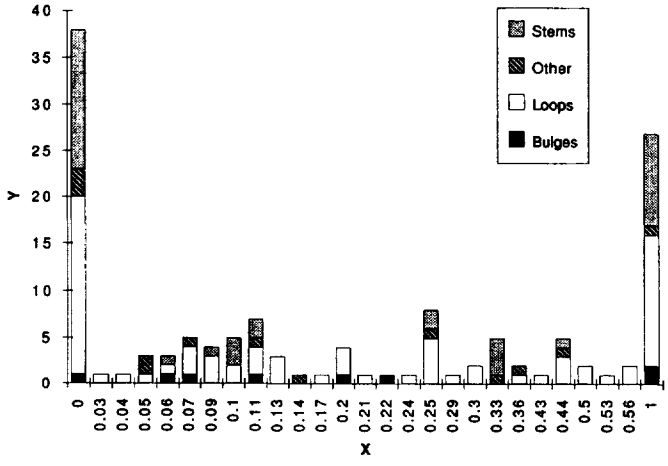

FIG. 9. Plot for frequency of positions (Y) vs character rescaled consistency index (RC) (X) calculated on the tree obtained with successive weighting applying the maximum value of RC (Fig. 7b). Sites are classified in stems, loops, bulges, and "other" according to the definitions by Vawter and Brown (1993) and based on the secondary structure reconstruction of the human sequence by Gutell and Fox (1988).

weight changes dynamically differed in their performance. L_{ij}, the homoplasy-correcting dynamic-weighting, yielded a tree that has a higher congruence with trees derived from nuclear and morphological data.

The third approach we employed, that of successive approximations to character weighting applying the RC, yielded a better solution than both dynamic weighting variants and successive weighting using the CI as criterion. This is again assessed by comparing the phylogenies resulting from morphological data and nuclear data mentioned above. Successive weighting does not take into account differential number of steps for different kinds of changes within those characters. So, in a position that contains both transitions and transversions, for example, where each has a different degree of homoplasy, a single value is assigned to both, the one that the position as a whole deserves, by its overall number of steps. But these cases are not so substantial in number in our sequences, and successive weighting seems to perform fairly well, compared to other methods. Though dynamic weighting is intended to correct the reliability of estimates for character changes calculated by successive weighting, in our data set the high percentage of characters containing only one kind of change (38%) seems to tilt the balance of compromise of the two methods toward successive weighting. The fact that the cost for each change in the T matrix is a single non-site-specific value applied across the whole data set may be the reason the method did not perform as well as successive weighting alone. The range of steps for some kinds of changes is very wide across sites, and although the C vector is applied simultaneously, the T matrix tends to average off the site-specific effect of the C vector.

The *a priori* method of trying different ratios for TS/TV would be an even more accentuated case of this problem, because changes are grouped into even broader categories. Overall and on average (across optimizations and sites), transversions, as expected, show fewer steps than transitions and therefore a higher reliability as phylogenetic indicators. However, we presume that the amount of sequence required to retrieve any phylogeny would be much smaller if we did not discard all transitions, or downweighted all of them unnecessarily, but instead performed a finer-grained weighting procedure.

The need for site specificity in weighting would also apply to variation in homoplasy within structural classes. For example, within loops are those sites that show the highest amount of homoplasy and hence deserve low weights, but also within this class is a large number of sites that show no homoplasy. Hence, a method sensitive to level of homoplasy in each site is desirable. *A posteriori* weighting methods do not discriminate sites accoding to their location on the secondary structure, but according to their degree of homoplasy, particularly so successive weighting, therefore they satisfy the need for site specificity. If all sites in loops were downweighted (as suggested by Miyamoto *et al.*, 1994), then the amount of sequence required to solve a set of relationships would be presumably larger (increasingly so for larger numbers of taxa), because informative sites are not that abundant throughout the nonloop structures, both because they are shorter and also more conserved (fewer variable sites per number of nucleotides and fewer character states per variable site). Another difficulty concerning differential weighting of structural classes is that, across taxa, the classes are not mutually exclusive but there is considerable intersection, i.e., in some taxa certain positions belong to stems and in other taxa to bulges. Only eight variable positions are in stems in all taxa, because they compensate for each other, preserving the pairing required to be classified as stems. Most of the phylogenetic information is in the sites that belong to different structural classes in different taxa. Since these sites cannot be classified into mutually exclusive classes, therefore it does not seem possible to weight them across a whole data set according to which class they belong to.

In conclusion, successive approximations weighting applying RC was the most successful method of phylogenetic analysis of the sequenced fragment of 16S mtDNA of New World monkeys. This was judged by comparing the topology of trees resulting from different weighting methods, as applied to this data set, with highly corroborated published results based on morphological characters and nuclear ε-globin DNA sequences. Differential *a priori* weighting of TS/TV or two variants of dynamic weighting were not satisfactory methods in this case. This is presumably because the range of degrees of homoplasy among sites in this

data set is so wide that a method more sensitive to local variation of homoplasy is more appropriate. *A priori* weighting of secondary-structure classes seemed not to be appropriate for the same reasons and also not possible to apply because a large number of variable sites presumably belonged to different structural classes in different taxa.

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