

**An analysis of Metachromism within the phylogeny of the Neotropical primate genus *Alouatta*, the howler monkeys.**

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## Abstract

Metachromism has been controversial ever since (HersHKovitz 1968) first proposed it as a key part of his taxonomic treatments of neotropical primates. Under critical testing, (Jacobs, Larson et al. 1995) found a near-perfect match between *Saguinus* (Callitrichidae) mtDNA- and metachromism-based phylogenies. A composite phylogeny of the genus *Alouatta* was constructed in order to do a similar test on an additional Neotropical primate taxon. Metachromism is consistently not the most parsimonious reconstruction of the changes in fur color in *Alouatta*, so it is not a promising source for predicting *Alouatta*'s phylogeny. Similar investigations should be attempted with other genera, especially where HersHKovitz evaluated the taxonomy using the principle of metachromism (eg. *Cacajao*, *Pithecia*).

## Introduction

### **Metachromism**

Metachromism is a principle of evolutionary change (HersHKovitz 1968; HersHKovitz 1977), under which many pelage colors are not adaptive but are the consequence of a predictable sequence of color changes through evolutionary time. Starting with a primitive agouti banded fur, over many generations one of two pathways may be followed within a chromatogenic region of the pelage: a) the agouti may saturate with eumelanin, giving a black-saturated agouti, the agouti banding disappears giving black, which then begins a bleaching process through brown, drab, and gray until the fur is white, with no pigment at all; b) the agouti may saturate with pheomelanin, giving a red-saturated agouti, the agouti banding disappears giving red, which then begins a bleaching process through orange, yellow, and cream until the fur loses all pigment and appears white. Since HersHKovitz construed these chromatic progressions as linear and irreversible, he applied metachromic pelage analysis during taxonomic work as a powerful tool for reconstructing biogeographic divergence events, and hence phylogenetic relationships over a wide range of Neotropical taxa including small primates (HersHKovitz 1977) and any of the genera of larger Neotropical primate (Cebidae, eg. *Ateles*, *Alouatta*, *Cacajao*, *Saimiri*). Despite his death in the late 1990's, many of HersHKovitz' taxonomic and phylogenetic conclusions still underpin Neotropical primatology even today. Given the importance of a clear taxonomy and phylogeny in setting conservation priorities, it is important to test the validity of any conclusions based on the principle of Metachromism.

HersHKovitz specified that each genus begins with an all-agouti ancestor, and may exhibit both pathways as species diverge within the genus. Different parts (chromatogenic regions) of the same animal might follow different pathways, and even be at different colors along a given pathway. He also stated that any chromatogenic region might leap to the white, non-pigmented state rapidly, from any intermediate color. He did not specify if within a genus, the same chromatogenic regions might follow different pathways in different species, although data from (Jacobs, Larson et al. 1995) and the current study show that this occurs frequently.

### ***Jacobs et al's work***

(Jacobs, Larson et al. 1995) used phylogenetic analysis of Neotropical tamarin monkeys (*Saguinus*) to test the value of metachromatic progression. Sixteen chromatogenic regions of *Saguinus* specimens were coded as complex, multi-state characters where the metachromatic progression was enforced by using a step-matrix. A phylogeny constructed from mtDNA was compared to a phylogeny constructed using these fur color characters, and with an implicit narrative phylogenetic tree from Hershkovitz' writings. Additionally, the fur color states were traced on the molecular tree, and the resulting cost evaluated to determine if assuming metachromism produces a more parsimonious tree than not assuming it. The results supported the predictive value of metachromism, although Jacobs et al. (p. 526) recommended the test be done with other taxa, as Hershkovitz largely developed the principle of metachromism based on his observations on *Saguinus*.

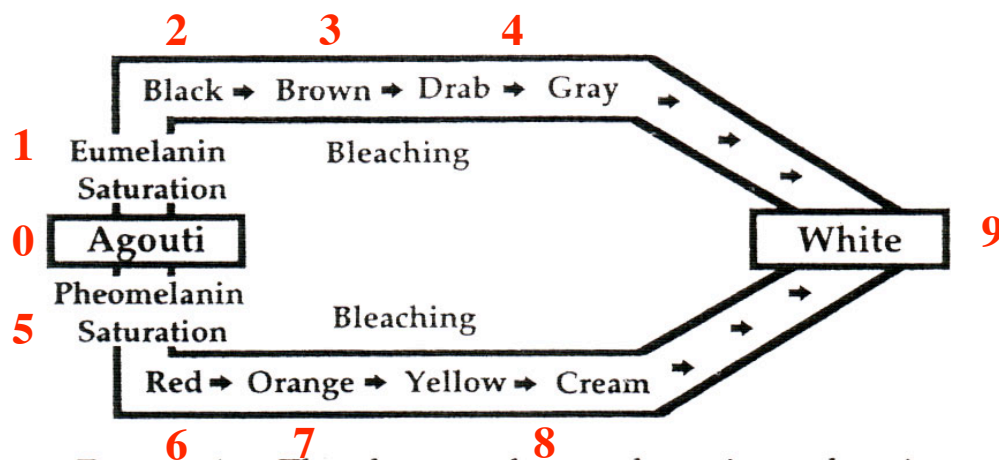


FIGURE 1. The theory of metachromism, showing the evolutionary progression of coat colors in the eumelanin and pheomelanin pathways. [Adapted from Hershkovitz, 1968, 1977.]

Jacobs et al. 1995

Figure 1. Modified reproduction of Fig. 1 from (Jacobs, Larson et al. 1995) showing the metachromatic pathways, and the character states assigned by Jacobs et al.

Adrian Barnett and Caroline Ross (pers. comm.) set themselves the task of extending Jacobs et al's work to the larger Neotropical primates (Cebidae) by tracing fur color data onto the composite phylogeny of Neotropical monkeys developed by (Purvis 1995). For this project, I selected to work with a promising subset of their data, specifically the genus *Alouatta* with 9 recognized taxa (Groves 2001).

## ***Previous phylogenetic work on Alouatta***

The absence of any intraspecific structure for *Alouatta* on (Purvis 1995)'s composite phylogeny reflects the paucity of phylogenetic work that has been done within the genus. Though he employed metachromism in his analysis of many other primate genera, Hershkovitz never visited the genus after having developed the principle of metachromism. The only evidence in the literature of an explicit morphological phylogenetic analysis in an unpublished Masters' thesis (Gregorin 1996) and an edited book (Mittermeier and Coimbra-Filho 1981) is mentioned in (Oliveira, Neusser et al. 2002), but has not been reviewed for this study. While substantial effort has been put into the location of the genus *Alouatta* within the Cebidae, or to the identification, status and naming of various taxa, only recently, as sophisticated molecular techniques have become available, has work been done on the phylogeny of the species within the genus as a whole (Meireles, Czelusniak et al. 1999; Bonvicino, Lemos et al. 2001; Oliveira, Neusser et al. 2002; Cortés-Ortiz, Bermingham et al. 2003). (Cortés-Ortiz, Bermingham et al. 2003) found that while mtDNA genes provide a well-resolved phylogeny, nuclear genes show almost no resolution. This probably reflects a very shallow divergence among the species, and (Schneider, Canavez et al. 2001) identifies the interesting chromosomal rearrangements within the genus as the most promising source of characters for resolving intraspecific phylogeny.

## ***Hypotheses/questions***

The purpose of this study is to evaluate the fit of an *Alouatta* phylogeny as predicted by metachromism with the phylogeny predicted from molecular sources.

## **Materials and Methods**

### ***Acquiring the Fur Color Data***

#### **Description of data – Barnett & Ross**

Following the pelage quantification methodology developed in (Jacobs, Larson et al. 1995), Barnett collected fur state data on Cebidae specimens in the collection of the Natural History Museum, London (BMNH) and the Museum of Vertebrate Zoology, U.C. Berkeley (MVZ). Data collection was restricted to museum specimens only. Experience with museum skins showed that these had subtleties of pelage features (e.g. banding and two-tone fur) that remain unrevealed by photographs, book illustrations and other remote methods.

Prior to analysis of cebid specimens, a series of specimens of the callitrichid species studied by Susan Jacobs were test analysed to ensure methodological and terminological consistency between the two studies. This set of specimens remained available as a reference source while the cebid specimens were being color-coded. Jacobs' original chromatogenic regions are crown, forehead, cheeks, mouth, neck, throat, shoulders, arms, hands, chest, back, belly, rump, thighs, feet and tail. Because cebids do not have the uniformly colored tails exhibited by callitrichids, Jacobs' original "tail" chromatogenic

region was divided into three regions: “tail base”, “tail middle” and “tail tip.” Additionally, to distinguish between missing data and actual absence of fur on the animals, a new character state 10 or A was introduced for “naked.” Loss of fur to “naked” follows after “white” on the metachromic progression.

The following criteria were considered when choosing cebid specimens for analysis:

- females only (to avoid ambiguity of sexually-selected inter-sex dichromism; see (Brooke 1998) for a similar approach).
- adults only (to avoid juvenile pelage differences; see (Brandon-Jones 1999), (Ross and Regan 2000))
- no aberrant individuals (e.g. leucistic or melanistic)
- most recent specimens (to avoid foxing, and long-term effects of preservatives on coat color; see (Coetzee 1985))
- no specimens from captive animals (to avoid possible influences of excess pigments from less heterogeneous captive diet)
- no animals of unknown provenance (to discourage ambiguity in the event of taxonomic uncertainty)
- no animals with missing parts (to ensure maximum comparability)

Data for the chromatogenic regions was recorded on a taxon-by-taxon basis, as a summary of the fur color state for the available specimens ( $N = 1$  to 6) of the taxon.

## **Parallel coordinate plot visualization**

In a separate study (Shapley 2004), the fur color data set was examined with parallel coordinate plot visualization software. The values for each chromatogenic region were plotted on separate axes. During exploration, various subsets of the lines were highlighted. A leap from 0 to 5 was visible on at least two axes as a gap in the plotted lines, this is because the phaeomenlanic metachromic pathway was encoded by the numbers 0-5-6-7-8-9. Subsets of lines corresponding to genera were highlighted and examined for behavior consistent with metachromic progressions, or other insights into the data.

Assuming that members of the same genus share regions where they exhibit color contrast, one prediction is that the paths across the axes by lines representing members of the same genus should be roughly parallel, showing a “signature” of the color pattern. Metachromism’s historical perspective isn’t inherently visible on a parallel coordinate plot, and a hypothesis of historical nature is necessary to falsify metachromism’s claim of

irreversibility of the color states. However, the parallel coordinate plot can distinguish if a common ancestor with a different color state, and therefore an additional step or a longer branch length, is required. In the simple case, if the vector of fur color values for one species is diverging from the vector for another species, the lines should be either identical, or the line of the diverging species should cross the axes at a higher value (farther down the pathway). If the lines are crossing each other as they move between axes, then a common ancestor from which both species have diverged must be invoked to explain the observed fur color vectors in a manner consistent with metachromic progression. Any particular order of the parallel axes is only one of the numerous possible ways to order 18 axes; therefore the presence or absence of species lines crossing is only a subset of the potential observations; this study used the subset resulting from the default ordering of the character states.

### ***Phylogenetic Analysis for Alouatta Using Fur Colors***

Phylogenetic analysis of the fur color data was conducted using PAUP\* (Swofford 2001). An “ancestor” with all-agouti fur was added to the list of OTUs, but was not formally designated as an outgroup in PAUP\*. Methods used for deriving trees included exhaustive-search parsimony, neighbor-joining, UPGMA, and star-decomposition. The strict or semi-strict consensus tree was used to summarize the topology of multiple trees.

First, trees were derived from the unordered character states using exhaustive-search parsimony, neighbor-joining, UPGMA, and star-decomposition. The strict consensus tree was taken where more than one tree resulted.

Next, the exhaustive search parsimony tree searches were repeated with two different step matrices, each specifying the ordered and irreversible behavior of the two metachromic pathways encoded within the character states. One step-matrix was nearly the same as used by (Jacobs, Larson et al. 1995), where the cost to move any number of steps along the pathway is the same. The 11<sup>th</sup> state, A, was added to the matrix (Table 1).

	0	1	2	3	4	5	6	7	8	9	A
[0]	.	1	1	1	1	1	1	1	1	1	1
[1]	i	.	1	1	1	i	i	i	i	1	1
[2]	i	i	.	1	1	i	i	i	i	1	1
[3]	i	i	i	.	1	i	i	i	i	1	1
[4]	i	i	i	i	.	i	i	i	i	1	1
[5]	i	i	i	i	i	.	1	1	1	1	1
[6]	i	i	i	i	i	i	.	1	1	1	1
[7]	i	i	i	i	i	i	i	.	1	1	1
[8]	i	i	i	i	i	i	i	i	.	1	1
[9]	i	i	i	i	i	i	i	i	i	.	1
[A]	i	i	i	i	i	i	i	i	i	i	.

**Table 1.** The step matrix from (Jacobs, Larson et al. 1995) with addition of the A (“naked”) character state, which is treated as identical to but follows character state 9. This matrix enforces the two pathways, the irreversibility of progression down each pathway, and the ability to skip to any step farther down the progression. Each step has the same cost.

Comparing the original metachromic pathways diagram (Fig. III.15, p. 95) from (HersHKovitz 1977) with the diagram used in (Jacobs, Larson et al. 1995) (Fig. 1, p. 516) suggested the development of an additional step-matrix which is perhaps more consistent with HersHKovitz's original description. HersHKovitz' original diagram has arrows, absent from Jacobs et al.'s version, indicating that the leap from any color to white can be immediate. In the second matrix, referred to as the strict-pathway matrix, the cost of jumping to any other color down the pathway is the same as the cumulative cost of going through the intermediate states, with the notable exception of the jump to white. (Table 2)

	0	1	2	3	4	5	6	7	8	9	A
[0]	.	1	2	3	4	1	2	3	4	1	2
[1]	i	.	1	2	3	i	i	i	i	1	2
[2]	i	i	.	1	2	i	i	i	i	1	2
[3]	i	i	i	.	1	i	i	i	i	1	2
[4]	i	i	i	i	.	i	i	i	i	1	2
[5]	i	i	i	i	i	.	1	2	3	1	2
[6]	i	i	i	i	i	i	.	1	2	1	2
[7]	i	i	i	i	i	i	i	.	1	1	2
[8]	i	i	i	i	i	i	i	i	.	1	2
[9]	i	i	i	i	i	i	i	i	i	.	1
[A]	i	i	i	i	i	i	i	i	i	i	.

**Table 2. The strict pathways step matrix, developed to enforce a greater cost for skipping states along the pathway. The cost for stepping to white from any state remains the same as moving to the next state on the pathway, supporting the arrows included in HersHKovitz's original diagram (HersHKovitz 1977).**

Finally, to compare the phylogenetic information in the step-matrices, the topology of the possible-tree-space near the optima under the two different step matrices, and to correct for differences in weighting, the lengths of trees on the short lists of near-most-parsimonious trees under the two step matrices were determined under the other step matrix, and their semi-strict consensus tree created.

## ***Composite Phylogeny of Alouatta***

### ***Establishing OTUs***

Four recently published works on the phylogeny of the genus *Alouatta* (Meireles, Czelusniak et al. 1999; Bonvicino, Lemos et al. 2001; Oliveira, Neusser et al. 2002; Cortés-Ortiz, Bermingham et al. 2003) were used to establish a composite phylogeny. There was no overlap of the semaphoronts used in these studies or in the fur color coding data. The combination of phylogenetic analyses from a variety of sources necessitated the use of composite operational taxonomic units (OTU's) based on species identifications provided by the authors. Synonymies were resolved using (Wilson and Reeder 1993) and (Groves 2001). Monophyletic semaphoront groupings on the published trees were collapsed to match the species-level specificity of the fur color data, or were pruned from the tree. Specifically:

- *A. villosa* (from fur color data) is a synonym for *A. pigra*;
- *A. fusca* (from fur color data, (Meireles, Czelusniak et al. 1999; Bonvicino, Lemos et al. 2001; Oliveira, Neusser et al. 2002)) is a synonym for *A. guariba*;
- *A. macconnelli* was recently elevated to species status, previously located within *A. seniculus*. Since *A. seniculus* was also represented on trees where *A. macconnelli* was included, *A. macconnelli* was simply pruned from those trees;
- *A. seniculus arctoidea* in (Oliveira, Neusser et al. 2002) was generalized to *A. seniculus*;
- The authors' inclusion of *A. palliata* with *A. coibensis* clarified that any specimens previously designated *A. palliata coibensis* had been sorted out before inclusion.

### ***Networks***

Noting that the Maximum Parsimony tree and the Neighbor-joining tree reported in (Bonvicino, Lemos et al. 2001) showed the same relationship but with a different rooting, the trees presented within the four papers were first converted to unrooted networks and examined for compatibility and conflict in their topologies.

### ***Sensitivity evaluation of cytochrome b topology***

The cytochrome b mtDNA sequences for *Alouatta* and the outgroups from both (Bonvicino, Lemos et al. 2001; Cortés-Ortiz, Bermingham et al. 2003) published papers with mitochondrial DNA analyses were downloaded from GenBank for a combined re-analysis. The sequences from (Cortés-Ortiz, Bermingham et al. 2003) were shorter (800 bp) and already aligned; longer sequences (1016 bp) from (Bonvicino, Lemos et al. 2001) were aligned by eye in MacClade (Maddison and Maddison 2003) by adding a few



appropriate gaps at the beginning of the sequences. The sequences showed high levels of agreement; the alignment was very straightforward and not ambiguous.

Maximum Parsimony with branch-and-bound searching in PAUP\* (Swofford 2001) was used to generate a combined cyt b tree, checking that the named taxa shared between the papers fell out in the appropriate places. To test the effect that the choice of outgroup has, the cyt b *Alouatta* sequences from (Bonvicino, Lemos et al. 2001) were run with the following taxa sequences as outgroups: a) their original *Brachyteles* and *Callicebus*; b) the *Cebus* and *Ateles* outgroup sequences from (Cortés-Ortiz, Bermingham et al. 2003); c) all four: *Cebus*, *Callicebus*, *Brachyteles*, and *Ateles*; d) *Ateles* only; e) *Cebus* only. The location of the root on the topology of the network was noted for each of the resulting trees.

### ***Evaluation of Chromosomal characters***

Because the network topology results indicated an incompatibility between the mitochondrial DNA and chromosomal analyses, the 98 discrete chromosomal characters encoded by (Oliveira, Neusser et al. 2002) were examined in more depth. The characters were examined for variability. The different distinct patterns of grouping information contained within the characters were identified, and then a compatibility analysis (Meacham 1981, *Taxon*; 1980, *Systematic Botany*) was performed by hand on these groupings. Finally, the characters were examined for representation of independent evolutionary events.

### ***Matrix Representation with Parsimony***

To resolve the incompatibilities between the topologies due to different sources and outgroups in order to select one tree with which to proceed, four published trees (Table 3) were encoded using matrix representation and analyzed with parsimony (Baum 1992; Ragan 1992). Because this composite phylogeny is used to trace the progression of fur color character states in the best-known phylogeny of *Alouatta* as a test of the existence of metachromic progression, the trees created with fur color data within this study were not included; similarly, the morphological/biogeographic phylogenetic arrangement (Gregorin 1996); (Mittermeier and Coimbra-Filho 1981) reported in (Oliveira, Neusser et al. 2002) was not included because the role of metachromic characters in the resulting topology is unknown.

As before, OTU's included in the coding were based on species which occurred within the fur color data. Because the species used as outgroups were almost entirely non-overlapping, they were conflated and coded as a generic "outgroup" OTU.

Source	Tree used
(Bonvicino, Lemos et al. 2001)	Maximum parsimony (Fig. 1, p. 243)
(Cortés-Ortiz, Bermingham et al. 2003)	Maximum parsimony (Fig. 3, p. 71) and Maximum likelihood (Fig 5, p. 73) trees (both have same topology for this study)
(Meireles, Czelusniak et al. 1999)	Maximum parsimony (Fig. 4, p. 341)
(Oliveira, Neusser et al. 2002)	Maximum parsimony (Fig. 5, p. 680)

**Table 3. Sources of trees used when generating the composite phylogeny of *Alouatta* with MRP.**

Nodes:	CO	CO	CO	CO	CO	CO	CO	CO	CO	CO	B1	B2	B3	B4	B5	dO	dO	dO	dO	dO	M1	M2	M3
	1	2	3	4	5	6	7	8	9							1	2	3	4	5			
A. guariba	1	0	1	0	0	1	1	0	0	1	1	1	1	0	1	1	0	1	0	1	1	1	1
A. belzebul	1	0	1	0	0	1	1	0	0	1	1	1	1	0	1	1	1	0	0	1	1	1	1
A. caraya	1	0	1	0	0	1	0	1	0	1	1	0	0	0	1	1	1	0	0	1	0	0	0
A. sara	1	0	1	0	0	1	0	1	1	?	?	?	?	?	1	1	0	1	1	?	?	?	?
A. seniculus	1	0	1	0	0	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	0
A. pigra	1	0	1	1	0	0	0	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?
A. palliata	1	0	1	1	1	0	0	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?
A. coibensis	1	0	1	1	1	0	0	0	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?
outgroup	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

**Table 4. Matrix Representation of trees from Table 3.**

### ***Phylogenetic Analysis***

The combined matrix was analyzed by exhaustive search under parsimony in PAUP\*. The most parsimonious tree was kept. A semi-strict consensus tree was created for the trees one step longer than the most parsimonious tree.

### ***Tracing Metachromic Progression on Alouatta Phylogeny***

The Trace feature in MacClade (Maddison and Maddison 2003) was used to create the most parsimonious reconstruction (MPR) of the character states on the most parsimonious composite tree, for all 18 chromatogenic regions. To test if the metachromic progression represents the most parsimonious reconstruction of the fur color data, the trace was first conducted without imposing any order to the fur color character states. The metachromic progression was then imposed, and any increase in the number of steps required was noted. The “outgroup” was given a fur color coding of 0, the agouti state, for all chromatogenic regions.

Chromatogenic regions:	c1	c2	c3	c4	c5	c6	c7	c8	c9	c10	c11	c12	c13	c14	c15	c16	c17	c18
A. guariba	2	0	6	2	0	6	2	5	1	A	0	1	0	0	1	1	0	5
A. belzebul	6	2	2	A	2	2	2	2	7	A	2	A	2	2	7	2	?	7
A. caraya	8	2	2	2	1	2	1	2	2	2	2	2	2	1	1	1	1	1
A. sara	6	6	6	2	6	6	7	6	6	6	7	6	6	6	6	6	6	6
A. seniculus	6	6	6	2	6	6	8	6	6	A	8	8	6	6	6	6	7	7
A. pigra	2	2	2	2	2	2	2	2	2	A	2	2	2	2	2	2	2	2
A. palliata	2	2	2	2	2	2	2	2	2	A	8	2	2	2	2	2	2	2
A. coibensis	2	1	2	2	1	2	1	1	2	2	1	8	8	1	2	2	2	2
outgroup	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

**Table 5. OTUs and corresponding fur color codings used for most parsimonious reconstruction character tracing in MacClade. Character state “A” was problematic within MacClade.**

## Unordered MPR

The MPR was done first without imposing any order to the fur color character states. Each potential MPR for each of the 18 characters was evaluated by eye for consistency with the metachromic progression. For example, if a reconstruction required a state 2 (black) to change to a state 0 (agouti) the MPR is not consistent with metachromism, because it requires a reversal. Similarly, if a reconstruction required a state 2 (black) to change to a state 7 (orange) the MPR is not consistent with metachromism, because it requires switching between pathways. For each chromatogenic region, the number of steps and the existence or absence of an MPR consistent with the metachromic progression was noted.

## MPR enforcing metachromism

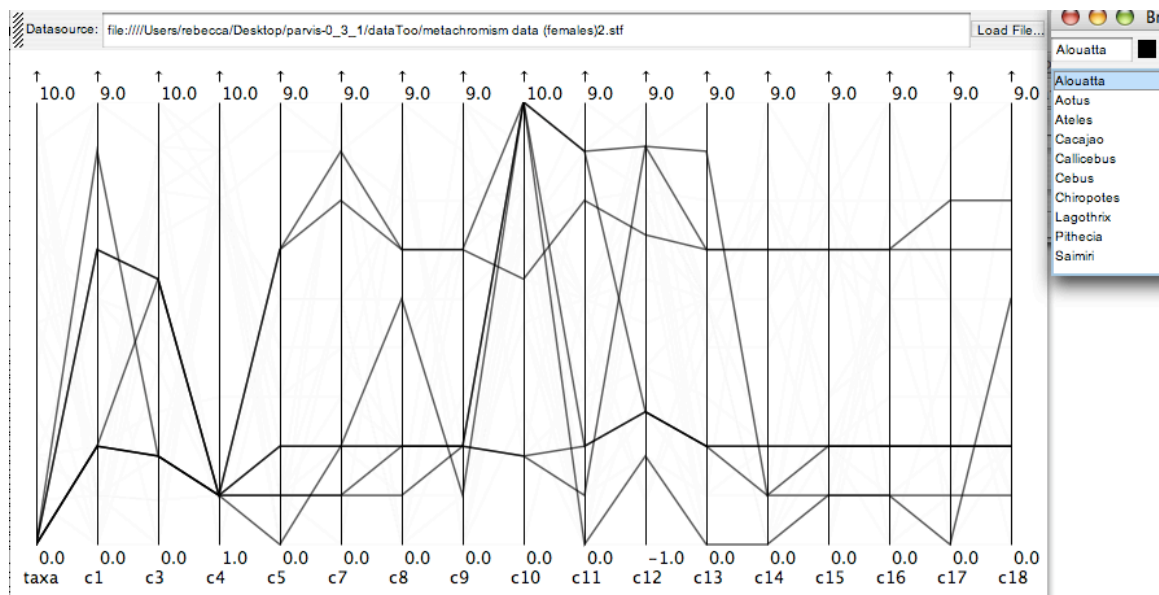
Next the MPR was done while enforcing metachromic progression using the Jacobs 95 step matrix. This matrix minimizes the weighting required to enforce metachromism: each acceptable transition has a weight of one, just like unordered steps, while unacceptable transitions are impossible. The number of steps required to achieve an MPR consistent with metachromism for each chromatogenic region was noted.

To determine how unusual these numbers are, MacClade’s “shuffle” feature was used on the fur color characters for the taxa 10 times, and the difference in the number of steps between an unordered MPR and the metachromically consistent MPR was noted. The outgroup was maintained as all-agouti.

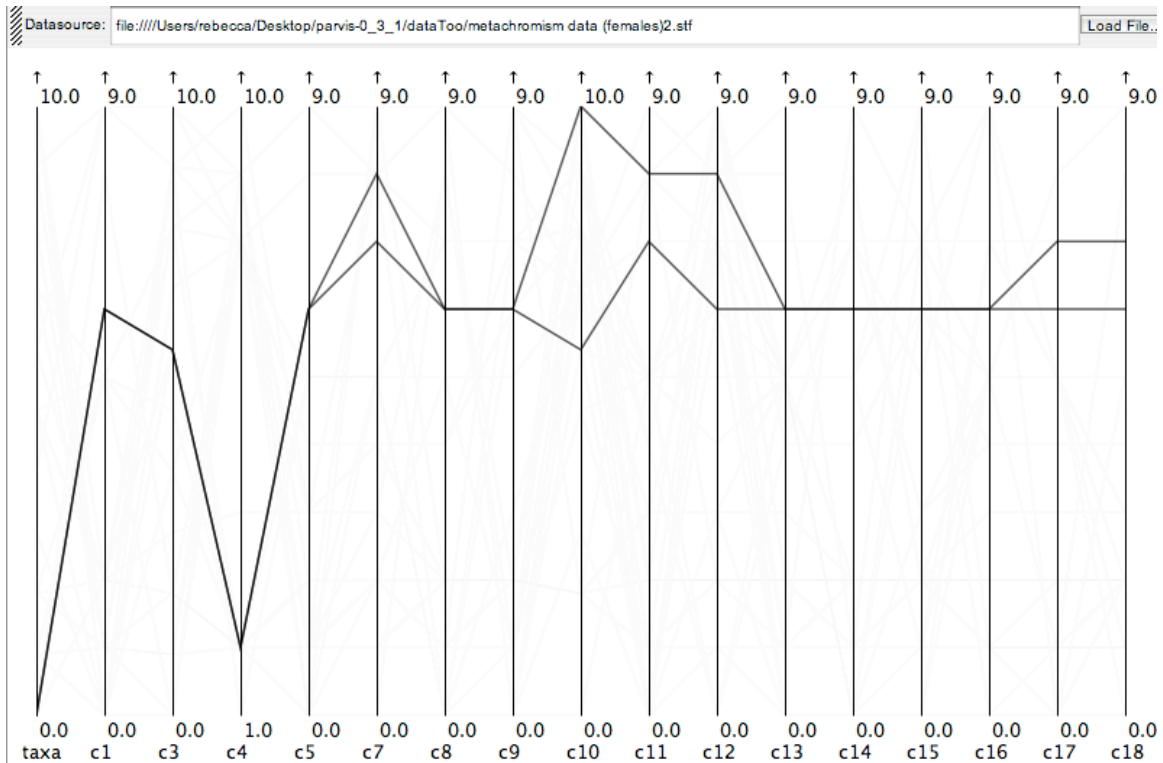
## Results

### Parallel coordinate plot visualization

In general, the visualization indicated that simple patterns fitting the metachromatic model were rare within the data. Highlighting lines by genus, it was possible to see what parts of the pathways were represented. The genera represented by fewer taxa were more likely to show a “signature” and have chromatogenic regions that occupy only one pathway; those with more taxa filled more of the possible character states. The signatures tended to be messier in the chromatogenic regions on the head, c1-c5. In *Alouatta*, all but one chromatogenic region had fur colors on both pathways (Figure 2).



**Figure 2.** The parallel coordinates visualization plot of fur color codings for *Alouatta*. Species have fur colors from both pathways for all chromatogenic regions except the mouth, c4.



**Figure 3. *Alouatta seniculus* and *A. sara* showing a simple divergence consistent with metachromism; *A. seniculus*'s line either plots to the same color state as *A. sara*, or to a higher value on the axes, therefore a color farther down the metachromic pathway.**

All genera showed both divergence patterns, those requiring a common ancestor (eg. *Alouatta*, Figure 4) and those not requiring one (eg. *Alouatta*, Figure 3) to be consistent with metachromatic progression. The parallel coordinates plots visualization indicates that the data tells a complex story from the metachromism perspective.

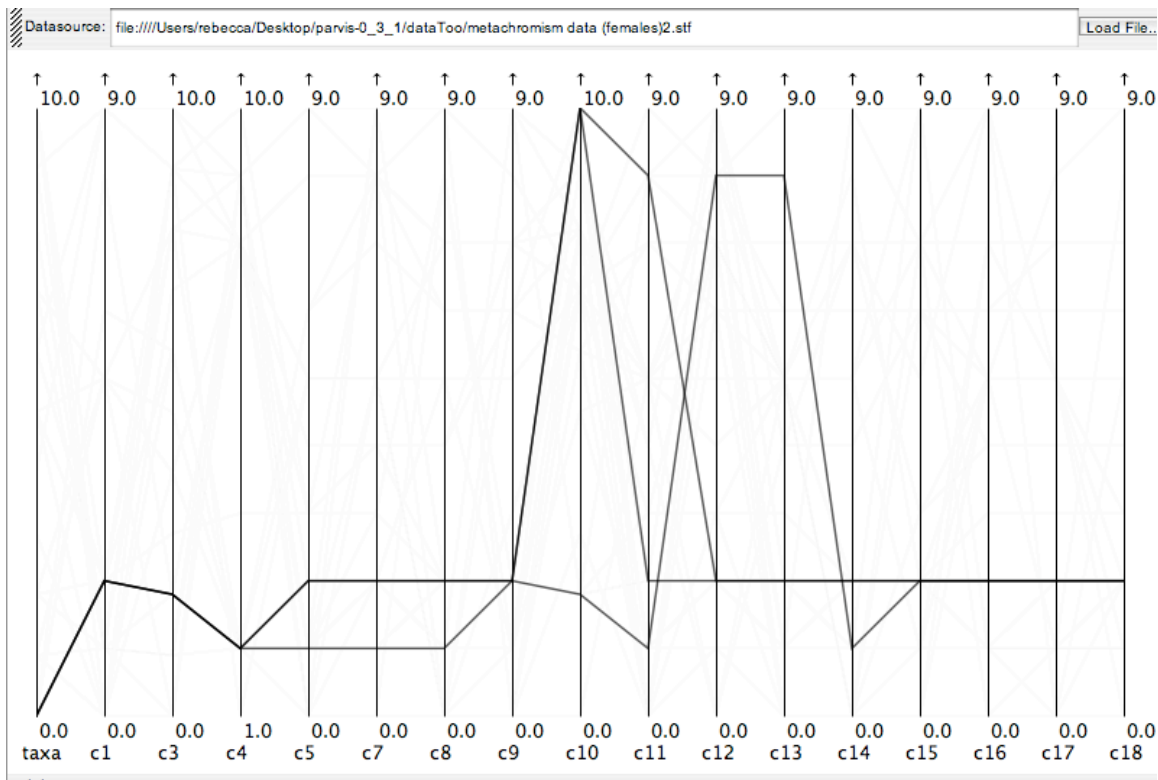


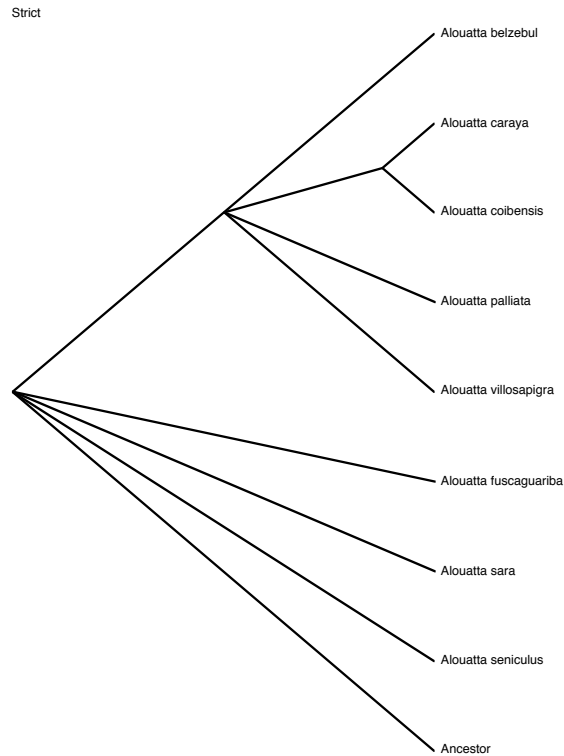
Figure 4. *Alouatta pigra*, *A. palliata*, and *A. coibensis* showing a divergence pattern inconsistent with simple metachromism. The lowest values on all axes are those of *A. coibensis*, with the exception of c12 and c13, belly and rump. Explaining this as a divergence consistent with metachromism requires invoking a now-absent common ancestor with an agouti (0) or black-saturated agouti (1) belly and rump. Note also that *A. palliata*'s yellow/cream (8) fur color state for c11, the back, is on a different pathway than *A. pigra*'s black (2) or *A. coibensis*'s black-saturated agouti (1), which also requires an agouti common ancestor to explain it under metachromatic progression.

## Phylogenetic Analysis for *Alouatta* Using Fur Colors

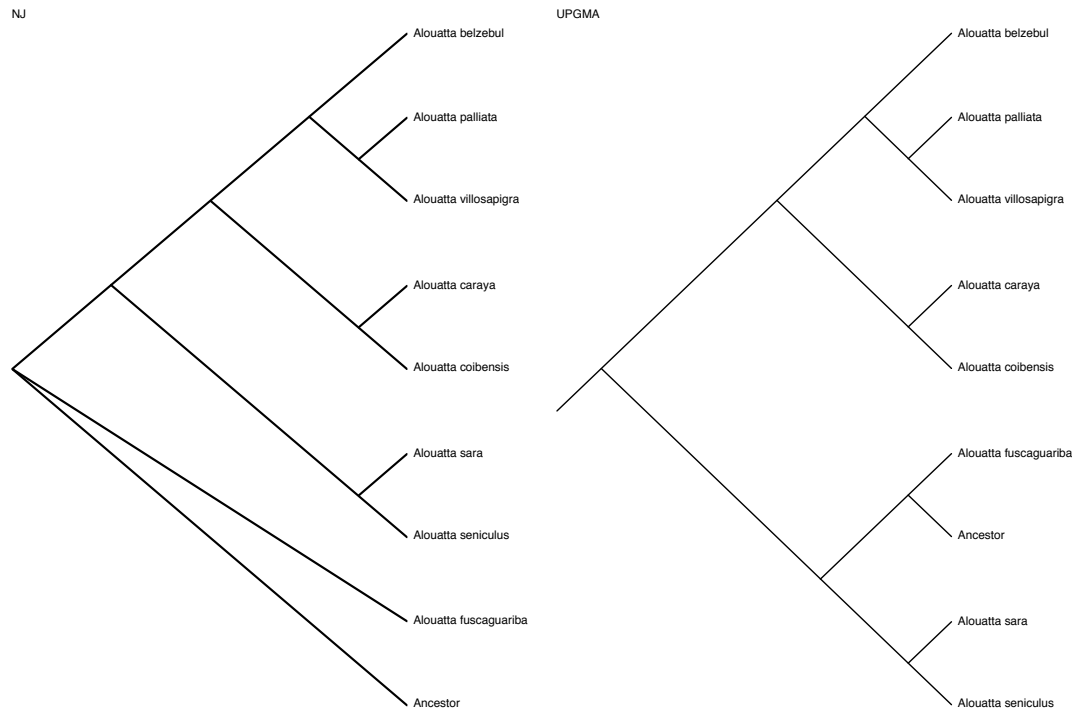
As unordered, unweighted characters, the fur color codes generated 18 most parsimonious trees of length 66. The strict consensus of these trees is shown in Figure 5, where the ancestor has been specified as the outgroup. The neighbor-joining and UPGMA trees are shown in Figure 6. The star-decomposition tree was not unique, and providing little insight, has not been included here.

Both of the step matrices produced a single most parsimonious tree (MPT), where the ancestor fell as the natural root (Figures 7 and 8). The length of the MPT under the Jacobs' step matrix was 80, and the same topology would have resulted in a tree of length 142 under the strict pathway matrix. The strict pathway step matrix MPT was 140 in length, and the same topology would have resulted in a tree of 82 under the Jacobs' step matrix. Exploration of the weightings of the trees up to two steps less parsimonious under the opposite step matrix shows that the optimality landscape is a slightly different shape

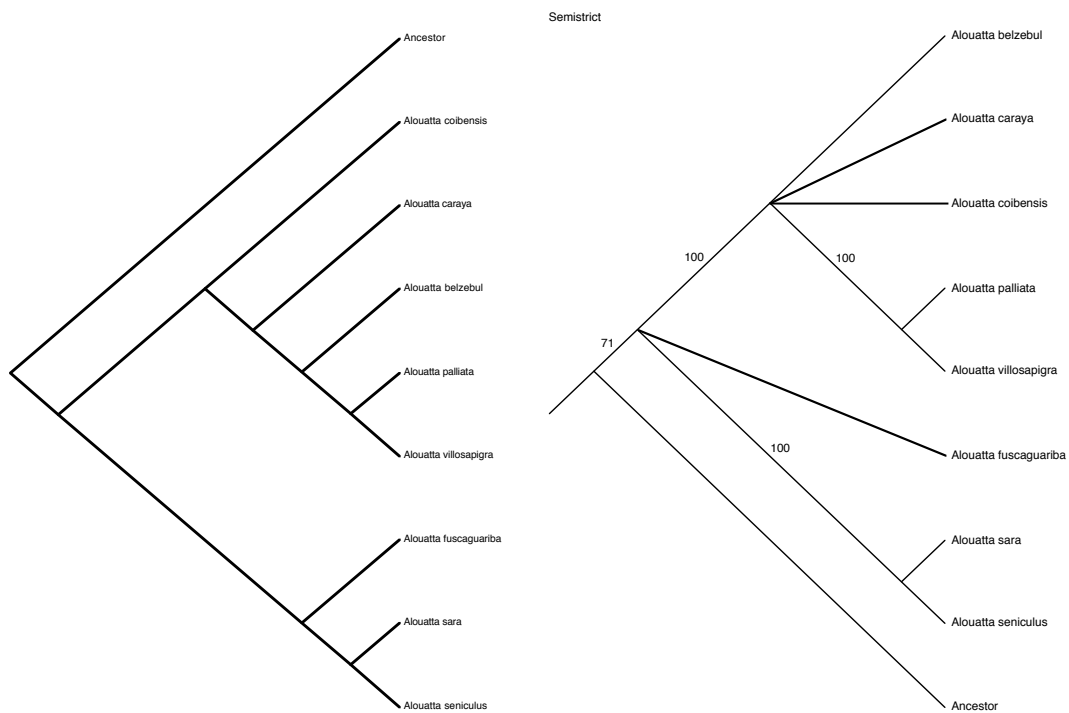
under the two step matrices; the short list of near-optimal trees does share 1-2 trees but is not the same under the two different matrices. However, the strict and semi-strict consensus trees for these groups of trees near the MPT agree, with the only difference being the resolution or not of *Alouatta guariba* as sister to the *A. sara*/*A. seniculus* clade.



**Figure 5.** The strict consensus tree of unordered fur color characters for *Alouatta* species, from 18 most parsimonious trees. The all-agouti ancestor was designated as the outgroup for the consensus tree.

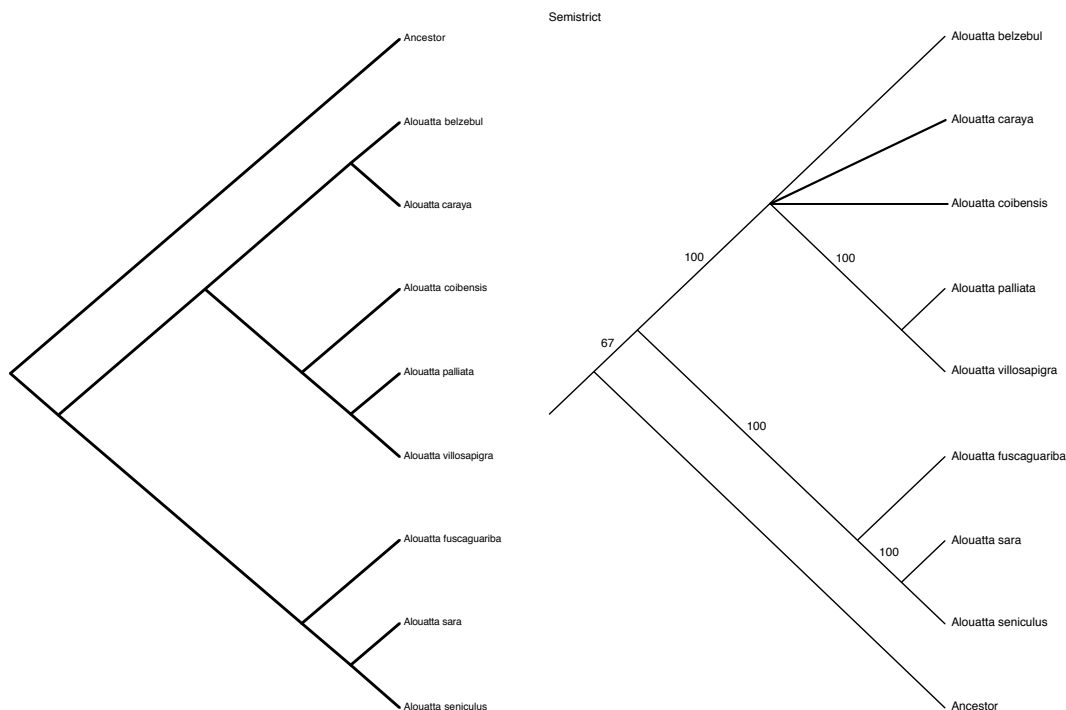


**Figure 6. The neighbor-joining and UPGMA trees for unordered fur color characters for *Alouatta* species.**



**Figure 7. The most parsimonious tree (length = 80) and the semi-strict consensus tree (lengths 80-82, 7 trees) of fur color characters for *Alouatta* species when ordered/weighted by the Jacobs step matrix.**





**Figure 8. The most parsimonious tree (length = 140) and the semi-strict consensus tree (lengths 140-143, 4 trees) of fur color characters for *Alouatta* species when ordered/weighted by the strict pathway step matrix.**

There is clearly a great deal of disagreement between the various methods for building trees from the fur color data. Except for the grouping together of *A. sara* and *A. seniculus*, it appears that difference methods, parsimony methods, and parsimony considering metachromic ordering can all arrive at different conclusions. The addition of metachromism-consistent ordering by using one of the step matrices clearly leads the tree in a particular direction; although the two matrices disagree on the optimal tree, their consensus of near-optimal trees shows a very similar topology.

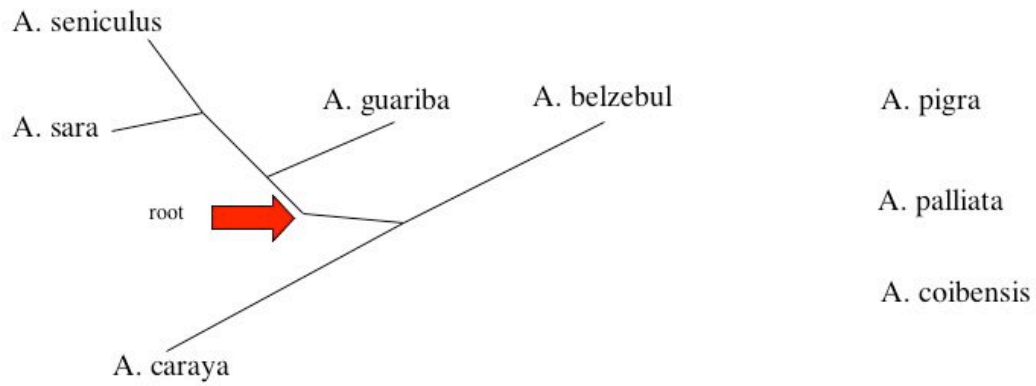
## ***Composite Phylogeny of Alouatta***

### ***Networks***

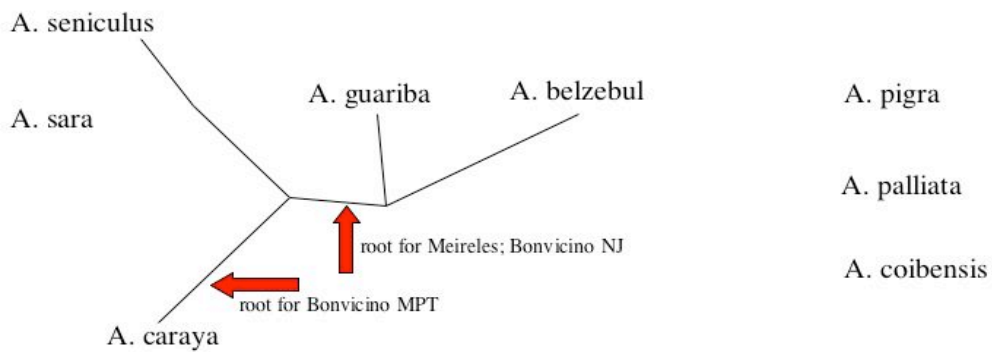
The network topologies from three previous mtDNA studies of the phylogeny within *Alouatta* (Meireles, Czelusniak et al. 1999; Bonvicino, Lemos et al. 2001; Cortés-Ortiz, Bermingham et al. 2003) are entirely compatible, while being incompatible the network from the chromosomal study (Oliveira, Neusser et al. 2002) and this study's fur color data (Figure 9). While each MPT from the two step matrices is individually compatible (rooted or unrooted) with the network from the chromosomal study, they are not compatible with each other as they each incorporate *A. coibensis*, *A. caraya*, and *A. belzebul* in a different fashion.

Incompatibility of phylogenetic signal between different character sources, such as morphology, nuclear molecular sequences, and organelle molecular sequences, is a known phenomenon in phylogenetic analyses (Marks 1994; Mishler 1994; Ruvolo 1994), and is usually accepted to be a result of such mechanisms as ancient polymorphisms, founder effects, allele sorting, hybridization and lineage sorting. Discrepancy in the phylogenetic signal between morphological/biogeographic and mtDNA sources in *Alouatta* has been noted by (Oliveira, Neusser et al. 2002); the phylogenetic arrangements proposed by the morphological/biogeographic studies unavailable for this study (Gregorin 1996);(Mittermeier and Coimbra-Filho 1981) provide an additional arrangement incompatible with those examined here.

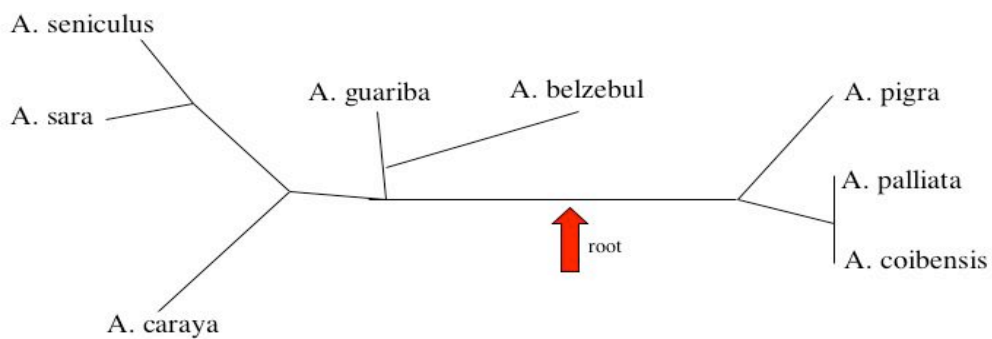
de Oliveira et al 2002



Meireles et al 1999, Bonvicino et al 2001



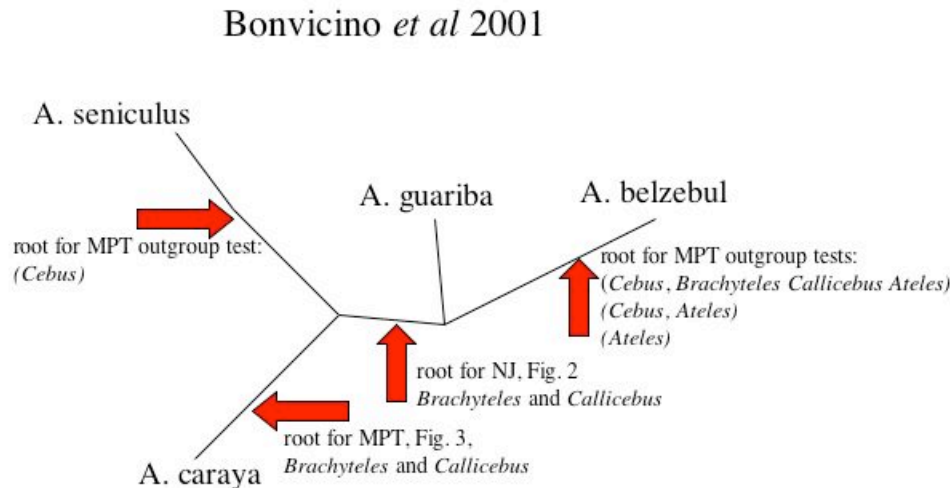
Cortes-Ortiz et al 2003



**Figure ?. Network diagrams for the four *Alouatta* phylogenies used in the composite phylogeny in this study. An attempt has been made to keep the taxa in the same location in order to make the figures easier to compare.**

### ***Sensitivity evaluation of cytochrome b topology***

The most parsimonious tree for each of the 5 different outgroup combinations provided the same network of relationships between the operational OTUs, but with the different outgroups suggesting that the network be rooted in a total of three different places (Figure 10). The neighbor-joining tree in the original paper specified a fourth place to root the network. The underlying relationships described by the cyt b sequences appear to be robust, but the rooting is unreliably sensitive to the choice of outgroup.



**Figure 10.** The network of relationships between the OTUs from (Bonvicino, Lemos *et al.* 2001) of interest within this paper, as rooted by various outgroup sequences during branch and bound search for the most parsimonious tree.

### ***Evaluation of Chromosomal characters***

Close examination of the coding of the chromosomal characters presented in (Oliveira, Neusser *et al.* 2002) showed some oddities. Twenty-four of the characters are coded “1” for all OTUs, including the outgroup. An additional thirty-three characters are autapomorphies, where only one OTU has the second state. These 57 characters fail the criteria of being variable within the study group, and because of long-branch attraction issues when using parsimony (Mishler 1994), would be better left out of the study. The remaining 41 are parsimoniously informative characters.

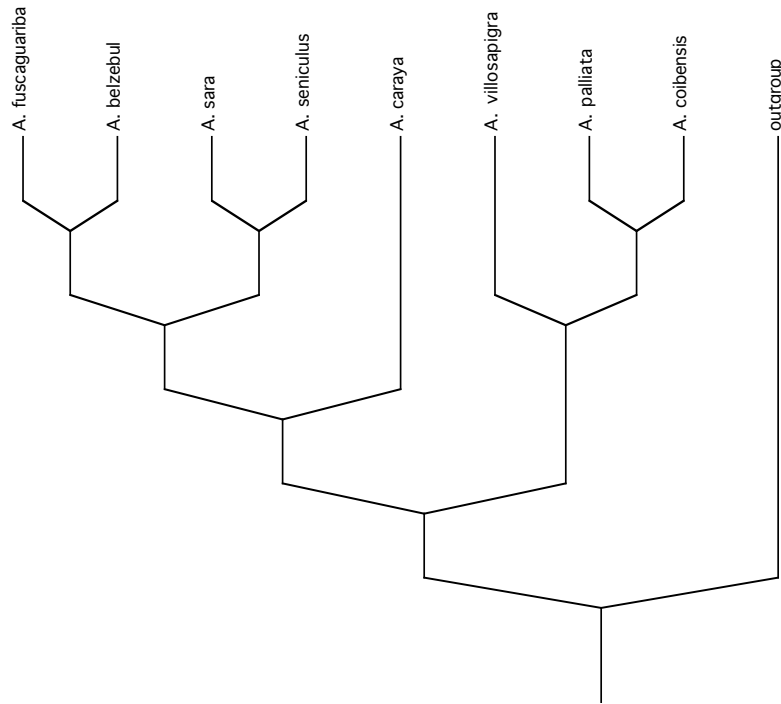
Eleven different grouping signals were identified within the 41 parsimoniously-informative chromosomal characters. Compatibility analysis indicated that four of these grouping signals, representing 24 of the characters, form a completely compatible clique. Another two grouping signals were nearly compatible, and the graph of all of the signals shows a high level of connectivity.

Finally, the events which the characters are coding were examined for evolutionary independence. At least one complex of characters failed the test of independence. For

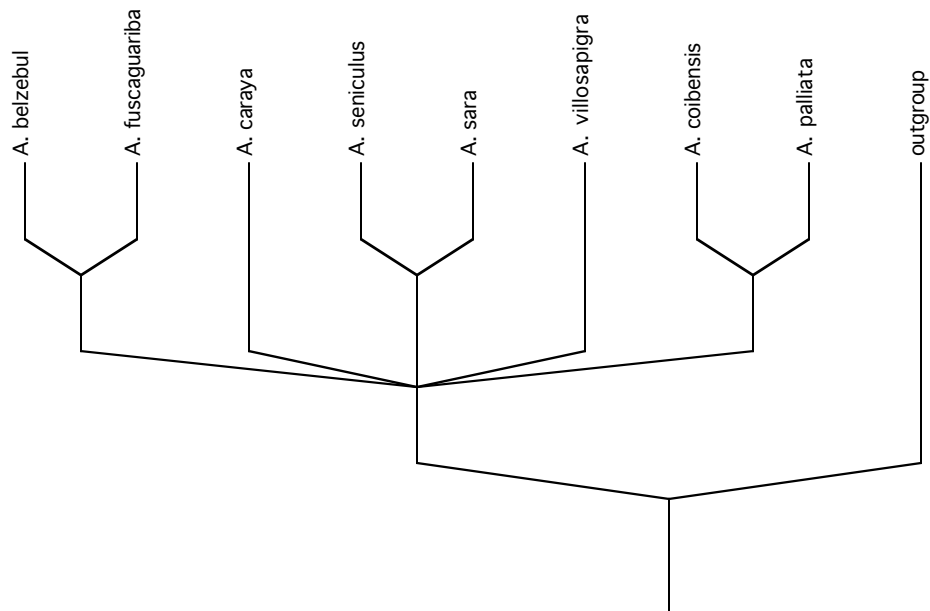
example, one character encodes the presence or absence of a particular association between two identified pieces of the chromosome. At the same time, a different character encodes the presence or absence of a different association which involves one of the first pieces with a second, different piece of chromosome. These characters are interrelated by being mutually exclusive – they cannot both have happened in the same OTU – or by being mutually dependent – one cannot have happened unless the fission creating the absence of the other has taken place. Analysis suggests that one character cluster could involve as many as 7 of the 98 characters, and confirms that this is not an example of binary encoding of a complex character. In order to ensure that characters in a matrix encode the occurrence of an evolutionary event once and only once, these character clusters should be coded as one multi-state character, where a step-matrix or character state tree captures the complexity of the possible combinations.

### ***Matrix Representation with Parsimony***

The single most parsimonious tree that incorporates the four previously published studies is shown in Figure 11. The semi-strict consensus tree between the trees one step longer is shown in Figure 12.



**Figure 11. The single most parsimonious tree that combines the four previously published studies on the phylogeny of *Alouatta*.**



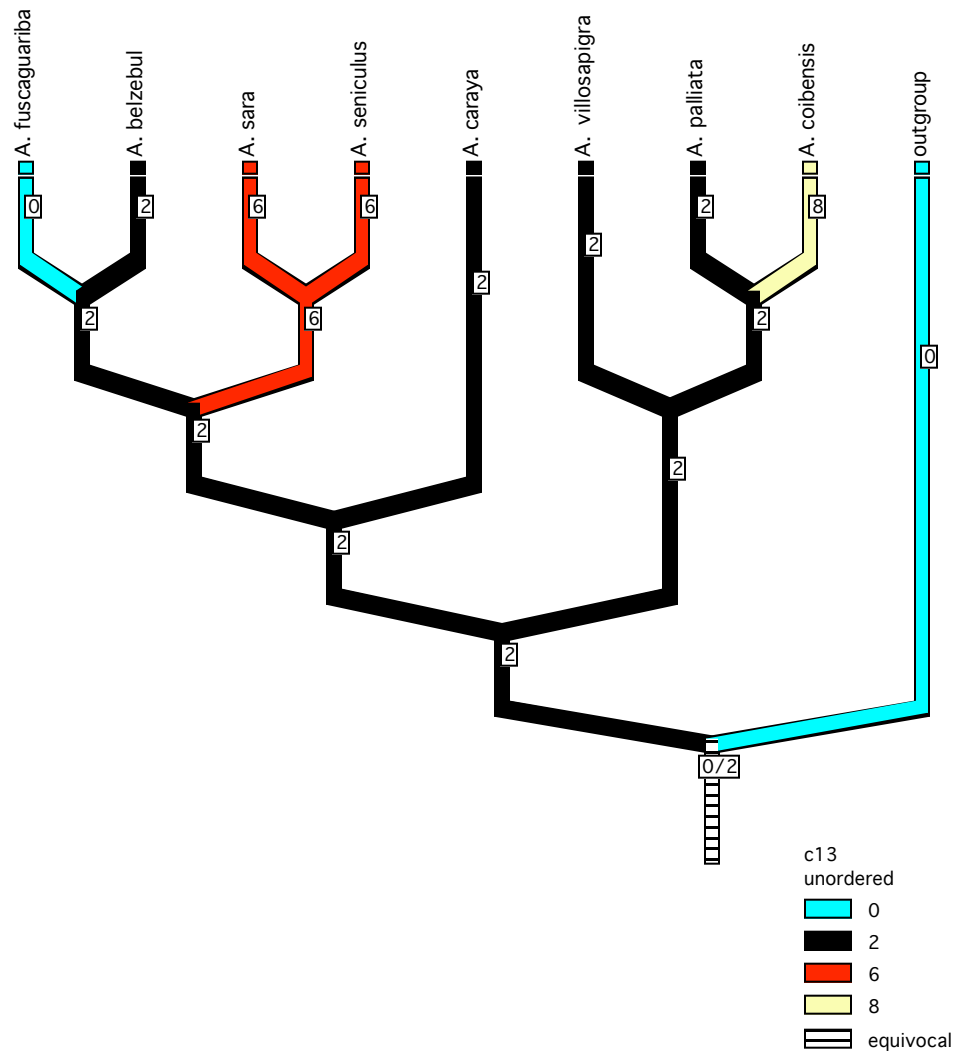
**Figure 12.** The semi-strict consensus tree of the trees one step longer than the MPT in Figure 11.

### ***Tracing Metachromic Progression on Alouatta Phylogeny***

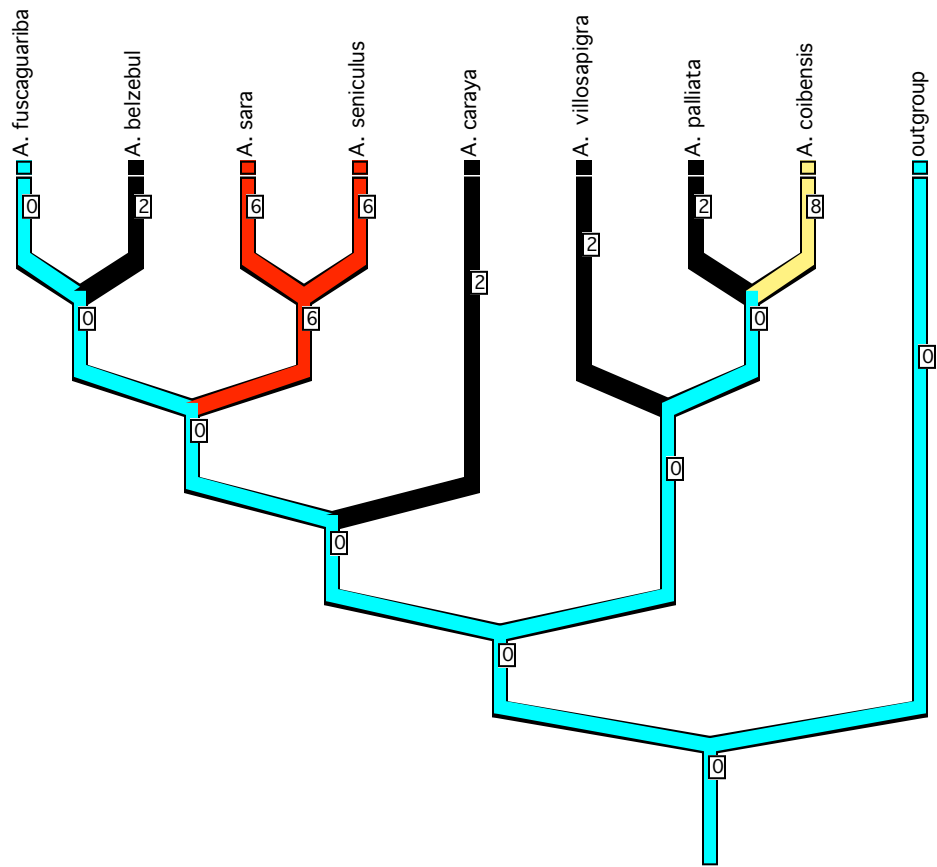
Color states were well represented among the 18 chromatogenic regions within *Alouatta*, with 17 regions having color states from both pathways, and 14 regions having 4 to 6 different color states.

The c4 chromatogenic region around the mouth changes only from black to naked within *Alouatta*. One of the four MPR of fur colors on the main section of the tail, c17, is consistent with the metachromic progression. For the remaining 16 chromatogenic regions, none of the equally MPRs of unordered fur color states are consistent with metachromic progression. Enforcing a state reconstruction that is consistent with metachromism adds a total of 31 additional steps to the tree, comprised of 1 to 4 additional steps for each of the 16 chromatogenic regions.

The results from tracing the actual fur color characters on the composite phylogeny were essentially indistinguishable from tracing shuffled fur color states. When the fur color characters were shuffled, an average of 34.8 (range 32-37) steps were added to the tree by an MPR consistent with the metachromic progression. On average, 1.0 chromatogenic regions did not add steps when metachromism was enforced, although this was not evenly distributed among the regions; this occurred 4 times at the thighs, 3 times by the main tail section, 2 times at the back, and once at the neck.



**Figure 13.** Example of an MPR trace of unordered character states for chromatogenic region c13, the rump. Note the transition from 2- black to 1-black-saturated-agouti that is inconsistent with the irreversible metachromic progression. The transition from 2-black to 6-red and from 2-black to 8-yellow/cream represents leaping from one pathway to the other.



**Figure 14.** An MPR trace where metachromic progression according to the Jacobs matrix has been enforced, for the same chromatogenic region as in Figure 13. Enforcing metachromic progression costs one additional step in this example.

## Discussion

There is a high degree of disagreement between phylogenetic data sources about the tree for the genus *Alouatta*. Future studies should address this disagreement by avoiding composite OTUs, and draw on as many data sources as possible. This includes using fur colors from male and female semaphoronts; finding valid morphological characters, perhaps by examining published morphological data; using molecular data from multiple organelles and chromosome painting all from the same semaphoront. Properly coded chromosomal characters look particularly promising for providing a strong phylogenetic signal about the evolution of *Alouatta* taxa.

In spite of the disagreement about the proper tree for *Alouatta*, the evidence from this study does not support the predictive value of metachromic progression for uncovering the evolutionary tree of *Alouatta*. By using a Jacobs' mild-enforcement step matrix and



an all-agouti outgroup, these tests were biased towards supporting metachromism, yet still found it a poor explanation of the evolutionary patterns formalized by the composite tree. Reconstruction of the metachromic progression on the composite *Alouatta* tree adds an average of 2 steps to the most parsimonious reconstruction, a result robust to the distribution of fur color characters among the taxa and therefore likely to be robust to different tree topologies.

The principle of metachromism is based on well-established mechanisms for the biosynthesis of fur color pigments. This study refutes primarily Hershkovitz' claim of the uniqueness and irreversibility of the two pathways. For example, a step matrix that allows the ability to switch between the pathways, from 2-black to 6-red, (which also allows 2->7, 2->8, 1->6, 1->7, and 1->8) is remarkably more consistent with the most parsimonious reconstructions of the characters on the composite *Alouatta* phylogeny. Indeed, Hershkovitz mentions the possibility of this transition between the saturated states in the caption to figure III.15 on p. 95 (Hershkovitz 1977). However, this interpretation of the metachromic progression allows so many different possible transitions that the model begins to lack predictive power.

Parsimony is the appropriate default optimality criterion in the absence of any specific justification for why particular lineages evolved through a less-than-parsimonious set of transitions. Given current technologies and knowledge of fur color genes in other mammals, studies of the genetic mechanisms underlying fur colors in Neotropical primates should be feasible, and may eventually indicate that the metachromic progression (or some variation thereof) is a better description of the constraints on the evolving lineages than parsimony. Current knowledge does not constitute this specific justification for why parsimony should be laid aside in favor of the metachromic progression for fur colors or associated genetic traits.

Most likely, the processes of banding, saturation and bleaching that Hershkovitz describes are indeed important, natural aspects of the biology of mammalian fur coloration, but the pathways and irreversibility that he postulated in the principle of metachromism are not valid on a large evolutionary scale. The parallel coordinates visualization plots suggest that stories consistent with the principle of metachromism can be told between two species, or within a small group of very closely related species. This study, however, suggests that even for genus-level phylogenetic analyses, to include the pathways and irreversibility as additional structuring information for multi-state fur color characters is spurious.

Metachromism is consistently not the most parsimonious reconstruction of the changes in fur color in *Alouatta*, so it is not a promising source for predicting *Alouatta*'s phylogeny. Similar investigations should be attempted with other genera, especially where Hershkovitz evaluated the taxonomy using the principle of metachromism (eg. *Cacajao*, *Pithecia*).

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