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Molecular cladistic markers in New World monkey phylogeny (Platyrrhini, Primates)

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Abstract

Transpositions of primate-specific Alu elements were applied as molecular cladistic markers in a phylogenetic analysis of South American primates. Seventy-four human and platyrrhine loci containing intronic Alu elements were PCR screened in various New World monkeys and the human outgroup to detect the presence of orthologous retrotransposons informative of New World monkey phylogeny. Six loci revealed size polymorphism in the amplification pattern, indicating a shared derived character state due to the presence of orthologous Alu elements confirmed by subsequent sequencing. Three markers corroborate (1) New World monkey monophyly and one marker supports each of the following callitrichine relationships: (2) *Callithrix* and *Cebuella* are more closely related to each other than to any other callitrichine, (3) the callitrichines form a monophyletic clade including *Callimico*, and (4) the next living relatives to the callitrichines are *Cebus*, *Saimiri*, and *Aotus*.

1. Introduction

Extant New World monkeys are commonly assigned to 16 genera or subgenera, 12 of which belong to three monophyletic groups: the Pitheciidae/Pitheciinae (three genera), the Atelidae/Atelinae (four genera), and the Callitrichidae/Callitrichinae (five genera). In accordance with the age-related taxonomic classification of Goodman et al. (1998), we address these groups as subfamilies (but see also Ford, 1986; Horovitz et al., 1998; Kay, 1990; Rosenberger, 1992). Less agreement exists with regard to the branching order between these three clades and the phylogenetic affiliations of the genera *Cebus*, *Saimiri*, *Callicebus*, and *Aotus*.

Taxonomic grouping of all New World primates to the infraorder Platyrrhini implies a monophyletic origin of South and Middle American primates, which is not indisputed. Few synapomorphic characters demonstrate New World monkey monophyly. Dental characters, originally supposed to be synapomorphies, are shown to be not restricted to South American primates only. Anatomical characteristics like the broad nose and possession of three premolars are primitive rather than derived features (Hofer, 1976, 1979; Rosenberger, 1977). Few cranial and postcranial synapomorphies remain to support platyrrhine monophyly (discussion in Ford, 1986). The presence of intraplacental maternal vessels and placental hematopoiesis are shared derived traits linking all platyrrhines (Luckett, 1980). The strongest evidence in favor of New World monkey monophyly, however, is provided by DNA sequence studies (Horovitz and Meyer, 1995; Horovitz et al., 1998; Porter et al., 1995, 1997; Schneider et al., 1993). Nevertheless, there is also contrary evidence from skin morphology, cytogenetics, and immunogenetics arguing in favor of a paraphyletic origin of the platyrrhines and double invasion of South America by primates (Bauer and Schreiber, 1997; Chiarelli, 1980; Perkins and Meyer, 1980).

The phylogenetic history of the smallest New World monkeys, the callitrichines, has always been a subject of controversy. The "Callitrichidae" traditionally comprise the four genera *Cebuella*, *Callithrix*, *Saguinus*, and *Leontopithecus*. Bearing claws on all digits, except the big toe and regularly giving birth to twins, they were long considered to represent the primitive ancestral simian state (e.g., Hershkovitz, 1977). Evidence, however, points out that these and other traits are derived

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specializations (for discussion see Ford, 1980; Garber et al., 1996; Martin, 1990; Rosenberger, 1981), which separate them from all other New World monkeys. Accumulating information places the monospecific *Callimico goeldii*, formerly considered an intermediate between the callitrichines and the other platyrrhines, at the centre of the callitrichines as a sister to *Callithrix* and *Cebuella* (Canavez et al., 1999; Chaves et al., 1999; Harada et al., 1995; Horovitz and Meyer, 1995; Horovitz et al., 1998; Pastorini et al., 1998; Porter et al., 1997; Schneider et al., 1996; von Dornum and Ruvolo, 1999). Growing evidence suggests that the genus status of *Cebuella* has to be revised and its only representative *Cebuella pygmaea* included in the genus *Callithrix* (Barroso et al., 1997; Canavez et al., 1996; Canavez et al., 1999; Chaves et al., 1999; Garber, 1992; Groves, 1989; Hugot, 1998; Meireles et al., 1998; Moreira and Seuánez, 1999; Nagamachi et al., 1999; Porter et al., 1997; Rosenberger and Coimbra-Filho, 1984; Tagliaro et al., 1997). The question of the next living relative to the callitrichines is one of the issues attracting most controversies and is closely linked to the question of the positions of *Cebus*, *Saimiri*, and *Aotus* in the New World monkey tree.

In the present paper, we address the issue of New World monkey monophyly and the questions on callitrichine phylogeny using a molecular cladistic approach. Shared derived transpositional events of Alu elements serve as cladistic markers to reveal molecular synapomorphies and to establish sister group relationships of taxa.

Alu elements are a primate-specific family of short interspersed nuclear elements (SINEs) of about 300 base pairs (bp) in length and an estimated copy number of up to one million per human genome (Li et al., 2001). They are composed of two 7SL RNA derived dimers forming two guanine–cytosine rich subunits connected via an adenine-rich linker and ending in a poly-adenine tail. SINEs are class I transposable elements (retrotransposons), which transpose replicatively via an RNA intermediate. Containing an internal promoter for RNA polymerase III but lacking a reverse transcriptase gene their transposition depends upon the enzymatic machinery of corresponding class II long interspersed nuclear elements (LINEs) (Ogiwara et al., 1999; Okada et al., 1997). During the endonuclease-mediated integration process, staggered end breaks are introduced into the target sequence, resulting in direct repeats (DRs) flanking the SINE-insertion.

SINE-transpositions can be considered unique, irreversible, and largely target-independent events (Cook and Tristem, 1997; Hamdi et al., 1999; Shedlock and Okada, 2000). There is no evidence to suggest that once inserted, a retroelement has ever been precisely re-excised introducing a reversal of the character state (Cook and Tristem, 1997; Shedlock and Okada, 2000). Thus, by identifying the target site of a SINE-integration in a certain taxon, which is duplicated upon integration, it is possible to clearly distinguish between an ancestral and a derived character state at the respective loci. Although there might be a preference for certain target structures depending upon the integration machinery (Cantrell et al., 2001; Jurka et al., 1998), the heterogeneity of the integration sites of known transpositions and the size of the primate nuclear genome support the random character of target choice. Therefore, copies of the same element in two different taxa provide evidence of an integration event that took place in the germ line of a common ancestor. Insertion events provide few high quality markers, whereas DNA sequence data, which are easily and quickly generated and more frequently applied in molecular phylogeny, deliver many low weight characters (Cook and Tristem, 1997). SINEs have already been used as phylogenetic markers in salmonid fish (Murata et al., 1993, 1996), cichlids (Takahashi et al., 1998), and cetartiodactyls (Nikaido et al., 1999; Shimamura et al., 1997, 1999). Alu elements, in particular, have been applied to review the phylogenetic relationships of the great apes (Hamdi et al., 1999) and to analyze the position of *Tarsius* in the primate tree (Schmitz et al., 2001).

2. Materials and methods

2.1. Samples and DNA extraction

Applying standard protocols (Sambrook et al., 1989), genomic DNA was extracted from human blood and tissue samples of the following New World monkeys: *Ateles fusciceps*, *Alouatta belzebul*, *Aotus azarai*, *Cacajao calvus*, *Callicebus cupreus*, *Callimico goeldii*, *Callithrix jacchus*, *C. geoffroyi*, *C. penicillata*, *Cebuella pygmaea*, *Cebus apella*, *Chiropotes satanas* x *albinasus*, *Lagothrix lagotricha*, *Leontopithecus chrysomelas*, *L. chrysopygus*, *L. rosalia*, *Pithecia pithecia*, *Saguinus bicolor*, *S. fuscicollis*, *S.f. lagonotus*, *S. labiatus*, *S. midas*, *S. oedipus*, and *Saimiri sciureus*. The tissues were provided by the German Primate Center, the Universities of Göttingen, Kassel, and Montpellier and the Zoological Gardens of Apeldoorn, Dresden, Eberswalde, Köln, Magdeburg, Münster, Osnabrück, and Wuppertal.

2.2. Primer design and PCR amplification

The GenBank database was queried for human and platyrrhine sequences appropriate for PCR-based presence/absence analyses of SINEs at orthologous loci. The loci to be analyzed were ideally chosen to contain an intronic Alu element in either the human or platyrrhine sequence, proving their general target structure suitability for transposition processes. Primers were located in conserved exon regions to facilitate successful

amplification of orthologs in evolutionary distant platyrrhine taxa and the outgroup *Homo sapiens*. For some loci, intronic primers were additionally created to facilitate PCR and sequencing procedures. In one case (marker VP, see below), only intronic primers could be constructed because the database entry was restricted to intronic sequence information. We included only loci for which the physical distance between the exonic regions allowed routine PCR typing with an upper size limit in the range of 1.5 kb.

In this way, 74 primer pairs were first tested in a basic New World monkey panel comprising one callitrichine, one pitheciin, one atelin, the four genera with uncertain phylogenetic affiliation (*Aotus*, *Callicebus*, *Cebus*, and *Saimiri*), and the human outgroup. Standard PCR amplification (Taq Polymerase Kit, Qiagen) was performed in a Biozym PTC 200 cycler under the following conditions: 120 s at 92 °C predenaturation, 30 cycles consisting of 40 s at 92 °C denaturation, 60 s at primer specific annealing temperature, and 60 s per 1 kb at 72 °C elongation. PCR products were analyzed by agarose gel electrophoresis (1% SeaKem, Biozym) and UV-visualized by ethidium-bromide staining.

2.3. Marker identification, cloning, and sequencing

PCR products were considered monomorphic if they differed less than 100 bp in size in all species investigated, taking into account the intronic sequence length variation due to non-specific insertions/deletions. In case the amplification products obtained from two or more platyrrhines exceeded the fragments of other taxa or the human outgroup by more than 100 bp in length (size range of monomeric and dimeric transposable elements), this presumably informative marker was submitted to further PCR analysis including all available New World monkey genera. Markers indicative of callitrichine phylogeny were confirmed in various species and/or individuals of the callitrichine genera at the PCR level.

Informative markers were then sequenced in one representative of each taxon in question and in an outgroup taxon. The PCR products were purified using QIAquick Gel Extraction Kit (Qiagen), ligated into pGEM-T vector system I (Promega), and electroporated into *Escherichia coli* TOP 10 cells (Invitrogen). Recombinants were screened by PCRs applying plasmid-specific primers. From positive clones, DNA was isolated (QIAprep Spin Miniprep Kit, Qiagen). Each of the three positive clones was sequenced on both strands with universal primers using an automated LI-COR DNA sequencer 4200 and the Thermo Sequenase Fluorescent Primer Labelled Cycle Sequencing Kit (Amersham) according to manufacturer's instructions.

Thus, orthologous partial sequences were obtained from three loci specifying the thymidine kinase (TK), LIM kinase (LIMK1), and intron 4 of visual pigment (VP) from *Aotus*, *Ateles*, *Callicebus*, *Cebus*, *Chiropotes*, and *Leontopithecus*.

Partial sequences were also obtained from three loci specifying the lysozyme (LYS), stem cell tyrosine kinase (STK1), and heparin-binding EGF-like growth factor precursor (HBGF). The respective orthologous sequences were determined for *Aotus*, *Ateles*, *Callimico*, *Callithrix*, *Cebuella*, *Cebus*, *Leontopithecus*, *Saguinus*, and *Saimiri*. All sequences are deposited in GenBank under Accession Nos. AF368141–AF368146, AF368153–AF368167, and AF489242–AF489265.

2.4. Analysis of Alu elements

Alu elements were detected and classified using the RepeatMasker software (Smit and Green, RepeatMasker at http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker). To ascertain that parts of the functional genes were amplified and to exclude unintentional analyses of possible pseudogenes, the reading frames of the exon sequences were verified and checked for possible frameshifts and stop codons by the sequence alignment editor (Se-Al) available from http://evolve.zoo.ox.ac.uk/. The orthology of inserted Alu elements was confirmed by identification of their flanking sequences.

2.5. Sequence data analysis

The intron sequences obtained from *Aotus*, *Ateles*, *Cebus*, *Chiropotes*, and *Leontopithecus* from the three loci, indicating platyrrhine monophyly, and the intron sequences obtained from *Ateles*, *Saguinus*, *Leontopithecus*, *Callimico*, *Callithrix*, and *Cebuella* from the three loci informative of callitrichine phylogeny were aligned to the human orthologs by CLUSTAL X (Thompson et al., 1997). In this way, two alignments of 3595 and 2268 bp in length, respectively, were produced excluding exons and Alu elements. To eliminate poorly alignable sequence parts and indels from further analysis, we applied the GBLOCKS 0.73b software with default settings for rDNA alignments, as outlined in Castresana (2000). We, thus created alignments of 3184 and 2071 bp for the concatenated datasets, indicative of platyrrhine monophyly and callitrichine phylogeny, respectively.

Phylogenetic reconstructions were performed applying three methods: maximum-parsimony (MP) included in PAUP* 4.0b4a (Swofford, 2000), LogDet distance transformation using PAUP* 4.0b4a (Swofford, 2000), and maximum likelihood (ML) as implemented in PUZZLE 4.0.2 (Strimmer and von Haeseler, 1996). Heuristic parsimony analyses were conducted with random taxon addition and tree bisection-reconnection (TBR) branch swapping. The ML analyses were carried out with the HKY model of sequence evolution

approximating a gamma distribution of rates across sites by introducing four rate categories. The respective gamma distribution parameter alpha was estimated from the datasets as well as the frequency of the nucleotides. Support of internal branches was either determined by bootstrap analysis (MP and distance) performed with 1000 replications or was indicated by the ML quartet puzzling support values (1000 puzzling steps).

3. Results

Altogether, 74 PCR primer pairs were tested in a standard New World monkey panel comprising representatives of all platyrrhine genera, with the exception of *Brachyteles* (see below). Primer pairs specific to exonic or intronic regions of the TK, LIMK1, VP, LYS, STK1, and HBGF-genes, respectively, displayed length variation of the PCR products indicating the presence of shared derived Alu-transpositions that originated during New World monkey phylogeny (Table 1). To prove the presumed orthology of the transposing elements and to exclude the analysis of independent transpositions that took place in the same intron at different positions, the fragments were sequenced. In this way, we determined the sequences adjacent to the transposing element including the DRs, which represent the transposition target site after integration. The physical appearence of Alu elements at orthologous sites yielded retropositional evidence that links: (1) all extant platyrrhine genera, (2) *Callithrix* and *Cebuella*, (3) all callitrichines including *Callimico*, and (4) the callitrichines with *Cebus*, *Saimiri*, and *Aotus*. Figs. 1 and 2 show the PCR patterns obtained by amplification of these marker loci and diagrams of the respective situations at the molecular level.

3.1. Markers supporting NWM monophyly

3.1.1. Marker TK (Fig. 1a)

PCR primers specific for the regions of exons 2 and 3 of the tyrosine kinase gene were created which amplify a 1.7 kb fragment in *H. sapiens* that contains two Alu elements. The corresponding PCR products of New World monkeys exceeded the human one by 300 bp in length. Initial sequencing of the 2 kb amplification products in some platyrrhines revealed an additional Alu repeat located between the two ancestral ones, which are also present in the human sequence. From the New World monkey sequences obtained, we deduced intronic primers yielding 1 kb fragments in all New World monkeys examined. Subsequent sequencing in representatives of the main platyrrhine groups and of taxa with questionable affiliation confirmed the presence of a platyrrhine-specific Alu element 76 bp 3′ from the first and 74 bp 5′ from the second plesiomorphic Alu repeats. Identical DRs can be traced at its boundaries and the unoccupied target site observable in *H. sapiens* differs in only one position (depicted in map in Fig. 1a). This corroborates the orthology of the element and its derived character status shared by all New World monkeys, providing strong evidence for their monophyletic origin.

3.1.2. Marker LIMK1 (Fig. 1b)

Primers were designed from human elastin which amplify an 850 bp fragment with an ancient Alu element in intron 8. The New World monkey ortholog contains an additional Alu element adjacent to the plesiomorphic one, which they share with the outgroup *H. sapiens* (indicated in map in Fig. 1b). Because the poly(A) tail of the ancient Alu repeat served as target sequence for the second

Table 1
Primers, their sequences and origins used for amplification of different loci in different genera

Primers	Primer sequences 5′ – 3′	Locus	GenBank Acc. No.	Genera sequenced	T_{Anneal}
TK1	CTCGGGCCGATGTTCTCAGG	Human thymidine kinase gene, exons 2/3	M15205	*Ateles*, *Chiropotes*, *Leontopithecus*	62 °C
TK2	TTAATTCCTACTCCCTTAATGTG				
TK3	CTCGGGCCGATGTTCTCAGG	Human thymidine kinase gene, intron 2	M15205	*Aotus*, *Ateles*, *Callicebus*, *Cebus*, *Chiropotes*, *Leontopithecus*	59 °C
TK4	TTAATTCCTACTCCCTTAATGTG				
LIMK11	GCAAGGACCTGGGTCGCTC	Human LIM kinase gene, exons 8/9	U63721	*Aotus*, *Ateles*, *Callicebus*, *Cebus*, *Chiropotes*, *Leontopithecus*	62 °C
LIMK12	CTGATCCGGTTCGACGAGGAG				
VP1	AATCAGTCCACTGAGACTAC	visual pigment gene, intron 4	AF092850–61 X88888–93	*Aotus*, *Ateles*, *Callicebus*, *Cebus*, *Chiropotes*, *Leontopithecus*	58 °C
VP2	AGCTAACAGA(CT)GGAACCAG				
LYS1	CACAAGGCATTAGAGCATG	Human lysozyme gene, exons 3/4	X14008	*Callithrix*, *Cebuella*, *Saguinus*	56 °C
LYS2	TTAATTCCTACTCCCTTAATGTG				
LYS3	CTTTGCTGCAAGATAACATC	Human lysozyme gene, exons 3/4	X14008	*Ateles*, *Callimico*, *Leontopithecus*	55 °C
LYS4	CATACTGACGGACATCTCTG				
STK1	TACAATTCCCTTGGCACATC	Human stem cell tyrosine kinase (STK-1) gene, exons 9/10	U82002	*Ateles*, *Callimico*, *Callithrix*, *Cebuella*, *Leontopithecus*, *Saguinus*	56 °C
STK2	ATGTTGTCTTGGATGAAAGG				
HBGF5	CTGT(C/T)TGTCTGCTGGTCATC	Human HBGF heparin-binding EGF-like growth factor precursor gene, exons 3/4	M31651	*Ateles*, *Aotus*, *Callimico*, *Callithrix*, *Cebuella*, *Cebus*, *Leontopithecus*, *Saguinus*, *Saimiri*	58 °C
HBGF6	CCACATCATAACCTCCTCTC				

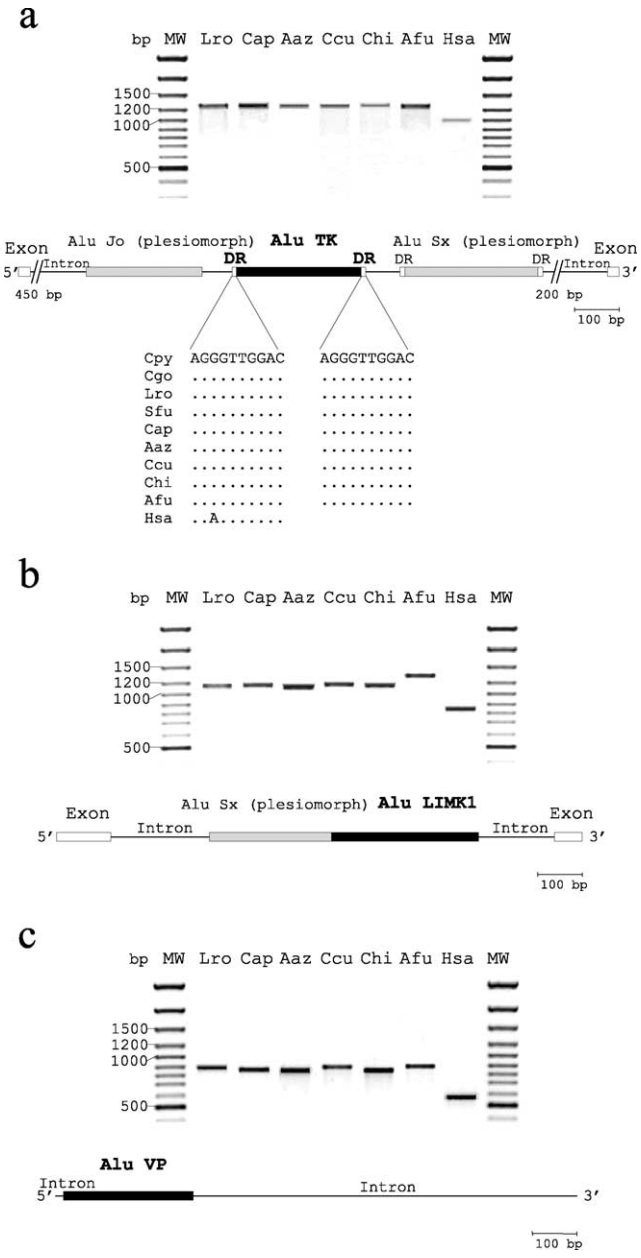

Fig. 1. PCR analyses of orthologous Alu elements supporting New World monkey monophyly: (a) TK, (b) LIMK1, and (c) VP. Their locations corresponding to the human sequence are depicted in the diagrams below (drawn to scale). Exons are depicted as open rectangles, plesiomorphic ALU markers as gray rectangles, and informative ALU markers as black rectangles. For the TK marker, DNA sequences of the direct repeats could be identified. Hsa *H. sapiens*, Afu *A. fusciceps*, Chi *C. satanas* x *albinasus*, Ccu *C. cupreus*, Aaz *A. azarai*, Cap *C. apella*, Lro *L. rosalia*, MW, molecular weight marker (100 bp DNA ladder).

transposition, the DRs and the unoccupied target sequence consist of unspecific A-runs. However, concordance of the intron sequences 3′ from the Alu insertion underlines the true orthology of the repeats that are found in all analyzed platyrrhine genera providing another argument for New World monkey monophyly.

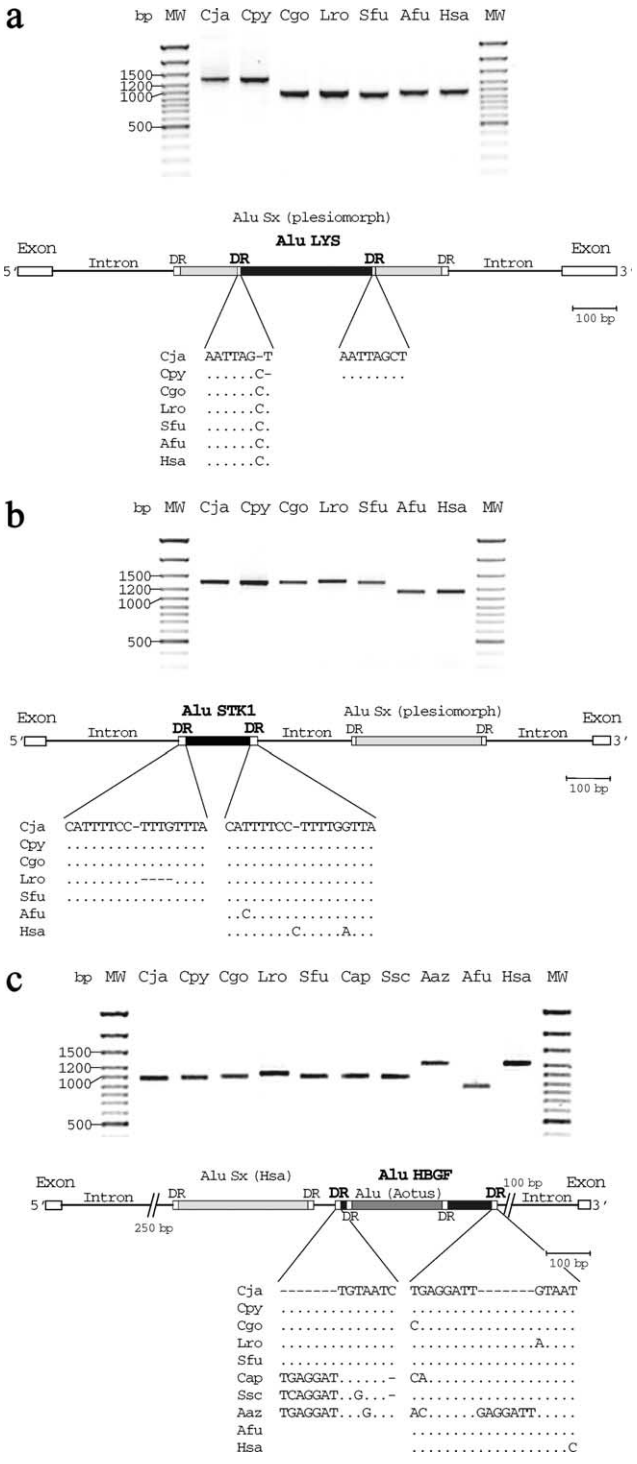

Fig. 2. PCR analyses of orthologous Alu repeats supporting the sister group relationship of *Callithrix* and *Cebuella* (a) LYS, callitrichine monophyly (b) STK1, and a monophyletic origin of *Aotus*, *Cebus*, *Saimiri*, and the callitrichines (c) HBGF. The Alu elements' locations corresponding to the human reference are shown diagramatically (drawn to scale). Exons are depicted as open rectangles, plesiomorphic ALU markers as gray rectangles, and informative ALU markers as black rectangles. The sequences of the direct repeats representing the duplicated target sites and the outgroups' unoccupied target sites are depicted. Hsa *H. sapiens*, Aaz *A. azarai*, Afu *A. fusciceps*, Cap *C. apella*, Cgo *C. goeldii*, Cja *C. jacchus*, Cpy *C. pygmaea*, Lro *L. rosalia*, Sfu *S. fuscicollis*, Ssc *S. sciureus*, MW, molecular weight marker (100 bp DNA ladder).

At the PCR level, length polymorphism within the callitrichines and longer fragments in atelines could be observed because of diverse independent transpositional events that occurred during their phylogenetic history (data not discussed in this paper).

3.1.3. Marker VP (Fig. 1c)

The respective primers, encompassing a 900 bp intronic sequence, were derived from information of intron 4 of the visual pigment gene of various New World monkeys (*Callithrix jacchus*, *Cebus nigrivittatus*, *Pithecia irrorata*, *Saguinus mystax*, *Saimiri sciureus*, and *Alouatta seniculus*) and human (see Boissinot et al., 1998). As shown earlier, New world monkeys, with the exception of *Alouatta*, possess only one X-chromosome-linked opsin gene (Boissinot et al., 1997, 1998) with various alleles. Based on opsin sequence comparisons, Boissinot et al. (1998) suggest that the X-linked opsin alleles and the duplicate opsin genes in *Alouatta* and Old World monkeys were derived from a common ancestral green opsin gene (''single origin hypothesis''). Moreover, all of the New World monkey intron 4 sequences described by Shyue et al. (1995) and Boissinot et al. (1997, 1998) harbored an Alu element that was lacking in the human red and green alleles, indicating it to be a monophyly marker for platyrrhines. To extend the existing dataset on each New World monkey genus available and to test this hypothetical marker locus, we constructed intronic primers to amplify intron 4 of VP. These primers were not allele specific.

In addition to the ones previously described, all platyrrhine genera investigated herein (*Aotus*, *Ateles*, *Callicebus*, *Chiropotes*, and *Leontopithecus*) exhibit a PCR product 300 bp longer than that of the human outgroup. This increase in length in the New World monkeys is due to insertion of a common Alu element, which is lacking in the human sequence (map in Fig. 1c). Unfortunately, DRs cannot clearly be recognized at its boundaries. Based on the presence of this Alu in every New World monkey allele and genus, this transposition must have occurred in the common ancestor of these alleles and the duplicated opsin genes of *Alouatta* in a common ancestor of all living platyrrhines after its separation from the Old World monkey stock. In addition to the two markers already described, this Alu repeat therefore provides further support to New World monkey monophyly.

3.2. Markers investigating branching patterns within and among NWM lineages

3.2.1. Marker LYS (Fig. 2a)

The informative Alu element is located between exons 3 and 4 of the lysozyme gene. In humans, the intronic region spans about 1 kb and carries an Alu element. PCR-products of the same size as in humans could be amplified in all platyrrhine genera tested (the results are exemplarily depicted for *Ateles*), with the exception of *Callithrix* and *Cebuella*. The latter exhibited a fragment exceeding the size of the other taxa analyzed by 300 bp. Sequence analysis revealed that the fragments of both, *Callithrix* and *Cebuella*, harbored the ancestral Alu element that is also present in humans. However, this ancestral Alu element served as a target for a second integration that is found in *Callithrix* and *Cebuella* only. This Alu-integration is encompassed by an 8 bp long DR in both, *Callithrix* and *Cebuella* (see map in Fig. 2a), corroborating the common origin of this transposition. In contrast, the unoccupied target site, representing the character state in the outgroup, is detectable in all other taxa considered. From this pattern, a scenario can be inferred with a first Alu-integration into this intron taking place in a common ancestor of humans and platyrrhines, followed by a second transposition before the divergence of *Callithrix* and *Cebuella* but after the other callitrichines split off. The latter renders *Callithrix* and *Cebuella* members of a monophylum to the exclusion of all other analyzed platyrrhines.

3.2.2. Marker STK1 (Fig. 2b)

The amplified product of this marker locus encompasses intron 9 of the stem cell tyrosine kinase gene. The products display a considerable length polymorphism in different New World monkeys due to transpositions of different Alu-fragments into this intron that occurred in the pitheciins, in *Saimiri*, and in *Cebus*, independently at different nucleotide positions (data not shown).

Upon sequencing the fragment in various species, it could be demonstrated that all New World monkeys tested and humans harbor a full-length Alu element in the 3′ part of the intron. Upstream of this Alu-sequence, a free right Alu-monomer (FRAM), exhibiting closest similarity to the Alu Sp, could be detected in all callitrichine genera including *Callimico*. This Alu-monomer is flanked by DRs, suggesting the common staggered end break mechanism of insertion. The unoccupied target site for this integration, represented by the monomeric form of the DR-sequence, is recognizable in both humans and *Ateles* and all remaining New World monkey genera (not shown). It represents the ancestral character state at this locus whereas the Alu-monomer links the traditional callitrichid genera *Callithrix*, *Cebuella*, *Saguinus*, and *Leontopithecus* with *Callimico*.

3.2.3. Marker HBGF (Fig. 2c)

The pair of primers constructed for this locus flanks the intron 3 of the heparin-binding EGF-like growth factor precursor gene. The sizes of the amplification products range from 850 bp to 1 kb in the New World monkeys analyzed. Both *Aotus* and humans display a fragment of about 1.3 kb. The fragment sizes of these

two species can be explained by two independent Alu-transpositions, one taking place on the lineage to humans (Alu Sx in Fig. 2d) and another autapomorphic transposition taking place on the lineage to *Aotus*. The latter Alu element integrated into an Alu-monomer, flanked by DR sequences that could be found in all callitrichine genera as well as in *Cebus*, *Saimiri*, and *Aotus*. PCR analyses show that all other New World monkey genera analyzed and the human outgroup lack this element (the results are displayed for *Ateles* and humans only) whereas the unduplicated target site for transposition could be recognized in *Ateles* and human therefore representing the ancestral character state.

3.2.4. Sequence analysis and phylogenetic reconstruction

To confirm these molecular cladistic results and to check if both presence/absence of Alu elements and substitutional sequence evolution result in a congruent interpretation of the branching pattern, the sequences of the six loci analyzed were compared among the platyrrhine representatives excluding exons and Alu elements. Two alignments were created, one for the concatenated sequences of the three markers depicting New World monkey monophyly and another one for the concatenated sequences of the three markers informative of callitrichine phylogeny. These two datasets comprised 3184 and 2071 bp after removing gaps, respectively (the alignments are available from the authors upon request). For phylogenetic reconstructions, *H. sapiens* and *A. fusciceps* were chosen as respective outgroups.

As a result, all reconstructions based on different algorithms basically yielded the same tree topologies with the monophyletic clades *Cebus–Saimiri–Aotus*–Callitrichinae (MP bootstrap support value (BS): 91, distance BS: 96, and quartet puzzling support value (QPS): 100) and Callitrichinae (MP BS: 100, distance BS: 99, QPS: 100). Within the callitrichines, *Callithrix jacchus* and *Cebuella* form a sister group (MP BS: 100, distance BS: 100, and QPS: 100). The next split leads to *Callimico* (MP BS: 93, distance BS: 92, and QPS: 92). The position of *Saguinus* in relation to that of *Leontopithecus* cannot be clearly resolved, neither can the sister group relationship of *Aotus*. *Ateles*, however, forms a weakly supported clade with the cebids to the exclusion of *Callicebus–Chiropotes* (MP BS: 64, distance BS: 57, and QPS: 78). The maximum likelihood trees are shown in Fig. 3 and the conclusions based on the presence/absence patterns of the six loci analyzed are depicted in the ML trees. TK, LIMK1, VP, LYS, STK1, and HBGF designate the marker loci informative of the respective clades. We found no conflicts between tree reconstruction based on the DNA sequences and the SINE markers.

4. Discussion

A thorough presence/absence analysis of orthologous retrotransposable elements in all major platyrrhine taxa is presented. Of the 74 loci checked for the presence of shared integrations of Alu elements in different New World monkey genera, three provided evidence for platyrrhine monophyly and three proved to be informative of consecutive branching events during callitrichine phylogeny. Though *Brachyteles* is missing in the analysis, we are convinced that New World monkey monophyly can

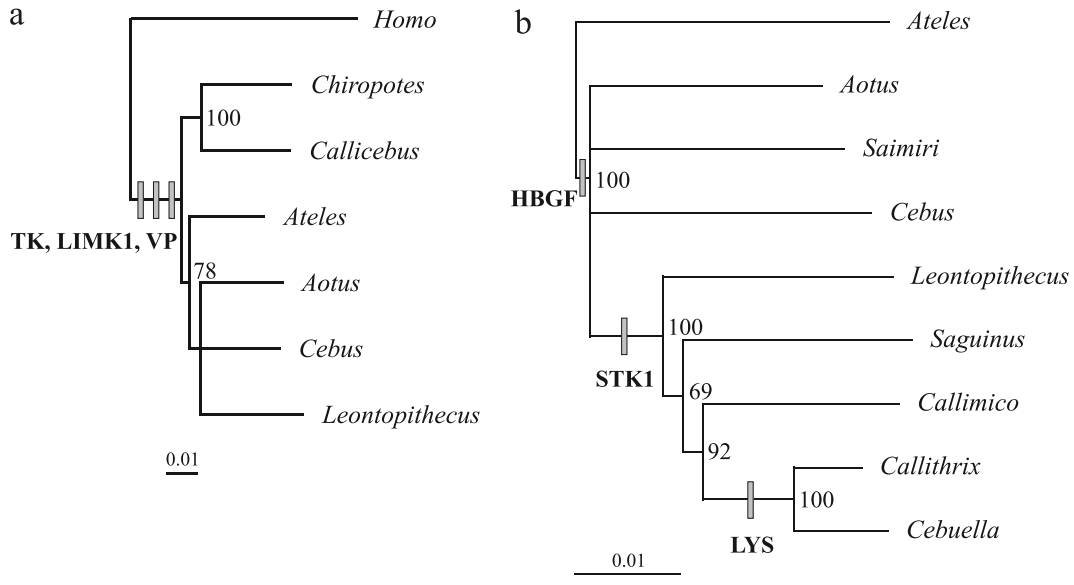

Fig. 3. Maximum likelihood reconstructions based on the concatenated intron sequences excluding Alu repeats of the three markers diagnostic for New World monkey monophyly (a) and characterizing callitrichine phylogeny (b). The evolutionary origins of the Alu integrations are indicated by bars. Values corresponding to the internal nodes represent quartet puzzling support values. Branch lengths represent nucleotide substitutions per site.

be reliably inferred from our data because *Brachyteles*' affiliation to the atelines is undoubted throughout the literature. We therefore assume that *Brachyteles* exhibits the same presence/absence patterns as the other atelines as far as markers outside the ateline branch are concerned.

The orthology of the six Alu elements present in the respective New World monkey groups was verified by sequencing the complete PCR products, and thus, obtaining the regions adjacent to the Alu elements. With the exception of the Alu element that transposed into the poly(A)-tail of an ancient Alu element in intron 8 of the elastin gene and the Alu element that was inserted into intron 4 of the opsin gene, the direct repeats could be unequivocally defined. Moreover, the target structures for the integrations could be clearly identified in the outgroup taxa.

With regard to the theory of Alu retroposition, it is remarkable that in the six loci analyzed in detail, three transpositional events occurred that involve Alu-monomers. Whilst a single insertional event of a monomeric Alu element could be deduced at the STK1-locus, two consecutive integrations of monomers took place at the HBGF-locus. The first insertion happened on the lineage to the most recent common ancestor of *Saimiri*, *Cebus*, *Aotus*, and the callitrichines. The second insertion occurred on the lineage leading to *Aotus*. This *Aotus*-specific Alu-monomer integrated into the pre-existing Alu-monomer that is present in the taxa mentioned above. According to a strict theory of Alu master genes (Shen et al., 1991), this is not expected, since monomeric Alu elements should have been retropositionally active, prior to the emergence of the typical dimeric Alu element, and therefore, prior to the divergence of the order Primates. In the cases described in this paper, however, direct repeats flanking all Alu-monomers could be determined as well as the unoccupied target sites in the outgroup taxa. This indicates an integration via staggered end breaks typical of retropositions mediated by a reverse transcriptase/endonuclease machinery provided in trans. This observation provides further evidence against a complete deactivation of Alu elements after the generally assumed time span of their transpositional activity and argues in favor of the existence of multiple Alu-source genes in primate evolution (Leeflang et al., 1992).

Apart from the unlikely scenario of convergent insertions of the same retropositional element at identical sites, incomplete lineage sorting has to be taken into account as a potentially misleading factor. Though this phenomenon is not a problem specific to SINE transpositions, rather affecting the analysis of any polymorphic marker, ideally multiple independent SINE insertions attributable to one single branch would eliminate any doubts related to lineage sorting. Lineage sorting phenomena become the more pronounced the closer consecutive splitting events occur, the higher the respective effective population sizes, and the longer the generation times of the taxa in question. While short splitting intervals can be assumed for New World monkey phylogeny, currently available data do not allow statements about population genetic parameters.

The fact that only one SINE insertion could be detected for a certain branch in callitrichine phylogeny requires an additional corroboration of the proposed branching pattern. For this purpose, we included the phylogenetic information from the sequences flanking the retroposing elements in our analysis. The sequences of the six loci exclusive of the exons and Alu repeats from the respective genera were used in two phylogenetic reconstructions. In this way, we specifically tested whether the presence/absence pattern gives rise to the same tree topology, as demonstrated by the phylogenetic analysis of the base substitutional pattern obtained from the same loci. It is obvious that molecular cladistic evidence and the base substitutional pattern perfectly agree with each other. The sequence information thus lends support to the notion that incomplete sorting of ancestral polymorphisms into progeny does not influence the presence/absence pattern in the lineages after speciation.

Thus, our data add to the evidence obtained from other molecular studies as well as morphological, cytogenetic, and etho-ecological data to support the following views.

4.1. Platyrrhines are monophyletic

Though generally assumed, New World monkey monophyly to date is not unequivocally proven. Though several morphological (see Ford, 1986) and anatomical features like intraplacental maternal vessels and placental hematopoiesis (Luckett, 1980) link all platyrrhines and they cluster together in DNA sequence analyses (Horovitz and Meyer, 1995; Horovitz et al., 1998; Porter et al., 1995, 1997; Schneider et al., 1993), the evidence is not unambiguous.

New World monkey polyphyly is proposed by skin histology and hair follicle arrangement (Perkins and Meyer, 1980) with a lemuriform origin of *Aotus* and *Callicebus*, whereas the other platyrrhines are assumed to be derived from a tarsiiform ancestor. Chiarelli (1980) doubted platyrrhine monophyly on karyological grounds. According to his data, the distinctive karyotypes of *Ateles*, *Brachyteles*, and *Lagothrix* disagree with a common origin of all living New World primates. Immunogenetic evidence from antigenic determinants of human serum proteins also favors a paraphyletic origin of platyrrhines. As a by-product of a comparative determinant analysis carried out to estimate the temporal scale of human phylogeny, Bauer and Schreiber (1997) found that the two New World monkey species included as outgroups did not form a common clade. Rather, *Lagothrix lagotricha* is united with the Old World primates to the exclusion of *Cebus albifrons*. The authors

therefore infer a double invasion of South America with an estimated divergence date of *Cebus* of 52 mya, whereas *Lagothrix* is assumed to have diverged only 45 mya from catarrhine ancestors.

Our transpositional evidence unambiguously corroborates arguments supporting a monophyletic origin of New World monkeys and a single invasion of the New World by primates. The three molecular cladistic markers contradict findings from skin morphology, karyology, and immunogenetics deducing a scenario of platyrrhine paraphyly. An incomplete lineage sorting of ancestral polymorphisms in the progeny lineages after splitting, which might confound the phylogenetic interpretation, can be excluded because of the consistent support provided by three independent transpositional events. The independencies of the three transpositions is secured further by their location in three different human chromosomes. According to GenBank entries, M15205, U63721, and X88888 TK is located on chromosome 17 (17q23.2-q25.3), LIMK1 on 7 (7q11.23), and VP on the X-gonosome.

4.2. *Callithrix and Cebuella are monophyletic*

The synapomorphic Alu element in the lysozyme locus reflects the long-standing monophyly of *Callithrix* and *Cebuella*. Genus status of the pygmy marmoset is questioned by morphological (Rosenberger and Coimbra-Filho, 1984), cytogenetic (Canavez et al., 1996; Nagamachi et al., 1999), and etho-ecological data (Garber, 1992). It can be maintained even less in the face of increasing DNA-sequence information (Barroso et al., 1997; Canavez et al., 1999; Chaves et al., 1999; Hugot, 1998; Moreira and Seuánez, 1999; Porter et al., 1997; Tagliaro et al., 1997), linking the Amazon *Callithrix* species of the *argentata* group more closely to *Cebuella pygmaea* than to the Atlantic *Callithrix* species of the *jacchus* group. As *Cebuella pygmaea* as well as *Callithrix jacchus* share the respective Alu markers, however, it does not allow further discrimination of the branching order within the *Callithrix*/*Cebuella* clade.

4.3. *Callimico falls within the traditional callitrichids*

Due to the mosaicism of original and derived traits, *Callimico goeldii* was occasionally placed in the Callitrichidae (Napier and Napier, 1967; Pocock, 1925; Szalay and Delson, 1979), the Cebidae (Martin, 1990; Simons, 1972; Simpson, 1945) or its own family, the Callimiconidae (Chiarelli, 1972; Hershkovitz, 1977). Morphological (Ford, 1986; Kay, 1990; Rosenberger et al., 1990) as well as cytogenetic (Chiarelli, 1980) and socio-ecological data (Garber, 1994) support a sister group relationship of *Callimico* to all other callitrichids. Molecular sequence data, however, point out that *Callimico* is the sister group to *Callithrix*/*Cebuella* (Canavez et al., 1999; Chaves et al., 1999; Harada et al., 1995; Horovitz and Meyer, 1995; Horovitz et al., 1998; Pastorini et al., 1998; Porter et al., 1997; Schneider et al., 1996; von Dornum and Ruvolo, 1999).

Retropositional evidence from the marker in the stem cell tyrosine kinase gene underlines the monophyly of *Callimico* and the traditional callitrichid genera but is unable to elucidate the exact position of *Callimico*.

4.4. *Cebus, Saimiri, and Aotus are the closest living relatives to the callitrichines*

The molecular cladistic marker information from the HBGF-locus is indicative of the phylogenetic affiliations of the callitrichines to other platyrrhines. Who is the next living relative to the callitrichines is a matter of much dispute. Morphological data support a sister group relation of the callitrichines to a pitheciine–ateline (Ford, 1986) or a *Cebus–Saimiri* (Rosenberger, 1992) clade. Kay's dental data (1990) link them with *Saimiri*, being part of an unresolved trichotomy with *Aotus* and the atelines, whereas *Callicebus* represents the most basal split followed by a *Cebus* split. Ford (1986) places *Cebus* at the base of all platyrrhines, with *Saimiri* being either sister to *Cebus* or to *Aotus–Callicebus*. Rosenberger (1992) propose *Aotus–Callicebus* to be the sister taxon to the pitheciins. Cytogenetic data (Chiarelli, 1980) also support the sister group relationships of *Cebus–Saimiri* and *Callicebus–Aotus*, the latter being next to the pitheciids/pitheciins. Most molecular data pair *Callicebus* with the pitheciins and *Cebus* with *Saimiri* (Harada et al., 1995; Horovitz et al., 1998; Porter et al., 1997; Porter et al., 1999; Schneider et al., 1996). The callitrichines are considered sister to *Aotus* (Harada et al., 1995; Porter et al., 1997; Porter et al., 1999), the resulting clade being grouped with a *Cebus–Saimiri* clade, or sister to *Cebus–Saimiri*, together forming a clade with *Aotus* (Horovitz et al., 1998; Schneider et al., 1996).

In summary, the placement of *Cebus–Saimiri* with *Aotus* and the callitrichines is strongly supported on molecular grounds. However, evidence for the branching order within that clade is not so strong. The molecular cladistic data from this study reflect this overall pattern by grouping the callitrichines with *Cebus*, *Saimiri*, and *Aotus* in an unresolved polytomy.

The New World monkeys represent a broad radiation of simian primates that occupied a wide range of ecological niches during the past 40–25 million years and generated a likewise morphological and etho-ecological variety. In the absence of other primates, the first New World monkeys reaching South America some 40 mya could diverge dramatically. This rapid radiation with splitting events following each other in quick succession may be the reason why we found relatively few retrotranspositional markers character-

izing the phylogenetic history of the New World monkeys as compared to that of the Old World monkeys. Lineage sorting may therefore be a more serious problem in platyrrhine than in catarrhine evolutionary history. Moreover, we found a number of autapomorphic characters on the branches to *Saimiri*, *Cebus*, and *Aotus*, namely the genera whose affiliations to the major platyrrhine groups are hard to determine through other data as well. The molecular cladistic data and respective conclusions presented herein therefore represent a first starting point that needs to be complemented in the future. However, due to the huge amount of incoming sequence data from other primates and the enormous potential of retropositional markers to solve long-standing phylogenetic questions, an undisputed phylogenetic framework for the New World monkeys seems to be an achievable goal for the near future.

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