Evolution of Cyclophilin A and *TRIMCyp* Retrotransposition in New World Primates

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Host cell factors modulate retroviral infections. Among those, cyclophilin A (CypA) promotes virus infectivity by facilitating virus uncoating or capsid unfolding or by preventing retroviral capsid interaction with cellular restriction factors. In *Aotus* species, a retrotransposed copy of *CypA* inserted into the tripartite motif 5 (*TRIM5*) gene encodes a fusion protein which may block human immunodeficiency virus type 1 by targeting the incoming virus to ubiquitin-ligated degradation or by interfering with normal uncoating of the incoming particle, rendering those monkeys resistant to infection. In this study, we have extensively analyzed representative specimens from all New World primate genera and shown that the retrotransposed *CypA* copy is only present in *Aotus*. We have shown that this inserted copy diverged from its original counterpart and that this occurred prior to *Aotus* radiation, although no positive selection was observed. Finally, our data underscores the need for a precise taxonomic identification of primate species used as models for retroviral infections and novel antiviral approaches.

The zoonotic events involved in the transmission of human immunodeficiency virus type 1 (HIV-1) and HIV-2 from chimpanzees and sooty mangabeys to humans demonstrate the ability of lentiviruses in crossing species barriers (21). Although numerous examples of retroviral transmission across species have been reported to date (5, 13, 23), retroviral host ranges, *in natura*, are limited by restriction or enhancing factors in new hosts, which may block or promote the early steps of the life cycle of retroviruses inside cells (6). Some of these blocking factors include members of the tripartite motif 5 (TRIM5) protein family (25), contrary to factors that enhance infection, as is the case of cyclophilin A (CypA) (22). TRIM5 and CypA interact with the capsid (CA) portion of the Gag protein (3, 20). It has been suggested that CypA may facilitate disassembling of the virus capsid for reverse transcription (1). Conversely, one of the TRIM5 isoforms, TRIM5α, may target viral capsid to proteasome degradation before nuclear import or interfere with the uncoating of the incoming virus, while it has been postulated that CypA may promote infection by preventing the association of TRIM5α with the viral capsid (6).

An increasing amount of data on lentiviral infectivity has been provided by the lentiviral modulating factors of New World Primates (NWP), a group that, apparently, does not harbor lentiviruses *in natura*. NWP cells are only moderately infected by HIV-1 and SIVmac; owl monkey (*Aotus trivirgatus*) cells are not infected by HIV-1, while squirrel monkey cells are resistant to SIVmac (12). More recently, HIV-1 blockage in *Aotus* cells was explained by presence of a complete, in-frame insertion of *CypA* cDNA, between exons 7 and 8, in the *TRIM5* locus originating a chimeric gene encoding a TRIM-CypA fusion protein (14, 18) capable of blocking HIV-1 replication in *Aotus* cells and in human cells transiently expressing its coding cDNA (14).

Although it has been postulated that this *CypA* insertion must have occurred by a transposition event that took place after the divergence of NWP from the Old World Primates (OWP) (18), the precise origin and timing of this event could not be traced along the NWP phylogeny because this chimeric gene has not been investigated in representative species of all NWP genera. Moreover, the extent of this retrotransposition in congeneric *Aotus* species and within species has not been analyzed. In fact, *Aotus* is a complex genus that was initially considered to be monotypic until Hershkovitz (9) recognized nine allopatric species divided into two groups: the gray-neck species group with *A. brunbacki*, *A. trivirgatus*, *A. vociferans*, and *A. lemurinus* (with two subspecies, *A. lemurinus lemurinus* and *A. lemurinus griseimembra*), and the red-neck group with *A. nancymae*, *A. miconax*, *A. infulatus*, *A. azarae* (with two subspecies, *A. azarae azarae* and *A. azarae boliviensis*), and *A. nigriceps*. Later, Groves (7) recognized a new species, *A. hershkovitzi*, belonging to the gray-neck species group.

In this study, we analyzed representatives of all NWP genera, including representatives of several taxonomically characterized *Aotus* species of known geographic origin which were wild caught in different regions. For detecting the *CypA* insert, a single-round PCR was designed, resulting in fragments of different size from those observed when the insert was absent (Fig. 1). This was carried out with a forward primer (5′CAACGCTACTGGGGTAAGGAGA3′) annealing at the 3′ end of

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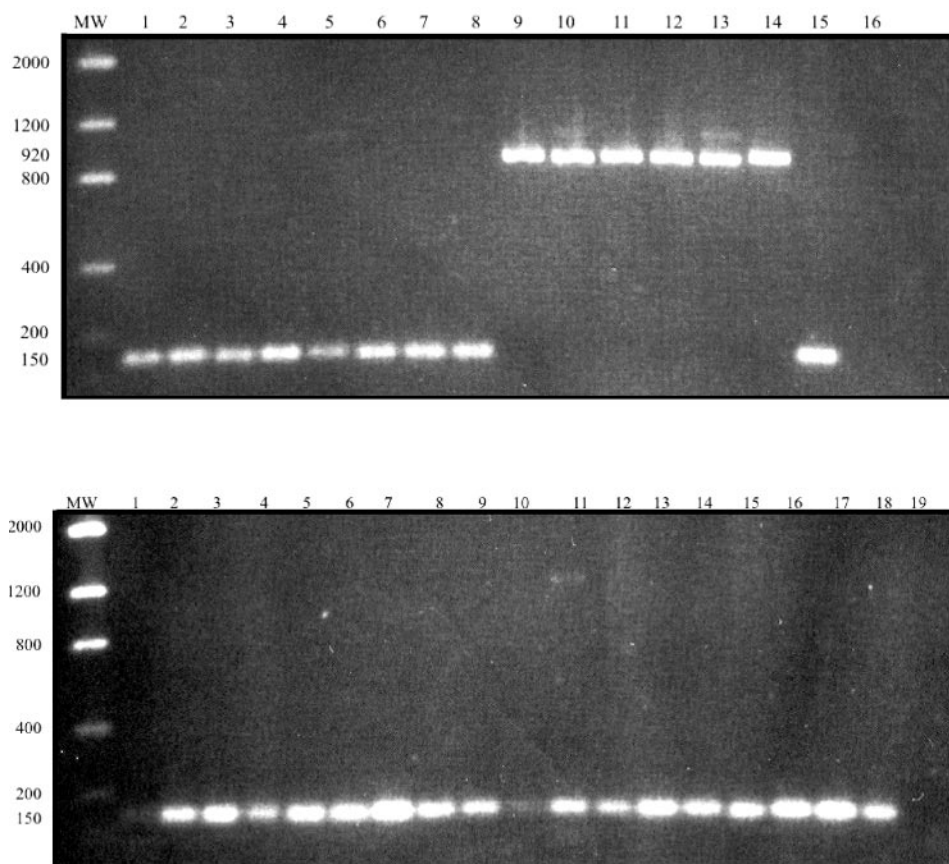
FIG. 1. PCR amplification of TRIM-CypA retrotransposition of representative New World monkey genera. Top panel, lanes: 1, *Saimiri ustus*; 2, *Saimiri sciureus*; 3, *Cebus* sp.; 4, *Cebus* sp.; 5, *Cebus albifrons*; 6, *Cebus apella apella*; 7, *Cacajao melanocephalus*; 8, *Brachyteles arachnoides*; 9, *Aotus infulatus*; 10, *Aotus azarae boliviensis*; 11, *Aotus trivirgatus*; 12, *Aotus infulatus*; 13, *Aotus trivirgatus*; 14, *Aotus azarae*; 15, *Homo sapiens*; 16, negative control. MW, molecular weight marker. Bottom panel, lanes: 1, *Callithrix penicillata*; 2, *Callithrix geoffroyi*; 3, *Callithrix jacchus*; 4, *Callithrix kuhli*; 5, *Callithrix emiliae*; 6, *Callithrix argentata*; 7, *Callithrix humeralifer*; 8, *Callithrix (Cebuella) pygmaea*; 9, *Leontopithecus chrysopygus*; 10, *Leontopithecus rosalia*; 11, *Leontophitecus chrysomelas*; 12, *Saguinus fuscicollis*; 13, *Saguinus imperator*; 14, *Saguinus bicolor*; 15, *Alouatta seniculus*; 16, *Callicebus moloch*; 17, *Callicebus personatus*; 18, *Callicebus torquatus*; 19, negative control.

TRIM5 exon 7, and a reverse primer (5′CATGTTTTAAGA TTTATATTTCTTCTTC3′) annealing at the 5′end of *trim5* exon 8. When the *CypA* insert was present, a fragment of approximately 900 bp (corresponding to cyclophilin A cDNA) was amplified, while a smaller fragment of approximately 100 bp was observed in agarose gels when the insert was not present. When studying representatives of all NWP genera (Table 1), the *CypA* insert was detected in all specimens of *Aotus* (*A. infulatus*, *A. azarae*, *A. trivirgatus*, and *A. lemurinus*) but was absent in the representatives of all other 15 genera of NWP. Identification of *CypA* inserts was confirmed by two-strand, direct DNA sequencing; reactions were carried out with the same primers used in the PCR assay and two internal primers (F1, 5′CAACGCTACTGGGGTAAGGAGA3′, and F2, 5′CATGTTTTAAGA TTTATATTTCTTCTTC3′). Samples were run in an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA); sequences were edited, translated into predicted amino acid sequences, and aligned with ClustalW (10).

Nucleotide sequence accession numbers. All nucleotide sequences were deposited in the GenBank database and were assigned the accession numbers DQ098851 to DQ098874.

Few amino acid substitutions were observed when comparing the transposed and nontransposed CypA sequences of NWP with the non-transposed sequences of OWP, indicating a very strong evolutionary conservation of this protein in the primate order. Interestingly, all *Aotus* CypA proteins showed an E84D substitution and lacked the last amino acid (E165) although we do not know whether these changes might represent signatures of *Aotus* CypA proteins or of all NWP genera. Other substitutions, like V29L, R69H and E81V, were also observed in several owl monkeys, with the exception of the previously reported *A. trivirgatus* sequences (AY646198, AY646199 and AY684997) and of *A. lemurinus* characterized herein. In fact, changes V2I and V20I were present only in specimens identified as *A. trivirgatus sensu* Hershkovitz (9), with a diploid chromosome number of 2n = 50, and captured in a locality within the geographic distribution of this species. Most interestingly, however, were changes V117A, I138V, S147C, and N149Y, which were present in all sequences derived from the retrotransposed *CypA* gene, but not in the sequence derived from the mRNA of the original (nontransposed) *CypA* gene of *Aotus trivirgatus* (Fig. 2). This finding confirmed a previous report showing sequence differences be-

TABLE 1. New World monkey species analyzed for
TRIMCyp retrotransposition

Species	No. of specimens analyzed/species
1. *Callithrix penicillata*	3
2. *Callithrix geoffroyi*	4
3. *Callithrix jacchus*	2
4. *Callithrix kuhli*	4
5. *Callithrix emiliae*	3
6. *Callithrix argentata*	1
7. *Callithrix humeralifer*	4
8. *Callithrix (Cebuella) pygmaea*	1
9. *Leontopithecus chrysopygus*	3
10. *Leontophitecus rosalia*	3
11. *Leontophitecus chrysomelas*	4
12. *Saguinus fuscicollis*	1
13. *Saguinus imperator*	1
14. *Saguinus bicolor*	1
15. *Callimico goeldii*	1
16. *Cebus* sp.	2
17. *Cebus albifrons*	2
18. *Cebus apella apella*	2
19. *Saimiri ustus*	3
20. *Saimiri sciureus*	2
21. *Aotus infulatus*	2
22. *Aotus azarae boliviensis*	3
23. *Aotus trivirgatus*	2
24. *Aotus lemurinus*	4
25. *Callicebus personatus*	2
26. *Callicebus torquatus*	2
27. *Callicebus moloch*	2
28. *Pithecia irrorata*	3
29. *Chiropotes* sp.	1
30. *Cacajao melanocephalus*	1
31. *Alouatta seniculus*	1
32. *Ateles paniscus*	1
33. *Brachyteles arachnoides*	2
34. *Lagothrix lagothrica*	1
35. *Homo sapiens*[a]	

[a] Negative control.

tween the *CypA* gene and the retrotransposed insert of a same cell line (14), indicating that the retrotransposed gene evolved subsequently to the insertion event. Among the retrotransposed CypA substitutions, S147C created a new cysteine residue, which could alter potential intra- or intermolecular disulfide bonds. All amino acid residues previously associated in interactions with HIV-1 CA (H54, R55, N71, N102, and H126) and with cyclosporine (W121) (2, 4) were strictly conserved in all primate species, suggesting that all retrotransposed sequences are biologically functional.

A phylogenetic *CypA* topology using six OWP sequences as outgroups, and including previous data on *Aotus CypA*, did not group specimens accordingly to their taxonomic status or geographic distribution. Retrotransposed *CypA* genes were tightly grouped in a well-supported clade divided in two branches. The divergence of the upper branch clearly indicated that *A. infulatus* specimens captured in Tucurui (3°45′S, 49°40′W) and *A. azarae boliviensis* specimens captured in the Samuel dam reservoir (8°45′S, 63°26′W) were clearly paraphyletic despite the fact that these animals were captured in regions that are very distant from one another. Moreover, two specimens of *A. trivirgatus sensu* Hershkovitz (9), captured north of the Rio Negro (Brazil), were also included in the upper branch. Con-

versely, the lower (retrotransposed) *CypA* branch showed seven collapsing, identical branches. These comprised (i) the previously reported cDNA insert of *A. trivirgatus* (AY646198); (ii) the previously reported *A. trivirgatus* insert (AY646199) isolated from genomic DNA of the same animal; (iii) the previously reported *A. trivirgatus* insert (AY684997) isolated from DNA of a kidney cell line (OMK); and (iv) the *CypA* sequences of four *A. lemurinus* from Colombia herein analyzed.

The possibility of positive selection at differing sites was tested using the CODEML program of the PAML package (24). Three alternative topologies were constructed using the OWP topology of Page and Goodman (15) and the parsimony topologies obtained with selected *Aotus* cyclophilin A sequence data (excluding specimens with identical sequences): 1- (((((Homo sapiens, Pan troglodytes), Pongo pygmaeus), (Papio hamadryas, Macaca mulatta)), Cercopithecus aethiops), (A. trivirgatus-AY646200 CypA mRNA, (A. trivirgatus AY684997-TRIMCyp, (A. azarae 04-TRIMCyp, ((A. infulatus 02-TRIMCyp, A. infulatus 01-TRIMCyp), (A. trivirgatus 01-TRIMCyp, A. trivirgatus 02-TRIMCyp)))))); 2- (((((Homo sapiens, Pan troglodytes), Pongo pygmaeus), (Papio hamadryas, Macaca mulatta)), Cercopithecus aethiops), (A. trivirgatus -AY646200 CypA mRNA, (A. trivirgatus AY684997-TRIMCyp, (A. azarae 04-TRIMCyp, A. infulatus 02-TRIMCyp, A. infulatus 01-TRIMCyp, (A. trivirgatus 01-TRIMCyp, A. trivirgatus 02-TRIMCyp))))); 3- (((((Homo sapiens, Pan troglodytes), Pongo pygmaeus), (Papio hamadryas, Macaca mulatta)), Cercopithecus aethiops), (A. trivirgatus -AY646200 CypA mRNA, (A. trivirgatus AY684997-TRIMCyp, ((A. azarae 04-TRIMCyp, A. infulatus 02-TRIMCyp, A. infulatus 01-TRIMCyp), (A. trivirgatus 01-TRIMCyp, A. trivirgatus 02-TRIMCyp))))). These topologies were tested assuming the same nonsynonymous/synonymous (dN/dS) substitution ratio for all branches (one-ratio model) and different dN/dS for branches (free-ratio model). No significant differences were observed between likelihood values obtained under these models, indicating absence of positive selection. Additionally, no evidence of positive selection for individual codon sites was found with CODEML using different codon substitution models (M0, M1, M2a, M3, M7, and M8).

Phylogenetic topologies relating the four *Aotus* species herein analyzed were clearly different when analyzing cytochrome *b* (1,140 bp) because a distance tree showed that the *A. trivirgatus sensu* Hershkovitz (9) were included in the most basal offshoot, with a sister branch splitting in a dichotomy, comprising *A. lemurinus* in one lineage and *A. infulatus/ A. azarae boliviensis* in another (Fig. 3). These two species, although strongly grouped, showed a paraphyletic arrangement, indicating the presence of a trans-specific, ancestral cytochrome *b* polymorphism. It is noteworthy that *A. infulatus* and *A. azarae boliviensis* were found to be karyotypically identical, and that their taxonomic status, as separate species, has been questioned (16, 17). This topology clearly indicated, however, that *A. trivirgatus*, *A. lemurinus* and *A. infulatus/A. azarae boliviensis* comprise three separate evolutionary lineages distributed in different geographic regions of South America. Distance estimates, considering synonymous substitutions, allowed for calculating the time when these *Aotus* lineages diverged from one another in approximately 4.5 million years
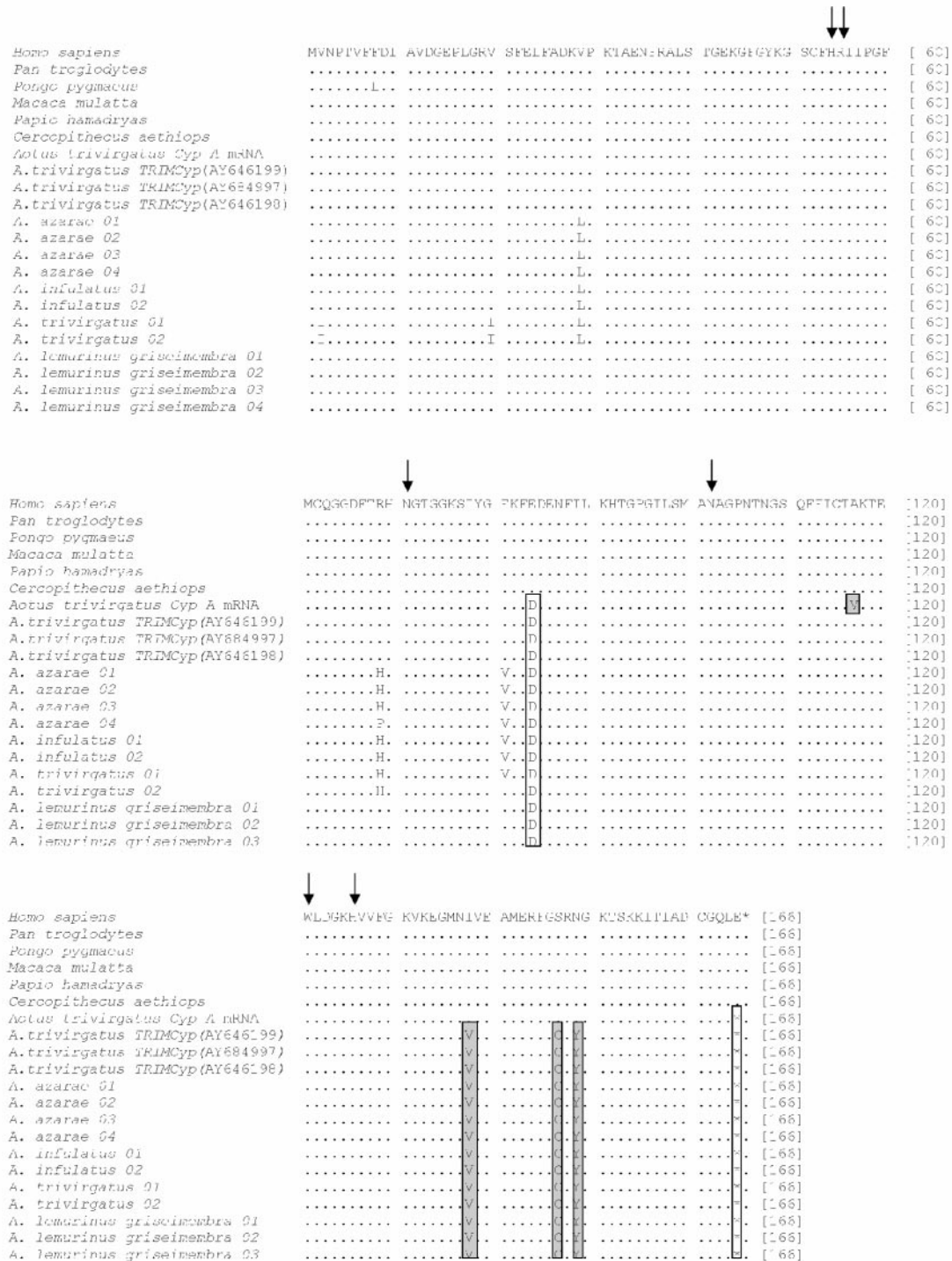
FIG. 2. Amino acid alignment of CypA proteins derived from sequences of original or retrotransposed *CypA* copies from New World monkeys (NWM) and representative species of OWP. Dots represent amino acid identities, whereas asterisks denote stop codons. Residues that are conserved among *Aotus* species (or NWM) are boxed in white, whereas residues which differ between the retrotransposed and the original CypA copy are boxed in gray. Residues which are known to interact with HIV-1 capsid or with cyclosporine are depicted by arrows.

before present (MYBP), in respect with human-chimpanzee synonymous cytochrome *b* substitutions and a divergence time of 6 MYBP.

The discordant *CypA* and cytochrome *b* topologies clearly

rule out that these sequences have co-evolved in the *Aotus* genome. In fact, the subfamilies Cebinae and Aotinae (*sensu* Schneider [19]) split some 22 MYBP, which represents the upper time limit when the *CypA* retrotransposition might have
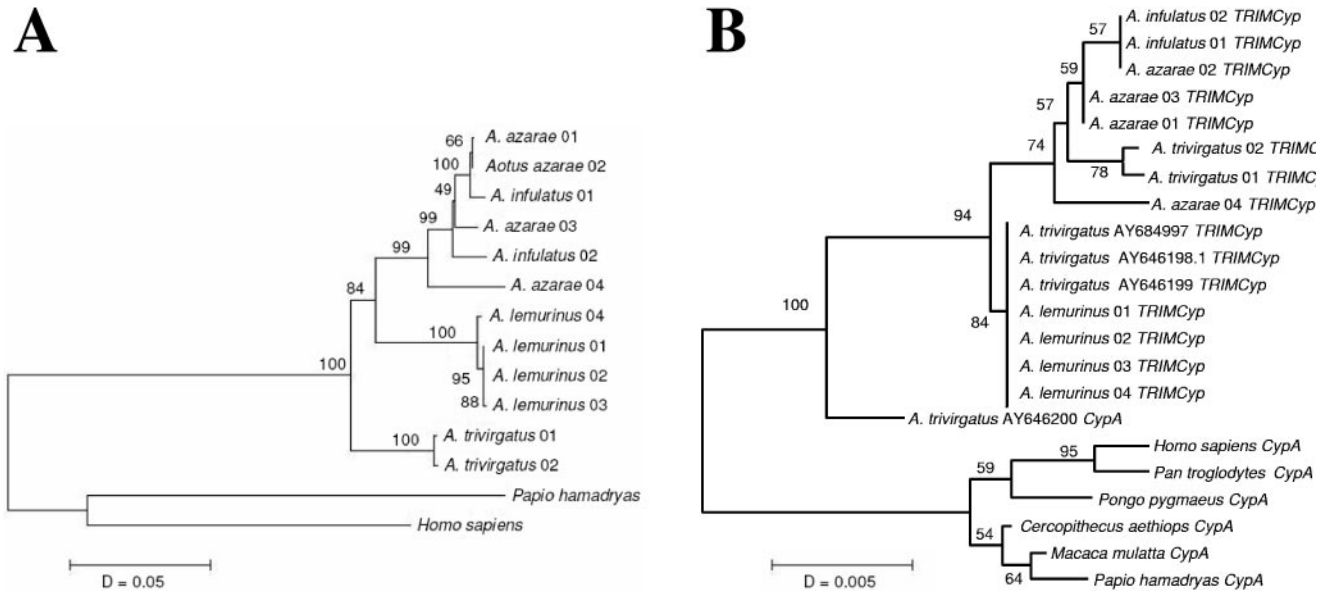
A

B

FIG. 3. Neighbor-joining nucleotide trees of New World monkey cytochrome *b* (A) and CypA/TRIM-CypA (B). Bootstrap values are based on 1,000 replicates. Representative species of OWP were used as outgroups.

occurred contrary to a lower limit of 4.5 MYBP. Once inserted, it is apparent that nucleotide substitutions at the *CypA* insert must have taken place before *Aotus* radiation, representing an ancestral polymorphism unrelated to the divergence of the three evolutionary lineages shown by the cytochrome *b* topology. This could also be due to the fact that mitochondrial DNA is more prone to be evolved by genetic drift than to the fact that it is maternally transmitted (11).

In summary, we have demonstrated that *Aotus* is the only genus of NWP harboring a *CypA* retrotransposition in *TRIM5*. Our results also indicate that the *CypA* retrotransposed copy present in *Aotus* has evolved subsequently to this insertion but prior to *Aotus* radiation. Although no positive selection was detected in the *CypA* gene as a whole or at specific codons, it is possible that ancient retroelements other than lentiviruses (such as endogenous retroviruses or retrotransposons) might have selected for amino acid substitutions seen today when comparing the original and the retrotansposed *CypA* copies. In addition, our data highlight the importance of an accurate classification of *Aotus* species in the study of restriction factors. Although it has been stated that *Aotus trivirgatus* is particularly resistant to HIV-1 infection (12), the specimens analyzed in this report grouped with *A. lemurinus* rather than with *A. trivirgatus sensu* Hershkovitz (9). It is noteworthy that the striking variability among *Aotus* species accounts for different susceptibilities to the development of malaria upon infection by different *Plasmodium* variants (8). Therefore, a precise characterization of primate species with distinctive susceptibility patterns to lentiviruses will enable a faster and better understanding of the processes controlling retroviral restriction and their application in antiviral strategies.

REFERENCES

1. **Braaten, D., E. K. Franke, and J. Luban.** 1996. Cyclophilin A is required for an early step in the life cycle of human immunodeficiency virus type 1 before the initiation of reverse transcription. J. Virol. **70:**3551–3560.
2. **Braaten, D., H. Ansari, and J. Luban.** 1997. The hydrophobic pocket of cyclophilin is the binding site for the human immunodeficiency virus type 1 Gag polyprotein. J. Virol. **71:**2107–2113.
3. **Colgan, J., H. E. Yuan, E. K. Franke, and J. Luban.** 1996. Binding of the human immunodeficiency virus type 1 Gag polyprotein to cyclophilin A is mediated by the central region of capsid and requires Gag dimerization. J. Virol. **70:**4299–4310.
4. **Dorfman, T., A. Weimann, A. Borsetti, C. T. Walsh, and H. G. Gottlinger.** 1997. Active site residues of cyclophilin A are crucial for its incorporation into human immunodeficiency virus type 1 virions. J. Virol. **71:**7110–7113.
5. **Gao, F., E. Bailes, D. L. Robertson, Y. Chen, C. M. Rodenburg, S. F. Michael, L. B. Cummins, L. O. Arthur, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 1999. Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. Nature **397:**436–441.
6. **Goff, S. P.** 2004. Genetic control of retrovirus susceptibility in mammalian cells. Annu. Rev. Genet. **38:**61–85.
7. **Groves, C. P.** 1993. Order Primates, p. 243–277. *In* D. E. Wilson and D. M. Reeder (ed.), Mammal species of the world: a taxonomic and geographic reference. Smithsonian Institution Press, Washington, D.C.
8. **Herrera, S., B. L. Perlaza, A. Bonelo, and M. Arévalo-Herrera.** 2002. Aotus monkeys: their great value for anti-malaria vaccines and drug testing. Int. J. Parasitol. **32:**1625–1635.
9. **Hershkovitz, P.** 1983. Two new species of night monkeys, genus *Aotus* (Cebidae, Platyrrhini): a preliminary report on *Aotus* taxonomy. Am. J. Primatol. **4:**209–243.
10. **Higgins, D., J. Thompson, T. Gibson, J. D. Thompson, D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
11. **Hoelzer, G. A.** 1997. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene revisited. Evolution **51:**622–626.
12. **Hofmann, W., D. Schubert, J. LaBonte, L. Munson, S. Scammell, P. Ferrigno, and J. Sodroski.** 1999. Species-specific, postentry barriers to primate immunodeficiency virus infection. J. Virol. **73:**10020–10028.
13. **Jin, M. J., J. Rogers, J. E. Phillips-Conroy, J. S. Allan, R. C. Desrosiers, G. M. Shaw, P. M. Sharp, and B. H. Hahn.** 1994. Infection of a yellow baboon with simian immunodeficiency virus from African green monkeys: evidence for cross-species transmission in the wild. J. Virol. **68:**8454–8860.
14. **Nisole, S., C. Lynch, J. P. Stoye, and M. W. Yap.** 2004. A Trim5-cyclophilin A fusion protein found in owl monkey kidney cells can restrict HIV-1. Proc. Natl. Acad. Sci. USA **101:**13324–13328.
15. **Page, S. L., and M. Goodman.** 2001. Catarrhine phylogeny: noncoding DNA

evidence for a diphyletic origin of the mangabeys and for a human-chimpanzee clade. Mol. Phylogenet. Evol. **18:**14–25.

16. **Pierczarka, J. C., and C. Y. Nagamachi.** 1988. Cytogenetic studies of *Aotus* from Eastern Amazonia: Y/autosome rearrangement. Am. J. Primatol. **14:** 255–263.

17. **Pieczarka, J. C., R. M. de S. Barros, F. M. Faria, and C. Y. Nagamachi.** 1993. *Aotus* from the Southwestern Amazon region is geographically and chromosomally intermediate between *A. azarae boliviensis* and *A. infulatus*. Primates **34:**197–204.

18. **Sayah, D. M., E. Sokolskaja, L. Berthoux, and J. Luban.** 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature **430:**569–573.

19. **Schneider, H., F. C. Canavez, I. Sampaio, M. A. M. Moreira, C. H. Tagliaro, and H. N. Seuánez.** 2001. Can. molecular data place each neotropical monkey in its own branch? Chromosoma **109:**515–523.

20. **Sebastian, S., and J. Luban.** 2005. Trim5a selectively binds a restriction-sensitive retroviral capsid. Retrovirology **2:**40.

21. **Sharp, P. M., E. Bailes, R. R. Chaudhuri, C. M. Rodenburg, M. O. Santiago, and B. H. Hahn.** 2001. The origins of acquired immune deficiency syndromeviruses: where and when? Philos. Trans. R. Soc. Lond. B Biol. Sci. **356:** 867–876.

22. **Sokolskaja, E., D. M. Sayah, and J. Luban.** 2004. Target cell cyclophilin A modulates human immunodeficiency virus type 1 infectivity. J. Virol. **78:** 12800–12808.

23. **van Rensburg, E. J., S. Engelbrecht, J. Mwenda, J. D. Laten, B. A. Robson, T. Stander, and G. K. Chege.** 1998. Simian immunodeficiency viruses (SIVs) from eastern and southern Africa: detection of a SIVagm variant from a chacma baboon. J. Gen. Virol. **79:**1809–1814.

24. **Yang, Z.** 1997. PALM: a program package for phylogenetic analysis by maximum likelihood. Comput. Appl. Biol. Sci. **13:**555–556.

25. **Yap, M. W., S. Nisole, C. Lynch, and J. P. Stoye.** 2004. Trim5alpha protein restricts both HIV-1 and murine leukemia virus. Proc. Natl. Acad. Sci. USA **101:**10786–10791.