

Loss of Urate Oxidase Activity in Hominoids and its Evolutionary Implications

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We have determined and compared the promoter, coding, and intronic sequences of the urate oxidase (*Uox*) gene of various primate species. Although we confirm the previous observation that the inactivation of the gene in the clade of the human and the great apes results from a single CGA to TGA nonsense mutation in exon 2, we find that the inactivation in the gibbon lineage results from an independent nonsense mutation at a different CGA codon in exon 2 or from either one-base deletion in exon 3 or one-base insertion in exon 5, contrary to the previous claim that the cause is a 13-bp deletion in exon 2. We also find that compared with other organisms, the primate functional *Uox* gene is exceptional in terms of usage of CGA codons which are prone to TGA nonsense mutations. Nevertheless, we demonstrate rather strong selective constraint against nonsynonymous sites of the functional *Uox* gene and argue that this observation is consistent with the fact that the *Uox* gene is unique in the genome and evolutionarily conserved not only among animals but also among eukaryotes. Another finding that there are a few substitutions in the *cis*-acting element or CAAT-box (or both) of primate functional *Uox* genes may explain the lowered transcriptional activity. We suggest that although the inactivation of the hominoid *Uox* gene was caused by independent nonsense or frameshift mutations, the gene has taken a two-step deterioration process, first in the promoter and second in the coding region during primate evolution. It is also argued that the high concentration of uric acid in the blood of humans and nonhuman primates has developed molecular coevolution with the xanthine oxidoreductase in purine metabolism. However, it remains to be answered whether loss of *Uox* activity in hominoids is related to protection from oxidative damage and the prolonged life span.

Introduction

Uricase or urate oxidase (*Uox*) is the copper-binding enzyme which catalyzes the oxidation of uric acid, a product of purine metabolism, to allantoin (Wu et al. 1994; Lehninger 1995, pp. 740–742; Chu et al. 1996). The origin of the enzyme is so ancient that it is present in both prokaryotes and eukaryotes. It is conceivable from its fundamental role in purine metabolism that the enzyme is functionally important and evolutionarily conserved. Lack of the enzyme in humans and chickens may result in hyperuricemia and is associated with gouty arthritis. In human blood, the concentration of uric acid is more than fifty times higher than in other mammals (Keilin 1959). Disruption of the *Uox* gene in mice caused pronounced hyperuricemia/urate nephropathy, and more than half of the mutant mice died before 4 weeks of age (Wu et al. 1994). Nonetheless, loss of *Uox* activity has been frequently observed in phylogenetically unrelated organisms, such as birds, terrestrial reptiles, Cyclostomata, insects except Diptera, and Annelida (Keilin 1959; Yeldandi et al. 1996), and has attracted considerable interest from developmental, physiological, and evolutionary perspectives (Haldane 1955; Orowan 1955; Friedman et al. 1985; Wu et al. 1989). In primates,

prosimians and Old World monkeys exhibit a moderate level of the enzymatic activity relative to mice and rabbits (Christen et al. 1970a; Friedman et al. 1985; Fujiwara, Nakashima, and Noguchi 1987). In contrast, hominoids and some New World monkeys do not show any detectable level of the activity (Christen et al. 1970b; Simkin 1971; Logan et al. 1976), although Wu et al. (1992) have shown that the nonfunctional *Uox* gene in the human is transcribed in liver. Christen et al. (1970a, 1970b) and Ames et al. (1981) presumed genetic changes in a primate ancestor resulted in reduced or labile *Uox* activity (or both) and proposed a model of the progressive loss during primate evolution. On the other hand, Friedman et al. (1985) applied a sensitive micro-radiochemical assay to a wide range of primates, concluding that a single mutational event silenced *Uox* activity in a common ancestor to the extant hominoids.

It has been proposed that because uric acid is a powerful scavenger of free radicals, it plays an important role in protecting hominoids from oxidative damage (Ames et al. 1981; Whiteman and Halliwell 1996). Recently, Scott and Hooper (2001) have further argued that the accumulation of uric acid in hominoids results from its capability to inhibit the peroxynitrite-dependent extravasation of inflammatory cells and thereby prevent oxidative damage to the increasingly complex brains. Another interesting postulate, which Haldane (1955) questioned, though, was made by Orowan (1955) on the basis of the structural similarity of uric acid to cerebral stimulants, caffeine and theobromine. According to this idea, loss of *Uox* activity might result in a quantum jump in intellectual capability and thus trigger emergence of man.

Wu et al. (1989, 1992) first isolated the human genomic DNA containing the *Uox* gene and sequenced the

Abbreviations: *Uox*, urate oxidase; *Xor*, xanthine oxidoreductase; Hosa, *Homo sapiens*; Patr, *Pan troglodytes*; Gogo, *Gorilla gorilla*; Popy, *Pongo pygmaeus*; Hyla, *Hylobates lar*; Hyag, *H. agilis*; Hymu, *H. muelleri*; Hyco, *H. concolor*; Hysy, *H. syndactylus*; Paha, *Papio hamadryas*; Mamu, *Macaca mulatta*; Mafa, *M. fascicularis*; Aotr, *Aotus trivirgatus*.

Key words: uric acid, purine metabolism, primates, codon bias.

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whole coding region. In addition, they examined the chimpanzee, orangutan, and gibbon orthologs regarding the presence or absence of three detrimental mutations that were found in the human (two nonsense mutations at codons 33 and 187 and one mutant splice acceptor signal of exon 3). They found that the nonsense mutation at codon 33 was shared by the chimpanzee and the orangutan but that at codon 187 existed only in the chimpanzee. Moreover, because none of these three detrimental mutations was present in the gibbon, they partially sequenced the gibbon exon 2 and found a 13-bp deletion between codons 72 and 76. On the basis of these findings, they came to the conclusion that two independent inactivations of the *Uox* gene occurred in hominoids: the nonsense mutation at codon 33 in the human, the chimpanzee, and the orangutan (see also Yeldandi et al. 1991) and the 13-bp deletion in the gibbon. However, these studies focused on exons 2 and 5 only in rather limited primate species.

We therefore decided to fully explore the evolutionary change of the *Uox* gene in five hominoids, three Old World monkeys (*Catarrhini*), and one New World monkey (*Platyrrhini*). We determined the DNA sequences of the entire coding region of 915 bp for all these nine species. We also sequenced the promoter region of about 1,400 bp to examine if the *Uox* gene was silenced progressively or suddenly and sequenced intron 4 of about 1,400 bp to measure relative selective constraint against the promoter and coding regions. Furthermore, to see if the 13-bp deletion is common among gibbons, we sequenced exons 2–5 for three subgenera of the family Hylobatidae. In this paper, we report comparative analyses of these sequences and discuss the evolutionary implications of loss of *Uox* activity in hominoids.

Materials and Methods

Genomic DNA Sources

Genomic DNAs from the human (*Homo sapiens*), the chimpanzee (*Pan troglodytes*), the gorilla (*Gorilla gorilla*), the orangutan (*Pongo pygmaeus*), the gibbon (*Hylobates lar*, *H. agilis*, *H. muelleri*, *H. concolor*, and *H. syndactylus*), the baboon (*Papio hamadryas*), and the rhesus monkey (*Macaca mulatta*) were provided or purchased from sources shown in Appendix I. For the crab-eating monkey (*M. fascicularis*) and the owl monkey (*Aotus trivirgatus*), DNAs were isolated from liver cells and cultured cells (CRL-1556) with genomic DNA purification kit (QIAGEN) according to the manufacturer's instruction. The sources of these cells are also listed in Appendix I.

PCR Amplification, Cloning, and Sequencing

For the human, chimpanzee, gorilla, orangutan, white-handed gibbon, baboon, rhesus monkey, crab-eating monkey, and owl monkey *Uox* genes, the entire eight exons of 915 bp, the promoter region of ca. 1,400 bp upstream of the initiation codon, and part of intron 4 of ca. 1,400 bp were amplified by polymerase chain reaction (PCR). In order to examine the inactivation pro-

cess of the *Uox* gene in the family Hylobatidae, similar PCR amplification was also carried out for exons 2–5 for another *H. lar*, as well as for *H. agilis*, *H. muelleri*, *H. concolor*, and *H. syndactylus*. On the basis of available human *Uox* genomic sequences (GenBank accession number S94095 and AL136113), PCR primers for each region were designed. Each PCR reaction was done in a 25- μ l volume. The reaction mixture contained 50–100 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of dNTPs, 1.5 U of TaKaRa EX Taq[®] polymerase (TaKaRa), and 0.4 μ M of the upper and lower primer. PCR primer sequences and conditions were adapted to each region and are available upon request. All PCR products were purified through QIAquick PCR Purification Kit (QIAGEN). For exons 3, 4, and 6–8 in the nine primates and exons 2–5 in additional gibbons, PCR products were directly sequenced. Other PCR products of the promoter region, exons 1, 2, and 5, and intron 4 were cloned into pCRII-TOPO through TOPO TA Cloning Kits (Invitrogen). Plasmid DNAs were purified with QIAprep Spin Miniprep (QIAGEN) and used as templates in sequencing reaction. Sequencing reaction was performed with ABI prism Dye-Deoxy Reaction Kit (PE Applied Biosystems) and analyzed on ABI PRISM 377 DNA sequencer (PE Applied Biosystems). To avoid sequencing errors, PCR products or plasmid DNAs were sequenced two to six times for each product or plasmid in both directions. Sequences were also confirmed by independent PCRs. These sequences were deposited in the DNA Data Bank of Japan (DDBJ), and their accession numbers are given in Appendix I.

Data Analysis

DNA sequence alignment with insertion of gaps was performed with CLUSTAL W (Thompson, Higgins, and Gibson 1994), and the aligned sequences were manually checked by eye (Appendix II). In the phylogenetic analysis, any site containing insertion or deletion (indel) was excluded. We used two tree-making methods: neighbor-joining (NJ) by Saitou and Nei (1987) and maximum parsimony (MP) by Fitch (1971) and Hartigan (1973) in PHYLIP version 3.57c (Felsenstein 1995). For the coding region, the synonymous and nonsynonymous sites and the numbers of nucleotide differences at these respective sites were counted by the method of Nei and Gojobori (1986) and by the modified version with transition bias ($R = 1.0$) in Nei and Kumar (2000, pp. 57–59). When necessary, multiple-hit substitutions were corrected based on either Kimura's two-parameter model (Kimura 1980, 1983, pp. 90–97) or Jukes–Cantor model (Jukes and Cantor 1969).

Results

Nucleotide Substitutions and Phylogeny

We have constructed the MP tree of the nine primate species based on the entire sequence information about the promoter, coding, and intronic regions (fig. 1). The MP tree contains four numbers along each branch, representing the estimated numbers of synonymous and

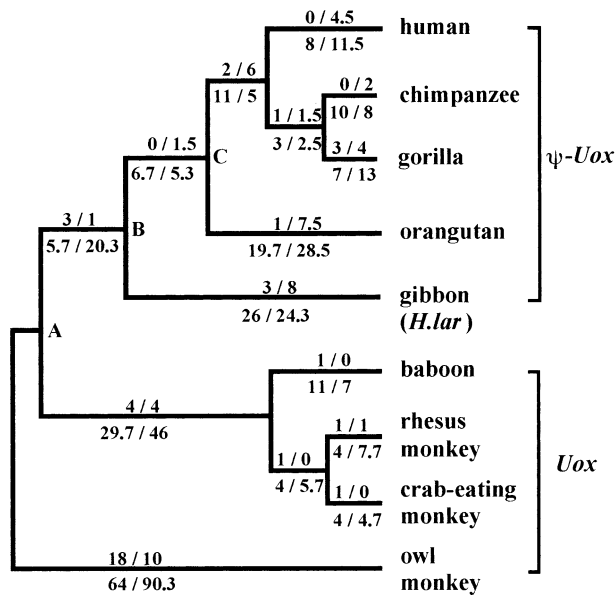


FIG. 1.—The MP tree of nine primate *Uox* genes based on the entire DNA sequences of 3,574 bp. Three ancestral nodes are labeled by A, B, and C. The numbers along each branch are estimated synonymous/nonsynonymous substitutions (above each line) and estimated substitutions in the promoter/intronic region (below each line). The bootstrap probability in 1,000 replications is 100% for all cases. The functional and nonfunctional groups of *Uox* DNA sequences are designated as *Uox* and ψ -*Uox*, respectively.

nonsynonymous substitutions in the coding region as well as the estimated numbers of nucleotide substitutions in the promoter region and intron 4. The NJ tree is essentially the same as the MP tree, except with a little long branch leading to the New World monkey. It is to be noted that the sister-relationship between the

chimpanzee and the gorilla to the exclusion of the human is supported by 100% bootstrap probability. In fact, there are six phylogenetically informative sites supporting the chimpanzee-gorilla clade but none supporting the alternatives among the human-chimpanzee-gorilla relationships. Because it is found that most variable sites among the hominoids are phylogenetically compatible with each other, the chimpanzee-gorilla clade is unlikely to result from parallel nucleotide substitutions. One exceptional change is a duplicated segment of GGGATGCC in intron 4 which is shared by the gorilla and the orangutan. However, because this change is phylogenetically incompatible with any of the three possible sister-relationships among the closely related trio of the human, the chimpanzee, and the gorilla, it might result from two independent duplications. Alternatively, though less likely, a single duplication occurred in the ancestral species of the great apes and had been polymorphic for a sufficiently long time to permit fixation of the duplicated form in the orangutan and the gorilla on one hand and loss in the human and the chimpanzee on the other hand. In any event, the chimpanzee-gorilla clade supported by the six compatible nucleotide substitutions is not unusual among the closely related trio. It is demonstrated that among 39 informative loci examined in the similar context, eight support the chimpanzee-gorilla clade (Satta, Klein, and Takahata 2000). It appears that this discordance between gene and species trees is caused by the high level of polymorphism in the ancestral species and by subsequent random sorting of polymorphism in descendant species.

The pairwise distance matrix is computed in terms of the proportion of nucleotide differences per site (table 1). It is immediately clear that the nonsynonymous sub-

Table 1
The Proportion of Nucleotide Differences per Site Among Nine Primate Species

| Species | Hosa | Patr | Gogo | Popy | Hyla | Paha | Mamu | Mafa | Aotr |
|--|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| A. Estimated per-site synonymous differences (above the diagonal) and per-site nonsynonymous differences (below the diagonal) ^a | | | | | | | | | |
| Hosa . . . | | 0.005 | 0.019 | 0.014 | 0.024 | 0.045 | 0.050 | 0.050 | 0.104 |
| Patr. . . . | 0.012 | | 0.014 | 0.019 | 0.028 | 0.050 | 0.055 | 0.055 | 0.100 |
| Gogo . . | 0.015 | 0.009 | | 0.033 | 0.043 | 0.064 | 0.069 | 0.069 | 0.114 |
| Popy . . . | 0.026 | 0.026 | 0.029 | | 0.019 | 0.043 | 0.047 | 0.047 | 0.102 |
| Hyla . . . | 0.030 | 0.027 | 0.030 | 0.026 | | 0.052 | 0.057 | 0.057 | 0.114 |
| Paha . . . | 0.027 | 0.024 | 0.027 | 0.021 | 0.020 | | 0.014 | 0.014 | 0.109 |
| Mamu . . | 0.028 | 0.025 | 0.028 | 0.023 | 0.021 | 0.002 | | 0.009 | 0.114 |
| Mafa. . . | 0.027 | 0.024 | 0.027 | 0.021 | 0.020 | 0.000 | 0.002 | | 0.114 |
| Aotr . . . | 0.036 | 0.033 | 0.036 | 0.031 | 0.029 | 0.021 | 0.023 | 0.021 | |
| B. Estimated nucleotide differences in intron 4 (above the diagonal) and in the promoter region (below the diagonal) ^b | | | | | | | | | |
| Hosa . . . | | 0.016 | 0.020 | 0.033 | 0.034 | 0.068 | 0.073 | 0.070 | 0.093 |
| Patr. . . . | 0.016 | | 0.016 | 0.033 | 0.031 | 0.067 | 0.071 | 0.069 | 0.092 |
| Gogo . . | 0.013 | 0.013 | | 0.035 | 0.036 | 0.072 | 0.076 | 0.073 | 0.097 |
| Popy . . . | 0.028 | 0.032 | 0.030 | | 0.042 | 0.079 | 0.083 | 0.080 | 0.103 |
| Hyla . . . | 0.038 | 0.042 | 0.040 | 0.038 | | 0.070 | 0.075 | 0.073 | 0.099 |
| Paha . . . | 0.052 | 0.054 | 0.052 | 0.053 | 0.052 | | 0.015 | 0.013 | 0.105 |
| Mamu . . | 0.050 | 0.052 | 0.050 | 0.049 | 0.050 | 0.014 | | 0.009 | 0.109 |
| Mafa. . . | 0.050 | 0.052 | 0.050 | 0.049 | 0.050 | 0.014 | 0.006 | | 0.109 |
| Aotr . . . | 0.069 | 0.070 | 0.068 | 0.069 | 0.070 | 0.078 | 0.075 | 0.074 | |

^a The average number of synonymous sites is 211, whereas the average number of nonsynonymous sites is 659.
^b The average number of sites in intron 4 is 1336, whereas the average number of sites in the promoter region is 1340.

stitutions have accumulated as rapidly as the synonymous substitutions among the five hominoids, but this is not the case among the three Old World monkeys and the New World monkey. Similarly, the proportion of nucleotide differences is computed regarding the promoter region and intron 4. For a given species pair among the hominoids, there is no significant difference in the proportion of nucleotide differences between the promoter region and intron 4, whereas for a species pair among the nonhominoid primates, the proportion is lower in the promoter region than in intron 4. After correcting multiple-hit substitutions by the two-parameter model (Kimura 1980, 1983, pp. 90–97), both synonymous and intron 4 nucleotide divergences between the Old World and New World monkeys became almost exactly the same as the silent sequence divergences of 13% which was previously obtained as the average value over 29 independent loci (Takahata 2001). Thus, there is no indication for functional conservation of this intron, and it is used as a neutral standard.

Functional Constraint and Nonsense Mutations

The degree of functional constraint against a particular region of a gene may or may not be altered by its dysfunctioning. We assume that intron 4 of 1,340 bp excluding indels is completely free from selective constraint so that gene dysfunctioning does not alter the rate (k) of nucleotide substitutions in the intron. This may also hold true at synonymous sites. In fact, for various species pairs, the nucleotide divergences per synonymous site are nearly the same as those per intron site. On the other hand, the promoter region of 1,336 bp excluding indels and the coding region represented by the nonsynonymous sites of 659 bp may be under negative selection in the functional *Uox* gene lineage. We measure the proportion of selective neutrality, which is inversely related to the degree of functional constraint (Kimura 1983, p. 313), by the ratio of the per-site nucleotide substitutions in a specified region to those in intron 4, and designate it by f and g if *Uox* is functional and nonfunctional (subsequently designated by ψ -*Uox*), respectively.

In the five hominoid ψ -*Uox* genes, there are several independent nonsense mutations in five CGA, one TGG, or one CAG codon (Appendix II). Furthermore, there exists one mutation in the splice acceptor signal of exon 3 shared by the human and the African apes (data not shown), one human-specific 26-bp deletion in the enhancer region, one gorilla-specific mutation in the initiation codon, and one gorilla-specific tandem duplication in exon 2. Of these nonsense mutations, three occur in species-specific manners, and only one at codon 33 in exon 2 is shared by the human and the great apes. As previously inferred by Wu et al. (1992), it is therefore reasonable to conclude that the ψ -*Uox* gene in the human and the great apes was caused by this shared nonsense mutation. Another nonsense mutation at codon 187 is shared by the human, the chimpanzee and the gorilla, so that it is most likely to have occurred in their ancestral species. The nonsense mutation (TGA) at co-

Table 2
Functional Constraints Against the Promoter Region and the Nonsynonymous Sites Relative to Intron 4

| | Number of Sites | Functional Lineages | Nonfunctional Lineages |
|--|-----------------|----------------------|------------------------|
| Intron 4 | 1336 | 181.7 ^a | 68.5 ^b |
| Promoter | 1340 | 122.4 ^a | 58.7 ^b |
| <i>G</i> values ^c | | 10.6 ($P < 0.005$) | 0.7 ($P > 0.1$) |
| Nonsynonymous sites . . . | 659 | 16 ^a | 25.5 ^b |
| <i>G</i> values ^d | | 64.8 ($P < 0.005$) | 1.5 ($P > 0.1$) |

^a The total number of nucleotide substitutions in the MP tree (fig. 1) excluding all the hominoid lineages that share the common ancestor B.

^b The total number of nucleotide substitutions within the clade of the human, chimpanzee, gorilla, and orangutan (fig. 1).

^c The *G* values for the difference between the MP estimated number of nucleotide substitutions in the promoter region and that in intron 4.

^d The *G* values for the difference between the MP estimated number of nucleotide substitutions at the nonsynonymous sites in the coding region and that in intron 4.

don 107 is, however, more complicated than others. It occurs in the gorilla, the orangutan, and the gibbon, and therefore requires multiple origins of this nonsense mutation. Overall, it is remarkable that, except one CGA codon in exon 6, all the other four CGA codons are converted to the TGA termination codon in all or some of the hominoids. Arginine (Arg) is a basic amino acid, and its usage varies greatly from gene to gene. However, in most organisms, Arg is generally coded by non-CGA codons (Wada et al. 1992; Osawa 1995, pp. 45–57). The fact that there are as many as five CGA among 12 Arg codons in the Old World monkey *Uox* is suggestive of the high likelihood of eventual dysfunctioning of this gene.

To obtain g in ψ -*Uox* gene lineages, we use the MP estimates for all branches that occur in the clade of the human and the great apes (table 2). The gibbon *Uox* is also nonfunctional, but we exclude it from the present calculation because its dysfunctioning occurred independently in that lineage and its timing is unknown (Wu et al. 1992). In total, there are 68.5 nucleotide substitutions in intron 4, 58.7 in the promoter region, and 25.5 at the nonsynonymous sites. We therefore have $g_p = (58.7/1340)/(68.5/1336) = 0.85$ for the former and $g_n = (25.5/659)/(68.5/1336) = 0.75$ for the latter. At face value, both g_p and g_n of ψ -*Uox* are smaller than 1. However, it is difficult to imagine any selective constraint against pseudogenes; in fact, the result of the *G*-test shows that the observed $g_p = 0.86$ and $g_n = 0.75$ values are not significantly different from 1 ($df = 1$, $P > 0.1$ in both cases). We thus conclude the absence of selective constraint against the promoter region (but see *Discussion*) as well as the nonsynonymous sites in the ψ -*Uox* gene lineage and subsequently assume that $g = 1$ in both regions.

For the functional *Uox* gene, we use all branches leading to Old World and New World monkeys as well as the internodal branch leading to the ancestral node B of hominoids as shown in figure 1. There are 181.7 nucleotide substitutions in intron 4, 122.4 in the promoter region, and only 16 at the nonsynonymous sites. The proportion of selective neutrality (f) is computed as f_p

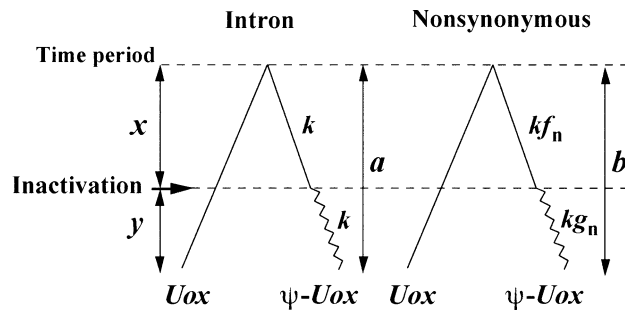


FIG. 2.—A schematic diagram for dating the inactivation time. A neutral standard, intron 4, is assumed to evolve at rate k throughout, whereas the nonsynonymous sites are assumed to evolve at rate kf_n while the *Uox* gene is functional and at rate kg_n when it becomes nonfunctional.

$= (122.4/1340)/(181.7/1336) = 0.67$ for the promoter region and $f_n = (16/659)/(181.7/1336) = 0.18$ for the nonsynonymous sites. Thus, in the functional gene lineage, the promoter region as a whole is somewhat constrained, and the nonsynonymous sites are fairly strongly constrained. The G -test confirms that relative to intron 4, the values $f_p = 0.67$ and $f_n = 0.18$ are significantly lower than 1 ($df = 1$, $P < 0.005$ in both cases). We have noted that the promoter region is not uniform with respect to the functional importance. The first 700 bp upstream region from the initiation codon is more selectively constrained in the functional *Uox* gene lineage and tends to accumulate nucleotide substitutions more rapidly in the ψ -*Uox* gene lineage than the remaining upstream region. In particular, it is interesting to note a *cis*-acting element (CAAAAATGTC) that was initially identified in rat by means of a transient transfection assay and thought to be important in transcription (Izuhara, Ito, and Takagi 1995). The nucleotide sequence of this *cis*-acting element (CAAAGATGTT) among primates is identical, but because it differs from that of rat at two underlined nucleotide positions, it may not be responsive to cAMP (Izuhara, Ito, and Takagi 1995).

Dating the Inactivation Event

A dramatic change in the degree of functional constraint at the nonsynonymous sites can be used to estimate when the *Uox* gene became nonfunctional. We separately treat two independent events of gene dysfunctioning in the hominoid lineage: one in the stem lineage of the human and the great apes and the other in the stem lineage of gibbons. We designate by x the time period during which the gene was functional since it diverged from the *Catarrhini* lineage and by y the time period of inactivation in the lineage leading to the human and the great apes or to the gibbon (fig. 2). As mentioned previously, the proportion of selective neutrality during these two time periods is given by f_n and g_n , respectively. If we denote the per-site substitution rate in intron 4 by k per unit time, we have

$$k(x + y) = a \quad \text{and} \quad k(f_n x + g_n y) = b \quad (1)$$

where a is the estimated number of nucleotide substitutions per site in intron 4 during the period of $x + y$

and b is that at the nonsynonymous sites during the same period of time. We have already had $f_n = 0.18$ and $g_n = 1$. Of interest is the ratio r of y to $x + y$. This ratio is independent of k and can be easily obtained from (1) as

$$r = \frac{y}{x + y} = \frac{b/a - f_n}{g_n - f_n}. \quad (2)$$

To compute a and b for the case of the human and the great apes, we first take the average number of nucleotide substitutions between node C and one of the four extant species (fig. 1). The average number is 20.3 in intron 4 and 9.8 at the nonsynonymous sites. These respective MP estimates between node A and node B are 20.3 and 1, whereas they are 5.3 and 1.5 between node B and node C. The height of node C relative to that of node A becomes $20.3/45.9 = 0.44$, and this value must be smaller than the ratio estimated from (2). In terms of the number of nucleotide substitutions in intron 4, the average height of node A becomes $45.9/1336 = 0.034$ and is used as an estimate of a . The average height of node A in terms of the number of nonsynonymous substitutions becomes 0.019 and is used as an estimate of b . The ratio of b/a is therefore given by 0.54. Using $g_n = 1$ and $f_n = 0.18$, we have $r = 0.44$. The comparison between this r value and the relative height of node C suggests that the inactivation of the human and the great ape *Uox* gene took place shortly before the splitting of their ancestral lineage from the orangutan lineage.

In the case of the gibbon lineage, the MP estimates of a and b become 0.033 and 0.014, respectively. The height of node B relative to node A becomes $24.3/44.6 = 0.54$ in intron 4. Again using the same estimates of f_n and g_n as before, we have $r = 0.28$ estimated from (2). This r value is considerably smaller than the relative height of node B and consistent with the finding that the gibbon *Uox* gene was inactivated independently of the human and great ape lineage (Wu et al. 1992). Although most species divergence times in primate phylogeny are still controversial (Martin 1993; Kumar and Hedges 1998; Takahata 2001), it is interesting to convert these ratios of r into the geological times. If we assume that *Catarrhini* and hominoids diverged 35 MYA, then $r = 0.44$ and 0.28 correspond to 15.4 and 9.8 MYA, respectively. Importantly, this relatively recent inactivation in the gibbon lineage suggests that some species in the family Hylobatidae may still possess the functional *Uox* gene. Although Friedman et al. (1985) were unable to detect *Uox* activity in the adults of *H. lar* and *H. concolor*, there is at present no information about subgenera *Bunopithecus* and *Symphalangus*. In order to examine this possibility and which mutation was responsible for the dysfunctioning of gibbon ψ -*Uox*, we have examined exon 2–5 DNA sequences for several gibbon species.

Inactivation in the Gibbon Lineage

There are seven seemingly detrimental mutations in the gibbon ψ -*Uox* gene: two in exon 2, three in exon 3, one in the splice donor site in intron 3, and one in exon 5. One such is the 13-bp deletion in exon 2, which

is previously thought to be responsible for the dysfunctioning of the gibbon *Uox* gene (Wu et al. 1992). In the present study, we have newly examined *H. agilis*, *H. muelleri*, *H. concolor*, and *H. syndactylus* in addition to two individuals of *H. lar*. The 13-bp deletion is indeed shown to be present in subgenus *Hylobates* ($2n = 44$) including *H. lar*, *H. agilis*, and *H. muelleri*. However, neither subgenus *Nomascus* ($2n = 52$) including *H. concolor* nor subgenus *Symphalangus* ($2n = 50$) including *H. syndactylus* possesses this deletion, and these subgenera exhibit the same stretch of AGAACACAGTTCA as in the human and the great apes. A parsimonious explanation is that the deletion took place in the common ancestral lineage of subgenus *Hylobates*. According to the recent study on the major *Hylobatid* divisions by Roos and Geissmann (2001), *H. nomascus* branched off first, followed by *Symphalangus*, and then by *Bunopithecus* and *Hylobates*. The *Uox* sequence differences between *Symphalangus* and *Hylobates* are smaller than those between the human and the orangutan, suggesting the relatively recent event of the 13-bp deletion.

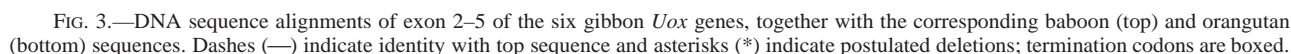
One nonsense mutation (CGA→TGA) is found in each of exon 2 and exon 3 (fig. 3). It turns out that the exon 2 mutation is shared by all gibbon species examined and is not found in any of the remaining primate species. It is therefore most likely that this mutation took place before the subgenus differentiation and inactivated the gibbon *Uox* gene altogether. If this is the case and if the inactivation resulting from the nonsense mutation occurred about 10 MYA as mentioned previously, the subgenus differentiation in extant gibbons must have taken place during the past 10 Myr (Roos and Geissmann 2001 and references therein). In contrast, the exon 3 mutation is not shared by *H. syndactylus* but by the gorilla and the orangutan. The origin of this mutation is therefore multiple and relatively recent in the gibbon lineage. The splice donor site mutation at intron 3 is shared by gibbons, except *H. syndactylus* (data not shown). This and other substitutions in the gibbon *Uox* gene support that *H. syndactylus* is the most distantly related species in the family (fig. 3). This conclusion is different from that of Roos and Geissman (2001), who studied the mitochondrial control region and Phe-tRNA. In addition to nonsense and splice donor site mutations, there are several indels. One such is a two-base deletion in exon 3 which occurs specifically to subgenus *Hylobates*. There are also one-base deletion in exon 3 and one-base insertion in exon 5, both of which are specific to all the gibbon lineages. The frameshift caused by the one-base deletion produces a termination codon in exon 4 and that caused by the one-base insertion produces a termination codon within exon 5. Because the active center of the enzyme is coded by codon 127, 129, and 131 in exon 4 (Wu et al. 1989; Chu et al. 1996), the one-base deletion may be more detrimental than the one-base insertion. However, this information alone seems insufficient to exclude the one-base insertion from the cause of the gene inactivation.

From these considerations, we conclude that neither 13 bp-deletion in exon 2 nor two-base deletion in exon 3 is the common cause of the gibbon ψ -*Uox*. Rath-

er, it is either the nonsense mutation at codon 18 or two one-base indels that dysfunctioned the gibbon *Uox* gene. Although it is difficult to decide which change occurred first, it is interesting to note that the exon 2 nonsense mutation (CGA→TGA) is the same as that found in ψ -*Uox* in the human and the great apes. One possibility for such coincidence may be attributed to a high transition rate from C to T in a CGA codon. Chen and Li (2001) found that the observed frequency of CpG is much lower in noncoding regions (0.7%) than in coding regions (2.8%) and argued that pseudogenes, relatively rich in CpG, may evolve faster than noncoding or intronic regions. To substantiate this argument, we compared the dinucleotide frequency and the number of nucleotide substitutions between the coding and intronic regions. In the human and great ape clade in which the gene is dead, there are 32.5 substitutions in the 870-bp coding region and 68.5 substitutions in the 1,336-bp intron 4. It turned out that the number of nucleotide substitutions per site is not significantly different between the two regions ($G = 2.18$, $df = 1$, $P > 0.05$). There is also no significant difference in the dinucleotide frequency between the two regions: 0.7% in the coding region and 0.9% in intron 4. Thus, the expected biased occurrence of the dinucleotide is not observed between the coding and intronic regions and these have evolved at almost the same rate. This does not exclude the possibility of a high transition rate of C to T in CpG. However, it is well known that CpG is most underrepresented on the genomic scale (Ohno 1988; Karlin and Burge 1995; Shioiri and Takahata 2001). In the ψ -*Uox* gene, the expected frequency of CpG is about 4.7% in both the coding and intronic region, but the observed frequency is only 15%–19% of it. Because of this underrepresentation, the high C to T transition in CpG, if present, cannot greatly contribute to an increase of the overall substitution rate.

Discussion

In the hominoid lineage, we have found eight independent nonsense mutations (fig. 1 and Appendix II). Of these, six are caused by C to T transitions in Arg CGA codons. The baboon *Uox* gene uses 12 Arg codons among 305, and its usage is not particularly high or low compared with the average over various genes (Wada et al. 1992). However, it may be unusual to use five CGA codons among 12 Arg codons and to mutate four of them to TGA codons independently in some hominoids. In most organisms, *Uox* is a single gene and it is expected to be conserved at the amino acid level. Exceptions to the single gene status are found in some plants (Takane, Tajima, and Kouchi 1997; Cheng et al. 2000) and *Drosophila virilis* (Lootens, Burnett, and Friedman 1993). In the latter case, however, duplicated *Uox* loci in tandem are polymorphic, and one copy is expressed very poorly. We have compared *Uox* amino acid sequences of mammals, fruit flies, plants, fungi, and a bacterium with special reference to the number of Arg and the number of CGA codons (fig. 4). In lower eukaryotes, the number of Arg is at variance; seven in *Candida utilis*



do not code Arg in other organisms. Thus, preferential use of CGA becomes apparent in mammals and notably in primates. Although the biological reason is not clear, increasing use of such an Arg codon is highly associated with frequent loss of Uox enzymatic activity in hominoids. In the current DNA database, no information

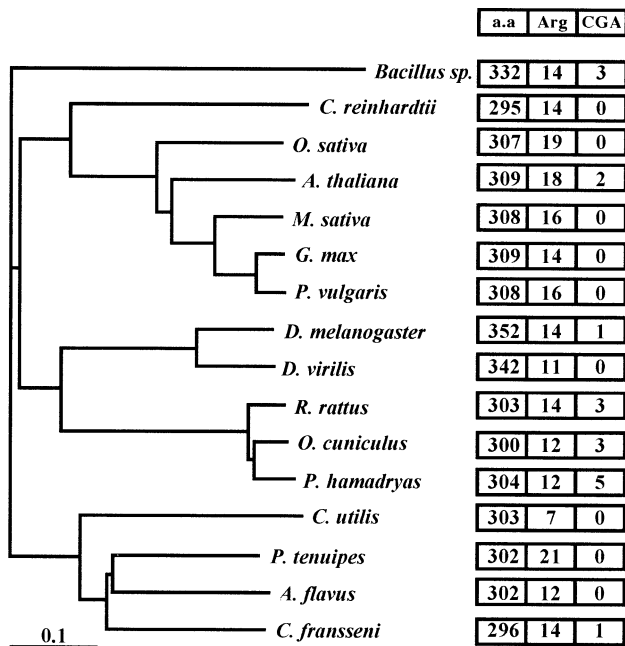


FIG. 4.—The NJ tree of *Uox* amino acid sequences of bacterium, fungi, fruit flies, mammals, and plants without multiple-hit corrections. The numbers of amino acids, Arg, and CGA codons used for Arg are indicated in the three columns on the right-hand side. The accession number (GenBank) for each species is as follows—*Bacillus* sp.: D49974, *Chlamydomonas reinhardtii*: AF195795, *Oryza sativa*: AP003611, *Arabidopsis thaliana*: AC004484, *Medicago sativa*: AB028150, *Glycine max*: D86930, *Phaseolus vulgaris*: U72663, *Drosophila melanogaster*: X51940, *Drosophila virilis*: X57114, *Rattus rattus*: X13098, *Oryctolagus cuniculus*: X14522, *Papio hamadryas*: M27694, *Candida utilis*: D32043, *Paecilomyces tenuipes*: AB038709, *Aspergillus flavus*: X61766, and *Cerataphis fransseni*: AB038706.

about the *Uox* gene is available for birds, terrestrial snakes, and lizards which lost their *Uox* activities (Keilin 1959; Yeldandi et al. 1996). If our interpretation is correct, frequent use of CGA codon for Arg is not restricted to mammals but it may be extended to these vertebrates too.

As briefly mentioned earlier, compared with the rat, the *cis*-acting element (−221 to −212 bp) of primates possibly involved in glucagon induction via cAMP (Izuhara, Ito, and Takagi 1995) differs by two point mutations. There are also two point mutations in each of the CAAT-box (−117 to −112 bp) and the palindromic sequence (−216 to −207 bp). It is possible that some of these point mutations have lowered the transcriptional activity in the early evolution of primates. This possibility is consistent with the observation that the *Uox* enzymatic activity of Old World and New World monkeys is known to be two- to fourfold lower than that of mice and rabbits (Friedman et al. 1985). Although dysfunctioning of the *Uox* gene in the human and great ape clade stems from a nonsense mutation in exon 2 or in the gibbon clade stems from a nonsense mutation in exon 2 or one-base indels, the promoter might have already been deteriorated by harmful mutations before *Catarrhini* and *Platyrrhini* diverged from each other. It therefore appears that the stepwise loss of *Uox* activity

is more reasonable than the single step loss during primate evolution.

It has been recently reported that the *Uox* gene and the *DLAD* (DNase II-Like Acid DNase) gene lie in a head-to-head orientation (Shiokawa and Tanuma 2001) and that exon 1 followed by large intron 1 of the *DLAD* gene is located from −776 to −900 bp in the promoter region of the *Uox* gene (Appendix II). Because of this, the promoter region of the *DLAD* gene, though not yet well characterized, should overlap with that of the *Uox* gene. Because the *DLAD* gene is presumably functional in the hominoids (Shiokawa and Tanuma 2001), this overlap can explain why the promoter region of the ψ -*Uox* gene in the hominoids is not completely free from selective constraint (table 2). The fact that the constraint in the region proximal to the initiation codon of the ψ -*Uox* gene (−1 to −400 bp) is more apparent than in the distal region (−401 to −700 bp) also suggests that this proximal region still plays important roles in *DLAD* transcription.

Loss of *Uox* activity in hominoids results in >10-fold higher concentration of uric acid in blood of humans and most primates than in other mammals (Keilin 1959). Although uric acid as a powerful antioxidant may provide a basis for the increase of life span and decrease cancer rates (Ames et al. 1981), the high concentration predisposes to crystalline deposition (Slot 1994). One way to reduce the risk is to decrease the xanthine oxidoreductase (*Xor*) gene activity (Xu, LaVallee, and Hoidal 2000). It is known that the *Xor* enzymatic activity in humans is 100 times lower than that in bovine, rats, and mice (Abadeh et al. 1992). Xu, LaVallee, and Hoidal (2000) demonstrated that transcription and core promoter activity of the human *Xor* are repressed. It appears that the purine metabolic system in humans has found a way to avoid the overproduction of uric acid by down-regulating the *Xor* gene expression, the product converting hypoxanthine to xanthine and further xanthine to uric acid. However, it is not known how and when this coevolution began. If the *Uox* gene expression has been repressed since the early evolution of primates, the repression of *Xor* gene expression might also be favored by natural selection from that time on. On the other hand, if the downregulation of *Xor* gene expression is hominoid-specific, the coevolution must be relatively recent. In this case, however, there is no reason to think that both great and lesser apes could downregulate the *Xor* gene expression in the same way. Whichever is the case, the purine metabolic system should provide an excellent example for studying molecular coevolution, and examination of the *Xor* regulatory elements in nonhuman primates will be of particular interest in this regard.

Acknowledgments

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Appendix I
List of Data Bank of Japan Accession Numbers and DNA Sources

| Species | Promoter | Accession Number Coding | Intron 4 | Sources |
|------------------------------------|----------|-------------------------------|----------|-----------------------------|
| <i>Homo sapiens</i> | AB074093 | AB074319-26 | AB074102 | Novagen |
| <i>Pan troglodytes</i> | AB074094 | AB074327-34 | AB074103 | Ueda ^a |
| <i>Gorilla gorilla</i> | AB074095 | AB074335-42 | AB074104 | O'hUigin ^b |
| <i>Pongo pygmaeus</i> | AB074096 | AB074343-50 | AB074105 | O'hUigin |
| <i>Hylobates lar</i> (1) | AB074097 | AB074351-58 | AB074106 | O'hUigin |
| <i>Hylobates lar</i> (2) | — | AB074391-94 | — | PRI ^c |
| <i>H. agilis</i> | — | AB074395-98 | — | PRI |
| <i>H. muelleri</i> | — | AB074399-402 | — | PRI |
| <i>H. concolor</i> | — | AB074403-06 | — | PRI |
| <i>H. syndactylus</i> | — | AB074407-10 | — | PRI |
| <i>Papio hamadryas</i> | AB074098 | AB074359-66 | AB074107 | PRI |
| <i>Macaca mulatta</i> | AB074099 | AB074367-74 | AB074108 | CLONTECH laboratories, Inc. |
| <i>M. fascicularis</i> | AB074100 | AB074375-82 | AB074109 | In Vitro Technologies, Inc. |
| <i>Aotus trivirgatus</i> | AB074101 | AB074383-90 | AB074110 | ATCC |

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^c Primate Research Institute, Kyoto University, Inuyama.

NOTE.—All other sources of DNA or cells were purchased from companies as indicated in the last column.

APPENDIX II

DNA sequence alignments of the *Uox* genes of human (Hosa), chimpanzee (Patr), gorilla (Gogo), orangutan (Popy), gibbon (Hyla), baboon (Paha), rhesus monkey (Mamu), crab-eating monkey (Mafa), and owl monkey (Aotr).

Dashes (—) indicate identity with top sequence, and asterisks (*) indicate postulated deletions. The promoter region, coding region, and intron 4 are presented in this order separately. In the promoter region, the rat ortholog (D50689) is also aligned from the initiation

codon to −320 bp; the enhancer region is underlined, the 5' untranslated region is doubly underlined, and the *cis*-acting element, palindromic sequence, and CAAT-box are boxed. In the coding region, the mutation in the initiation codon in the gorilla is shaded, and the termination codons produced by nonsense mutations are boxed. Three TGA nonsense mutations at codon 107 appear to occur independently in the gorilla, the orangutan, and the gibbon. However, in the orangutan, codon 107 is polymorphic with respect to TGA and CGA, whereas in the *H. syndactylus*, codon 107 is occupied by CGA.

Promoter region (1400 bp)

5'UTR

TATA-box

Coding region (915 bp)

Exon 1

1 10 20 30

Hosa ATG GCC CAC TAC CAT AAC AAC TAT AAA AAG AAT GAT GAG GTG GAG TTT GTC CGA ACT GGC TAT GGG AAG GAA ATG GTA AAA GTT CTC CAT

Patr

Gogo

Popy

Hyla

Paha

Mamu

Mafa

Aotr

Exon 2

40 50

Hosa ATT CAG TGA GAT GGA AAA TAT CAC AGC ATT AAA GAG GTG GCA ACT TCA GTG CAA CTT ACT CTA AGT TCC AAA AAA *** **

Patr

Gogo

Popy

Hyla

Paha

Mamu

Mafa

Aotr

Exon 3

80 90 100

Hosa GCA AAG TTT AAA GAA ATC AAA AGC ATA GAA GCC TTT GGT GTG AAT ATT TGT GAG CAT TTT CTT TCT TCT TTT AAC CAT GTA ATC CGA GCT

Patr

Gogo

Popy

Hyla

Paha

Mamu

Mafa

Aotr

Exon 4

110 120 130

Hosa CAA GTC TAC ATG GAA GAA ATC CCT TGG AAG CAT CTT GGA AAG AAT GGA GTT AAG CAT GTC CAT GCA TTT ATT CAC ACT CCC ACT GGA ACA

Patr

Gogo

Popy

Hyla

Paha

Mamu

Mafa

Aotr

Exon 5

140 150 160

Hosa CAC TTC TGT GAA GTT GAA CAG CTG ACA AGT GGA CCC CAA GTC ATT * CAT TCT GGA ATC AAA GAC CTC AAG GTC TTG TAC TGC AAG TGC

Patr

Gogo

Popy

Hyla

Paha

Mamu

Mafa

Aotr

Exon 6

200 210 220

Hosa CGC TAC CAC CAG TGC AGG GAT GTG GAC TTC AAG GCT ACC TGG GAC ACC ATT CGG GAC CTT GTC ATG GAG AAA TCT GCT GGG CCC TAT GAC

Patr

Gogo

Popy

Hyla

Paha

Mamu

Mafa

Aotr

Exon 7

230 240 250

Hosa AAA GGT GAA TAC TTG ACC TCT GTG CAG AAG ACC CTC TGT GAT ATC CAG GTG CTC TCC CTG AGC CGA GTT CCT GCG ATA GAA GAT ATG GAA

Patr

Gogo

Popy

Hyla

Paha

Mamu

Mafa

Aotr

Exon 8

260 270 280

Hosa ATC AGC CTG CCA AAC ATT CAC TAC TTC AAC ATA GAC ATG TCC AAA ATG GGT CTG ATC AAC AAG GAA GAG GTC TTG CTG CCA TTA GAC AAT

Patr

Gogo

Popy

Hyla

Paha

Mamu

Mafa

Aotr

Exon 9

290 300

Hosa CCA TAT GGA AAA ATT ACT GGT ACA GTC AAG AGG AAG TTG TCT TCA AGA CTG TGA

Patr

Gogo

Popy

Hyla

Paha

Mamu

Mafa

Aotr

APPENDIX II CONTINUED.

Intron 4 (1419 bp)

| | 1 | 100 | 200 | 300 | 400 | 500 | 600 | 700 | 800 | 900 | 1000 | 1100 | 1200 |
|-------|------------|------------|------------|------------|------------|-------------|-------------|------------|-------------|------------|------------|------------|------------|
| Ho sa | ATGTAGCTTC | CAGTAAGTAC | TTCCGAAAGT | TAAACAAGGA | TCATTTCAG | TTTC*ACTGC | ATTATCCTGG | GACACTCTGC | ACAGAAATATC | TTCTTTAATC | AGGACATATT | CAATCAGCAG | |
| Patr | ----- | ----- | A----- | ----- | ----- | *-C- | ----- | ----- | ----- | ----- | ----- | ----- | T----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | *-C- | ----- | ----- | ----- | ----- | ----- | ----- | T----- |
| Popy | -A----- | ----- | A----- | ----- | ----- | *-C- | ----- | ----- | ----- | ----- | ----- | ----- | T----- |
| Hyla | ----- | -C----- | A----- | -C----- | ----- | *-C- | ----- | ----- | ----- | ----- | T----- | T----- | T----- |
| Paha | ----- | ----- | A----- | ----- | -G----- | *----- | ----- | A----- | T----- | ----- | T----- | C----- | TG--T----- |
| Mamu | ----- | ----- | A----- | ----- | -G----- | *----- | ----- | A----- | T----- | ----- | ----- | C----- | TG--T----- |
| Mafa | ----- | ----- | A----- | ----- | -G----- | *----- | ----- | A----- | T----- | ----- | ----- | C----- | TG--T----- |
| Aotr | C-A----- | -A----- | -G----- | -A----- | -G----- | -A----- | -T----- | -TTG--T | ----- | A-TG-- | ----- | ----- | T----- |
| Ho sa | ATCTACCACT | AACCACTGGC | TATTGTCTCT | TCTGATAGGA | GTATACTGAA | GCATGGAAAA | *ACAGAAATA | CAGACTTTAT | TTACCTACTT | TTGAAACTCC | ACAGCTCTCG | AGTTAACTGT | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | A----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | ***** | ***** | *-A----- | T-A----- | ----- | T----- | ----- | T-G----- | G----- | T----- | ----- | ----- | ----- |
| Mamu | ***** | ***** | *-A----- | T-A----- | ----- | T----- | ----- | T----- | ----- | ----- | ----- | ----- | ----- |
| Mafa | ***** | ***** | *-A----- | T-A----- | ----- | T----- | ----- | T----- | ----- | ----- | ----- | ----- | ----- |
| Aotr | ----- | ----- | -G----- | ----- | -G----- | -T----- | -A----- | T----- | -C----- | ----- | -A----- | ----- | ----- |
| Ho sa | AGCAATAGAA | ACCACACAGG | TGATGTAAAC | CATTGGATT | TTAAGACGTT | CCTTGGATT | TAATTGAGGA | TCATGAGTTA | TATTCTTGTT | CTTCAACTAA | GACCATGTGT | GTGGTGATAC | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | ----- | -G----- | ----- | ----- | -C----- | CA----- | ----- | ----- | ----- | ----- | ----- | ----- | AA----- |
| Mamu | ----- | -G----- | ----- | ----- | -C----- | CA----- | ----- | ----- | ----- | ----- | ----- | ----- | AA----- |
| Mafa | ----- | -G----- | ----- | ----- | -C----- | CA----- | ----- | ----- | ----- | ----- | ----- | ----- | AA----- |
| Aotr | ----- | ----- | -T----- | ----- | -T-A----- | A----- | ----- | -A----- | -A----- | ----- | -G----- | ----- | AA----- |
| Ho sa | CTGACATCTC | TGGCCCTTGA | TTTTAGACT | GAACAGTCCA | AGAAGCATAG | ATATTGGTTC | GAACATAACAA | AGAGACTGAA | AAGAGG**** | *****TGATC | TTAATAATCG | TGCCATGGCA | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | ----- | -C----- | ----- | ----- | A----- | A----- | ----- | ----- | ----- | ----- | -G----- | TA----- | T----- |
| Mamu | C----- | -C----- | -T----- | -T-A----- | -A----- | -A----- | -C----- | ----- | -A-A-GTTT | TTTT***** | *A----- | A----- | C----- |
| Mafa | C----- | -C----- | -T----- | -T-A----- | -A----- | -A----- | -C----- | ----- | -A-A-GTTT | TTTT***** | *A----- | A----- | C----- |
| Aotr | -C----- | -AA----- | ----- | A----- | G-A----- | -G----- | -CA----- | -TG----- | ----- | ----- | ----- | -A----- | -G----- |
| Ho sa | CTTTGCTATT | AGGGTTTGCC | AAGTGCTCCC | GGAATGTCCC | CAATCCTTGC | CAGCTGTGTC | AGACAAATAA | AGTCATAGTG | ATGTTAATAT | TGTTCTTTTT | TAGATGATGC | CTGTCAACCT | |
| Patr | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | ----- | -T----- | ----- | A----- | ----- | -T----- | ----- | T----- | -A----- | -C----- | -N----- | -CA----- | T----- |
| Mamu | ----- | -T----- | ----- | A----- | ----- | -T----- | ----- | T----- | -A----- | -C----- | -N----- | -CA----- | T----- |
| Mafa | ----- | -T----- | ----- | A----- | ----- | -T----- | ----- | T----- | -A----- | -C----- | -N----- | -CA----- | T----- |
| Aotr | ----- | ----- | -T----- | T----- | ----- | ----- | ----- | -G----- | -G----- | -A----- | -GC----- | -G----- | ----- |
| Ho sa | TCCACCTGCC | CCATTACCTT | ATGAAAGCAC | AGCAGAGACA | GAAGGAGAGC | CATATCATTC | CCTCCATTGT | TTTCAACTCT | CCCTTCTCTC | CCCCCAGGTA | CCCATGAACC | AAGTTCGTCC | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | -N----- | ----- | -G-GG----- | -T----- | ----- | -C----- | -T----- | ----- | -C----- | -C----- | -T----- | -A----- | -C----- |
| Mamu | -N----- | -T----- | -G-GG----- | -T----- | ----- | -G----- | -C----- | -T----- | -C----- | -C----- | -T----- | -A----- | -C----- |
| Mafa | ----- | -T----- | -G-GG----- | -T----- | ----- | -G----- | -C----- | -T----- | -C----- | -C----- | -T----- | -A----- | -C----- |
| Aotr | ----- | -* | -G-GG----- | -T----- | ----- | -C----- | -T----- | -TG----- | -C-C----- | -C----- | -* | -T----- | -C----- |
| Ho sa | CTTCTCTTC | AGCAGAAAAA | ACAAAAGCCT | TTATAGATAT | CAACTTAGGC | AGATCTATCC | CTTGTGAGTC | ACCAACTCCA | TCTTCCCTGT | CTTTATTAGG | CTCAGCCAGG | CCCACGCTGC | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | -T----- | G----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mamu | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mafa | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Aotr | -G----- | ----- | -T----- | ----- | -C----- | ----- | ----- | -G----- | -T----- | -A-A----- | ----- | -T----- | ----- |
| Ho sa | TACCCATTTA | TGAAGTTGCC | TGGTTTGTCT | GAGCTGATAA | CCGTAAGGGA | ACTGGGATGC | TGGGATGCC* | *****ATC | A*GGGCTTAC | TGGAGATTGG | GATCATCAGC | TACTGTAGAT | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | -G----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mamu | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mafa | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Aotr | -A----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Ho sa | GATCAGCATG | AGGACTACCC | CCAGTAAAGG | GGTCACAGTA | GCAGGTAGAG | GAATGGCTCT | TAAGGGCCGT | ATTAACACAT | TTACAGCATG | TCACCTGTTG | GAGGAGATGG | AGCTCAAAT | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mamu | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mafa | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Aotr | ----- | ----- | -G----- | A----- | ----- | ----- | ----- | AGTT----- | -G----- | -AG----- | ----- | A----- | ----- |
| Ho sa | ACATAAGGTG | AGTTGGCCAA | GAAGCAGCTC | CGGCTTGTAG | TTGAGAGGAA | TGTATTTCATT | TCTGTGGCCT | TCAATACGGA | ATGGAGGTTA | TTCTTAGAGC | TTAGATTTTT | CGAGGGCAAG | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | -A----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | -G----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mamu | -G----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mafa | -G----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Aotr | ----- | -A----- | -A-TG----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Ho sa | ACAGGGCAGG | GAGTCATGGA | ATCCAGAT*G | GAAGCAAAGG | CACACCATCC | TCTTCACTTG | GACTTCAGAG | GAAATAGGAT | GACAGAAAAC | ACGGGCGGTG | ATGTAGTTTT | TGTACAGAA | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mamu | ----- | -A----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mafa | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Aotr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Ho sa | GTGTCTATGT | TTAATGACCA | GGATCCTCTG | TGCTAGGTAT | CAGCAACAAG | ACACTGTTT* | ****ATGACC | TCAGCCACTC | TTGACTTTCT | ACTTGAAAC | | | |
| Patr | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Gogo | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Popy | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Hyla | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Paha | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mamu | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Mafa | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |
| Aotr | ----- | -C----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |

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