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Positive selection along the evolution of primate mitogenomes

Albert N. Menezes [a], Maria Carolina Viana [a], Carolina Furtado [a], Carlos G. Schrago [b], Héctor N. Seuánez [a,b,*]

[a] Genetics Division, Instituto Nacional de Câncer, Rio de Janeiro, Brazil
[b] Department of Genetics, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

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ABSTRACT

The mitochondrial genomes of four neotropical primates, *Aotus infulatus*, *Chiropotes israelita*, *Callimico goeldii* and *Callicebus lugens* were sequenced and annotated. Phylogenetic reconstructions with mitochondrial genes of other 66 primates showed a similar arrangement to a topology based on nuclear genes. Screening for positive selection identified 15 codons in 7 genes along 9 independent lineages, three with two or more genes and five in internal nodes, ruling out false positive estimates. Mitochondrial genes of the electron transport chain (ETC.) complexes evolved with high substitution rates. A study of nuclear ETC. genes might elucidate whether they co-evolved with their mitochondrial counterparts.

1. Introduction

The proposition that mitochondrial genes (mt-genes) have been subjected to non-neutral evolution has been gaining support in the last decades (Ballard and Kreitman, 1995; Dowling et al., 2008; Nachman et al., 1996; Singh and Hale, 1990). This was the case of the model experiment with six generations of modified mice carrying proofreading-deficient mitochondrial DNA (mtDNA) polymerase, in which most non-synonymous mutations in the mitochondrial genome were rapidly eliminated, indicating a strong purifying selection (Stewart et al., 2008). Moreover, positive selection, or the rapid fixation of advantageous mutations, in mtDNA has been reported for some mammalian lineages (Foote et al., 2011; Hassanin et al., 2009; Mishmar et al., 2003; Shen et al., 2010), a finding that, though rare to present, might eventually prove to be more frequent than previously assumed.

On the other hand, some studies indicating positive mtDNA selection have been questioned (Ingman and Gyllensten, 2007; Mishmar et al., 2003; Sun et al., 2007), mostly because sampling problems or low genetic distances between the taxa under study lead to false positive estimates. Similarly, the proposed diversity of mitochondrial haplotypes of some human populations presumably resulting from positive selection related to climate (Mishmar et al., 2003) has not been confirmed and was subsequently ruled out by large scale sequencing of complete human mitochondrial genomes in other populations (Gunnarsdottir et al., 2011; Schonberg et al., 2011).

Positive selection has been previously indicated for genes of the electron transport chain complexes, including mitochondrial subunits in primates (Hughes and Friedman, 2008; Wright, 1990) but this was not confirmed to present by site-by-site, codon screening (Zhao et al., 2012). In view of the scarcity of evidence in favor of non-neutral evolution along evolutionary mammalian lineages, we have analyzed mtDNA genomes of 70 primate species. We herein report strong evidence of positive selection in at least four mitochondrial genes and nine evolutionary lineages.

2. Materials and methods

2.1. Preparation of biological samples and libraries

Peripheral blood samples were obtained from one captive *Callimico goeldii* kept in the Centro de Primatologia do Rio de Janeiro (CPRJ-INEA). DNA sample of *Aotus infulatus* was provided by Dr. Artur Silva from the Genetics Department of Universidade Federal do Pará, Belém, Brazil. Liver tissue from one *Chiropotes israelita* and one *Callicebus lugens* captured in the field were provided by Dr. Cibele R. Bonvicino.

Blood samples used in this study were part of the blood samples regularly collected for checkups and control of captive animals at CPRJ-INEA. Field and sample collections were carried out following the national guidelines and provisions of IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, Brazil; permanent license number 11375-1). The granting of this license by IBAMA followed approval by its Ethics Committee.

DNA from liver tissue was extracted with phenol chloroform (Sambrook et al., 1989) while DNA from fresh blood was extracted with QIAamp® DNA Mini and Blood Mini Kit (QIAGEN®). Sample

* Corresponding author at: Genetics Division, Instituto Nacional de Câncer, Rua André Cavalcanti, 37, 4th floor, 20231-050 Rio de Janeiro, R.J., Brazil. Tel.: +55 21 3207 6582; fax: +55 21 3207 6553.
E-mail addresses: albertmenezes@me.com (A.N. Menezes), mcarolviana@gmail.com (M.C. Viana), cfurtado@inca.gov.br (C. Furtado), guerra@biologia.ufrj.br (C.G. Schrago), hseuanez@inca.gov.br (H.N. Seuánez).

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quality was checked by both electrophoresis in agarose 1% gels and NanoDrop® 1.000 Spectrophotometer (Thermo Scientific) and quantified using a Qubit® 2.0 Fluorometer (Life Technologies™). Library preparation followed the Illumina Sequencing Workflow protocols (Nextera Kit or Truseq DNA Sample Prep) for the HiSeq2000 platform. DNA containing 800–900 bp fragments (with Nextera kit) and 300–400 bp (with Truseq Kit), checked with an Agilent Bioanalyzer 2100 DNA with a high sensitivity DNA chip, was subsequently tested by qPCR using a Library Quantification Kit for library validation (KAPA — KK4824). DNA cluster generation was prepared for 3 lanes of a PE Flowcell v.3 for *C. lugens*, 4 lanes for *C. goeldii* and one lane for *C. israelita* and *A. infulatus* following the manufacturer's protocol. A 97×96 pairend run was carried out in an Illumina HiSeq2000 platform for *C. lugens* (with a minimum of 83.6% registered base calls with Q30 quality score) and a 99×93 pairend run for *C. goeldii*, *C. israelita* and *A. infulatus* (with a minimum of 87.5% registered base calls with Q30 quality score).

2.2. Data analyses

Output data was converted to Fastq files using CASAVA v1.8.2 software (Illumina®) and contigs were obtained with De Novo Assembly analysis with CLC Genomics Workbench software (CLC Bio). Contigs over 15 kb and below 18 kb were run against non-human sequences on Blast (http://blast.ncbi.nlm.nih.gov) to check for mitochondrial sequence hits. Genes were mapped using the Mitos website (http://mitos.bioinf.uni-leipzig.de/help.py) and, subsequently, checked manually with MEGA 5.1 (Tamura et al., 2011). Sequence data from other primate species were obtained from Genbank (Supplemental Table 1) and datasets containing alignments for each gene were manually constructed with MEGA 5.1 (Tamura et al., 2011). A dataset (Dat-Con) containing concatenated mt-genes was also created and the best model of evolution was inferred with ModelGenerator v. 0.85 (Keane et al., 2006). Phylogenetic reconstructions with Dat-Con were performed with PHYML 3.0 (Guindon et al., 2010) for maximum likelihood (ML) and with MrBayes 3.2.1 (Ronquist and Huelsenbeck, 2003) for Bayesian analysis (BA), with the general time reversible model (GTR) with gamma distributed rate heterogeneity with invariable sites (I+G). In MrBayes, the Markov chain Monte Carlo (MCMC) algorithm was implemented with two independent runs with four chains each. The cold chains were sampled every 100th generation until 20,000 trees were obtained. A burn-in of 2000 was used.

Analysis of codon usage was carried out by estimating: (i) The improved effective number of codons index (ENC) providing an estimate of the departure of codon usage from usage of synonymous codons. An ENC estimate of 20 corresponds to the highest codon bias resulting from usage of one codon per synonymous codon family while an estimate of 61 indicates that all codons of all synonymous codon families are equally used (Sun et al., 2013; Wright, 1990); (ii) The improved codon adaptation index (CAI2) showing the relative adaptiveness of codon usage (Sharp and Li, 1987; Xia, 2007) by comparing codon usage of a given sequence with the relative synonymous codon usage estimates of very highly expressed genes of a reference species, in this case, *Homo sapiens*. The higher the similarity between the pattern of codon usage of a given sequence and the reference pattern, the higher CAI2 estimates will result; and (iii) The codon bias index (CBI), measuring the frequency with which any coding region uses a subset of preferred codons (Bennetzen and Hall, 1982). ENC and CAI2 were calculated with DAMBE (Xia and Xie, 2001) while CBI and GC content at second (GC_2), third (GC_3) and all positions (CG) were calculated with DnaSP 5.10 (Librado and Rozas, 2009). The association between codon usage and phylogeny was tested with the Bayesian tip-significant test (Parker et al., 2008) implemented in BaTS software (http://evolve.zoo.ox.ac.uk/evolve/BaTS.html). This analysis tested against the null hypothesis postulating that codon usage estimates were randomly distributed among the tips of the phylogeny. If higher (or lower) values tended to be associated with monophyletic groups at a rate greater than pure chance, the null hypothesis was rejected at significance level equal to 0.05.

Analysis of differential selection at codon sites was performed separately for each gene with the ML topology obtained with Dat-Con, using three algorithms available in the DataMonkey server (Delport et al., 2010), namely, SLAC, REL and FEL (Kosakovsky Pond and Frost, 2005). SLAC was used for inferring positive selection based on an ancestral state reconstruction of codon sites (Kosakovsky Pond and Frost, 2005). REL was used as a likelihood-based test for estimating the distribution of dN/dS ratios and subsequently assigning each codon site to a previously estimated dN/dS class, and empirical Bayes analysis for every codon site (Kosakovsky Pond and Frost, 2005). Finally, FEL is also likelihood-based for obtaining branch lengths and substitution rates, but the distribution of dN/dS ratios is not estimated and a likelihood ratio test is used site by site (Kosakovsky Pond and Frost, 2005). Simulation studies (Kosakovsky Pond and Frost, 2005) reported SLAC as the most conservative test, with REL and FEL showing a higher sensitivity for inferring positively selected codons. The codon model for each gene was also estimated with the DataMonkey server. Branch Site REL (Kosakovsky Pond et al., 2011) analysis was also run in DataMonkey to estimate branch-specific episodes of positive selection. A dataset containing only one species per genus was composed for verifying the robustness of findings and avoiding small tree branches that would increase the rate of false positive estimates.

3. Results and discussion

The mitochondrial genome of the species sequenced in our laboratory ranged from 16,523 bp to 16,689 bp (Supplemental Table 1). On average, coverage of mitochondrial genomes was 3,307× for *C. lugens*, 269× for *C. goeldii*, 777× for *A. infulatus* and 264× for *C. israelita*. Sequence and annotation data were deposited in Genbank with accession numbers KC592390–KC592393.

Bayesian and ML analyses produced an identical topology (Fig. 1). The topology herein described differed from a previous one based on nuclear genes (Perelman et al., 2011) although most differences occurred at low supported nodes or at nodes preceded by short branches. Our topology showed one incongruent grouping of taxa belonging to two different families, as was the case of *Perodicticus* (a member of the Lorisidae) grouping with the branch leading to *Galago* and *Otolemur* (members of the Galagidae) rather than with other Lorisidae (*Loris* and *Nycticebus*). Similarly, *Lepilemur* (family Lepilemuridae) was more basal with respect to *Propithecus* (family Indriidae). Within the neotropical clade, *Aotus* grouped with *Saimiri*/*Cebus* rather than with the callitrichids and, in the Hylobatidae, *Hylobates* grouped with *Symphalangus* rather than with *Nomascus*. In the Cercopithecidae, *Trachypithecus* grouped with *Presbytis* rather than with *Semnopithecus* and *Pygathrix* grouped with *Rhinopithecus* rather than *Nasalis*.

Differences between our mtDNA tree and the nuclear gene tree (Perelman et al., 2011) might have resulted from the different number of taxa included in analyses, changes in mutation rates, or selective forces. Interestingly, the most discordant arrangements were apparent in short branches, indicating that the time of speciation events had not been long enough for replicating a common phylogenetic signal for nuclear and mitochondrial genes.

Analysis of Dat-Con showed ENC estimates ranging from 43.06 to 48.16, with a mean equal to 45.47 (Supplementary Table 2), indicating departure of codon usage while estimates of CAI2 ranged from 0.66 to 0.71 with a mean equal to 0.68 (Supplementary Table 2) and CBI estimates ranged from 0.30 to 0.47 with a mean equal to 0.36. Codon usage indices differed between some clades (Fig. 1) and the Bayesian tip-significant test showed a significant association between phylogeny and codon usage ($p < 0.001$), indicating that the phylogenetic affinities

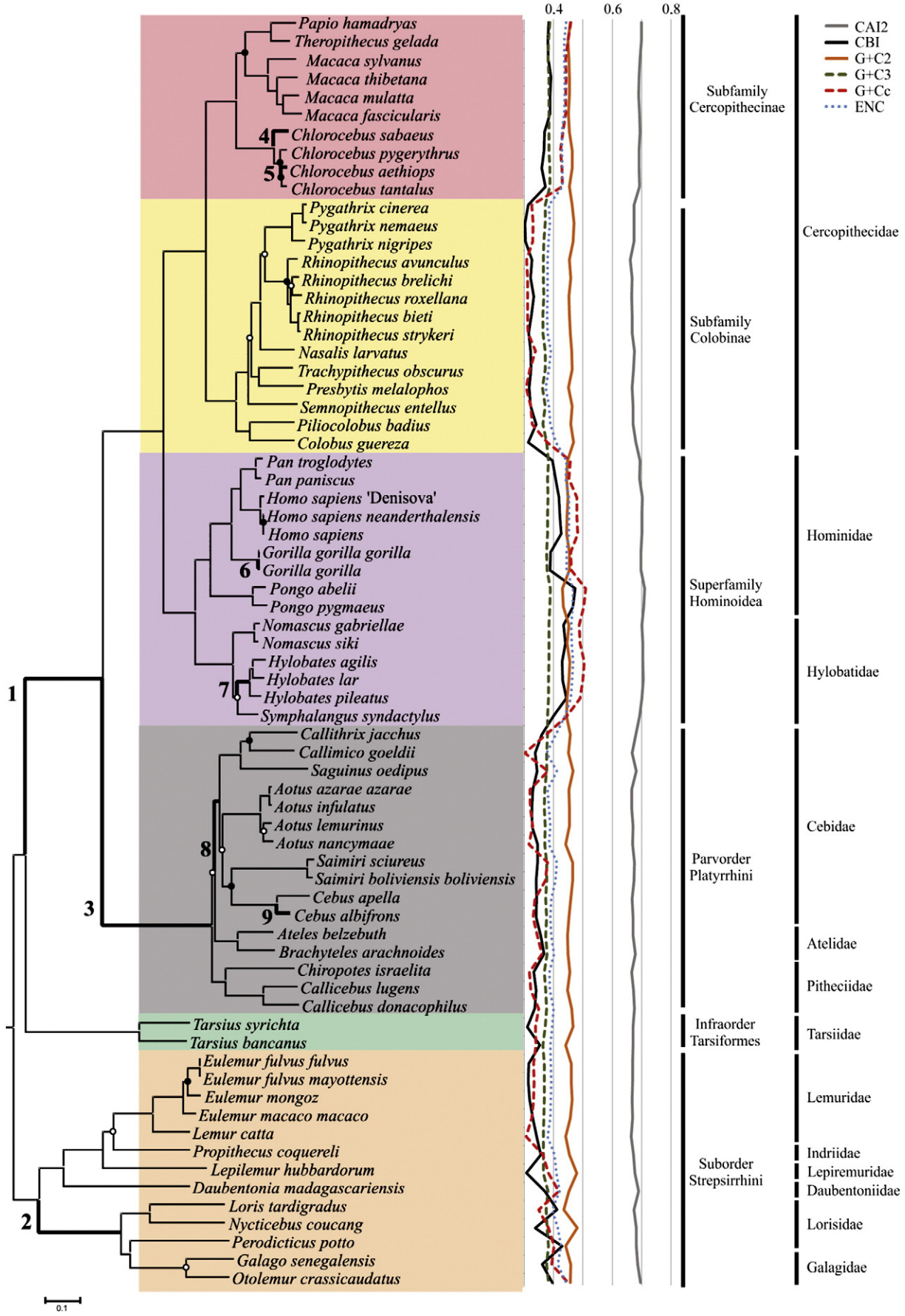
0.4
0.6
0.8
CAI2
CBI
G+C2
G+C3
G+Cc
ENC
Papio hamadryas
Theropithecus gelada
Macaca sylvanus
Macaca thibetana
Macaca mulatta
Macaca fascicularis
Chlorocebus sabaeus
Chlorocebus pygerythrus
Chlorocebus aethiops
Chlorocebus tantalus
Pygathrix cinerea
Pygathrix nemaeus
Pygathrix nigripes
Rhinopithecus avunculus
Rhinopithecus brelichi
Rhinopithecus roxellana
Rhinopithecus bieti
Rhinopithecus strykeri
Nasalis larvatus
Trachypithecus obscurus
Presbytis melalophos
Semnopithecus entellus
Piliocolobus badius
Colobus guereza
Pan troglodytes
Pan paniscus
Homo sapiens 'Denisova'
Homo sapiens neanderthalensis
Homo sapiens
Gorilla gorilla gorilla
Gorilla gorilla
Pongo abelii
Pongo pygmaeus
Nomascus gabriellae
Nomascus siki
Hylobates agilis
Hylobates lar
Hylobates pileatus
Symphalangus syndactylus
Callithrix jacchus
Callimico goeldii
Saguinus oedipus
Aotus azarae azarae
Aotus infulatus
Aotus lemurinus
Aotus nancymaae
Saimiri sciureus
Saimiri boliviensis boliviensis
Cebus apella
Cebus albifrons
Ateles belzebuth
Brachyteles arachnoides
Chiropotes israelita
Callicebus lugens
Callicebus donacophilus
Tarsius syrichta
Tarsius bancanus
Eulemur fulvus fulvus
Eulemur fulvus mayottensis
Eulemur mongoz
Eulemur macaco macaco
Lemur catta
Propithecus coquereli
Lepilemur hubbardorum
Daubentonia madagascariensis
Loris tardigradus
Nycticebus coucang
Perodicticus potto
Galago senegalensis
Otolemur crassicaudatus
1
2
3
4
5
6
7
8
9
0.1
Subfamily Cercopithecinae
Subfamily Colobinae
Cercopithecidae
Superfamily Hominoidea
Hominidae
Hylobatidae
Parvorder Platyrrhini
Cebidae
Atelidae
Pitheciidae
Infraorder Tarsiformes
Tarsiidae
Suborder Strepsirrhini
Lemuridae
Indriidae
Lepiremuridae
Daubentoniidae
Lorisidae
Galagidae

Table 1
List of codons under positive selection inferred by each algorithm.

Gene	SLAC analysis		FEL analysis		REL analysis		Total codon number under positive selection	Number and type[a] of amino acids per codon site
	Codon number under positive selection	dN–dS (p-value)	Codon number under positive selection	dN/dS (p-value)	Codon number under positive selection	Normalized dN/dS (posterior probability)		
ATP6					2	0.046 (0.660)	2	3 (D, N, T)
					7	0.065 (0.946)	7	3 (A, S, T)
ATP8	63	5.460 (0.006)	63	Infinite (0.001)	50	0.129 (0.972)	50	8 (I, K, L, M, N, S, T, V)
					60	0.134 (0.999)	60	2 (C, Y)
					61	0.124 (0.985)	61	2 (L, S)
					63	0.135 (1)	63	5 (H, L, P, S, Y)
CYB	11	4.966 (0.012)	11	Infinite (0.051)			11	6 (A, I, K, L, M, T)
			238	2.113 (0.074)			238	10 (A, C, F, I, L, M, P, S, T, V)
ND1			1	4.746 (0.079)	1	−0.012 (0.273)	1	4 (L, M, T, V)
					313	−0.370 (0.102)	313	7 (A, C, G, N, S, T, Y)
ND3	4	6.828 (0.034)					4	8 (A, I, L, M, P, S, T, V)
ND4L					1	0.174 (0.976)	1	2 (M, V)
ND6	4	4.828 (0.048)					4	6 (A, I, L, M, T, V)
	33	3.522 (0.023)					33	3 (I, T, V)
	46	3.068 (0.078)					46	4 (C, F, L, Y)

[a] A = Alanine; C = Cysteine; D = Aspartic Acid; F = Phenylalanine; G = Glycine; H = Histidine; I = Isoleucine; K = Lysine; L = Leucine; M = Methionine; N = Asparagine; P = Proline; S = Serine; T = Threonine; V = Valine; Y = Tyrosine.

of species were capable of predicting the codon usage statistics of tree terminals.

The subfamily Cercopithecinae and the superfamily Hominoidea showed the highest codon usage indices (Fig. 1). High estimates have been proposed to be associated to adverse selection considering that a tight control of codon usage would be a side effect of strong purifying selection (Resch et al., 2007). A positive correlation between codon usage and tRNA content has been reported for prokaryotic and eukaryotic organisms (Ikemura, 1985). It is thus possible that mutations in tRNA mt genes during primate radiation lead to codon usage signatures in different taxa due to their susceptibility to point mutations, in view that all RNA components necessary for mitochondrial translation are supplied in mitochondria (Suzuki et al., 2011).

Positive selection was inferred for 15 different codon sites in seven genes (*mt-ATP6*, *mt-ATP8*, *mt-CYB*, *mt-ND1*, *mt-ND3*, *mt-ND4L* and *mt-ND6*) by SLAC, REL and FEL algorithms (Table 1). Six codon sites in four genes (*mt-ATP8*, *mt-CYB*, *mt-ND3* and *mt-ND6*) showed to be under positive selection by SLAC, the most stringent algorithm, and the highest dN/dS ratio (6.828) was observed for codon 4 of *mt-ND3*. Four codon sites in three genes (*mt-ATP8*, *mt-CYB* and *mt-ND1*) showed to be under positive selection by FEL and nine codon sites in four genes (*mt-ATP6*, *mt-ATP8*, *mt-ND1* and *mt-ND4L*) by REL, a method based on more assumptions than the other two algorithms and susceptible to producing higher false positive rates (Kosakovsky Pond and Frost, 2005). Except for *mt-ATP8* (codon No 63), the inference of positively selected codon sites was not consistent across the three analyses, a finding that was expected because the model of codon substitution implemented in DataMonkey differs between algorithms. Although the occurrence of purifying selection has been reported to be widespread in the primate mitochondrial genome (Zhao et al., 2012), probably because the mitochondrion is responsible for most of the energy required by eukaryotic cells (Popadin et al., 2013), even the most conservative of the methods herein used, SLAC, was capable of estimating signals of positive selection in four mitochondrial genes, providing strong evidence that the primate mitochondria have undergone discontinuous episodes of diversifying evolution.

Although the three analyses (SLAC, FEL and REL) coincided in identifying codon 63 of *mt-ATP8* to be under positive selection, the Branch REL method did not show this gene as target of positive selection. This discrepancy occurs because Branch REL identifies lineages subject to episodic diversifying selection while the three other analyses search for individual codon sites under selection. On the other hand, positive selection of *mt-CYB* was found to occur in three independent evolutionary branches (Fig. 1; Table 2), all of which strongly-supported internal branches, ruling out the possibility of artifactual constructs resulting from short branching (Hughes and Friedman, 2008). The branch leading to the Lorisiformes and the one leading to the Platyrrhini also showed positive selection for other genes (*mt-ND3* or *mt-ND4*), indicating that these lineages have gone through adaptive molecular evolution. Although *Hylobates*, Platyrrhine and Lorisiform species inhabit tropical regions in Southeast Asia (Geissmann, 1995), South and Central America (Horovitz and Meyer, 1995) and Africa and South/Southeast Asia (Groves, 2005) respectively, positive selection cannot be attributed to climate in view that other primate species, also occurring in tropical environments, did not show evidence of positive mtDNA selection. Moreover, as drastic climate changes have occurred during primate radiation (Ivany et al., 2000), the association of mtDNA selection and climate cannot be firmly established.

It has also been postulated that the energy requirements of a larger brain could have resulted from the adaptive evolution of genes involved in aerobic energy metabolism (Hughes and Friedman, 2008; Shen et al., 2010). This, however, did not apparently occur in the lineage leading to the Hominidae, a group with the largest brain size among primates (Schillaci, 2006), because only the branch leading to *Gorilla* was under positive selection. Moreover, although platyrrhines have the smallest brains among simiformes (Schillaci, 2006), the evolutionary branch leading to this this group showed positive selection for two genes (*mt-CYB* and *mt-ND4*).

Our results indicated positive selection for *ATP8* and *ATP6*, two components of complex V of the electron transport chain (ETC.). As the nuclear subunit *ATP5J* of this complex was reported to be under positive selection in genomic comparisons of *Macaca fascicularis* and *H. sapiens* (Osada et al., 2002), our findings indicated that *mt-ATP6* and *mt-ATP8* might have coevolved with this nuclear ATP complex V subunit.

Fig. 1. Maximum likelihood topology of primate mt-genes. Bold branches (numbered) represent lineages under positive selection. All nodes were strongly supported (LRT = 1) except for nodes shown in black circles (LRT between 1 and 0.9) and white circles (below 0.9). Codon Usage statistics (see Supplemental Table 2) relationship to mt DNA phylogeny. Statistics include effective number of codons (ENC), codon adaptation index (CAI2), codon bias index (CBI) and GC content at second (G+C2), third (G+C3) and all positions (G+Cc). ENC values were divided by 100 to fit the graph scale.

Table 2
Lineages under positive selection indicated by Branch REL analysis, listing branch shown in Fig. 1, gene, ratio of synonymous to non-synonymous substitutions (ω) and p-value.

Branch	Gene	ω	p
1	*ND5*	10,000.00	<0.0001
2	*CYB*	51.69	0.019
	ND3	3333.11	0.002
3	*CYB*	9999.97	0.020
	ND4	16.01	0.010
4	*ND1*	5642.92	<0.001
	ND3	4146.43	<0.001
	ATP6	4942.05	<0.001
5	*ND5*	1000.00	<0.001
6	*ND4*	10,000.00	0.012
7	*CYB*	10,000.00	0.015
8	*CO2*	10,000.00	0.002
9	*CO2*	248.18	0.034

Coevolution of the nuclear and mitochondrial subunits of Cytochrome C Oxidase (ETC. complex IV) has been previously reported in primates (Grossman et al., 2004), with two mitochondrial and seven nuclear Cytochrome C Oxidase subunits showing accelerated evolution. Our findings showed that *CO2* had been under positive selection in the lineages leading to the *Cebidae* and *Cebus* (Fig. 1). Conversely, *CO1* did not appear to have been subjected to positive selection although the previously reported high rate of non-synonymous substitutions of this gene suggested that positive selection might have been operative (Grossman et al., 2004).

4. Conclusion

Finally, our analyses showed that mt-ND subunits, except for *mt-ND2*, have been subjected to positive selection, contrary to the single nuclear subunit *NDUFC2* that has been reported with accelerated rate of non-synonymous substitutions in primates (Osada et al., 2002). Analyses of ETC. nuclear subunits in a large number of primate species will be illuminating for unraveling their co-evolution with their mitochondrial counterparts.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mito.2013.06.001.

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