Evidence from Analyses of Intergenic Regions for Strand-specific Directional Mutation Pressure in Metazoan Mitochondrial DNA

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The dynamic forces that determine the evolution of metazoan mitochondrial DNA (mtDNA) are analyzed. Using 24 completely sequenced genomes, we show that metazoan mtDNA is extremely economical with respect to the number of redundant nucleotides and that the intergenic DNA, that is, the nucleotides not included in genes, control regions, putative light-strand replication origins, and putative promoter-like motifs, varies considerably with respect to the A+C content—the A+C content of the intergenic mtDNA of *Ascaris suum* is equal to 0.169 ± 0.040 (SD), whereas that of *Homo sapiens* is equal to 0.804 ± 0.053. In agreement with previous reports that focus on the nucleotide composition of various informative parts of the metazoan mtDNA, we conclude that a significant strand-specific directional mutation pressure ($P < 0.05$) is exerted on the mtDNA of some species. Strand-specific directional mutation pressure in metazoan mtDNA may invalidate the most commonly used methods for estimating nucleotide substitution rates because these assume an unbiased nucleotide composition.

Introduction

The metazoan mitochondrial genome, for which complete DNA sequences are known already from at least 24 species, has been used extensively in studies of phylogeny and evolution (e.g., DeSalle et al. 1987; Miyamoto and Boyle 1989; Meyer and Wilson 1990; Irwin et al. 1991; Cameron et al. 1992; Derr et al. 1992*a*, 1992*b*; Liu and Beckenbach 1992; Martin et al. 1992; Willis et al. 1992; Helm-Bychowski and Cracraft 1993; Kornegay et al. 1993; Kusmierski et al. 1993; Ma et al. 1993; Martin and Palumbi 1993; Jermiin and Crozier 1994). Nevertheless, the dynamic forces mediating the evolution of metazoan mtDNA, and how these forces interact, are still poorly understood. Neglect of these forces would seem likely to bias estimators of substitution rates and to interfere with phylogenetic analysis.

Recent studies of the nucleotide composition of various parts of the metazoan mtDNA (e.g., the control region, the silent and replacement sites of protein-coding genes, and the single-stranded parts of tRNA and rRNA genes) have shown, however, that some of the genomes have biased nucleotide compositions (Jukes and Bhushan 1986; Andersson and Kurland 1991; Asakawa et al. 1991, 1995; Osawa et al. 1992; Jermiin et al. 1994). Since this bias in some cases can be due only to a biased mutational pattern, it has been concluded that directional mutation pressure (first defined by Sueoka [1962] in terms of A+C and G+C biases) is exerted on some metazoan mitochondrial genomes (Jukes and Bhushan 1986; Andersson and Kurland 1991; Asakawa et al. 1991, 1995; Osawa et al. 1992; Jermiin et al. 1994).

The reports of directional mutation pressure on metazoan mtDNA differ substantially with respect to the direction of the mutation pressure and its amplitude. Many chordates, arthropods, nematodes, and echinoderms are subject to a symmetrical directional mutation pressure, and in almost all cases it is an A+T pressure (Jukes and Bhushan 1986; Jermiin et al. 1994). The mtDNA of arthropods and nematodes is subject to a stronger A+T pressure than that of chordates and echinoderms (Jermiin et al. 1994). Many chordates, arthropods, and echinoderms are also reported as being subject to a strand-specific, or asymmetrical, directional mutation pressure (Andersson and Kurland 1991; Asakawa et al. 1991, 1995; Osawa et al. 1992). For example, the strand-specific nucleotide composition in 11 completely sequenced metazoan mitochondrial genomes is biased toward A and C in third-codon positions on the L-strand (Asakawa et al. 1991). Because the reversed pattern occurs on the H-strand and since Asakawa et al. (1991) argued that this pattern could not be due to translational

Key words: Metazoa, mitochondrial DNA, nucleotide composition, strand-specific directional mutation pressure, A+C pressure, G+T pressure.

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Mol. Biol. Evol. 12(4):558–563. 1995.

0737-4038/95/1204-0004$02.00

selection (selection imposed by the translational mechanism), Osawa et al. (1992) and Asakawa et al. (1995) have suggested that the nucleotide bias may be due to strand-specific A+C or G+T pressure.

Although these studies seem convincing, they should be regarded with caution, because the regions studied are known to be subject to some degree to selection. Jukes and Bhushan (1986) and Jermiin et al. (1994) rely on the assumption of a selectively neutral control region; this is not true since all available data are consistent with the conclusion that motifs used for replicational and transcriptional initiation reside within the control region (Goddard and Wolstenholme 1978, 1980; Clayton 1992). On the other hand, the conclusions of Andersson and Kurland (1991) and of Asakawa et al. (1991, 1995) are based on empirical observations that have not been subjected to statistical assessment.

We here address one of the problems outlined above with respect to the evolutionary dynamics of mtDNA. Using as data all the available completely sequenced metazoan mtDNAs, we address the following question: Is strand-specific directional mutation pressure exerted on the mitochondrial genomes of various metazoan species, as reported by Andersson and Kurland (1991), Asakawa et al. (1991, 1995), and Osawa et al. (1992).

Material and Methods

Intergenic DNA

The complete mtDNA sequences of *Homo sapiens* (Anderson et al. 1981), *Mus musculus* (Bibb et al. 1981), *Bos taurus* (Anderson et al. 1982), *Xenopus laevis* (Roe et al. 1985), *Drosophila yakuba* (Clary and Wolstenholme 1985), *Strongylocentrotus purpuratus* (Jacobs et al. 1988), *Rattus norvegicus* (Gadaleta et al. 1989), *Paracentrotus lividus* (Cantatore et al. 1989), *Gallus gallus* (Desjardins and Morais 1990), *Balaenoptera physalus* (Árnason et al. 1991), *Ascaris suum, Caenorhabditis elegans* (Okimoto et al. 1992), *Phoca vitulina* (Árnason and Johnsson 1992), *Crossostoma lacustre* (Tzeng et al. 1992), *Anopheles gambiae* (Beard et al. 1993), *Apis mellifera* (Crozier and Crozier 1993), *Anopheles quadrimaculatus* (Mitchell et al. 1993), *Halichoerus grypus* (Árnason et al. 1993), *Balaenoptera musculus* (Árnason and Gullberg 1993), *Cyprinus carpio* (Chang et al. 1994), *Didelphis virginiana* (Janke et al. 1994), *Artemia franciscana* (Valverde et al. 1994), *Equus caballus* (Xu and Árnason 1994), and *Asterina pectinifera* (Asakawa et al. 1995) were obtained from the authors or from GenBank.

We extracted the intergenic DNA from each genome. This DNA is defined as those nucleotides that occur external to the selectively constrained components of the genomes, that is, not being included in the genes, the control region (i.e. the D-loop or A+T rich region), the putative light-strand replication origin, and the putative promoter-like motifs (these motifs are known only from the echinoderms; Jacobs et al. 1988; Cantatore et al. 1989; Asakawa et al. 1995). The intergenic DNA of *B. taurus,* for example, is therefore located at positions 3099–3100, 4195–4196, 5375, 5445, 5686, 7300–7304, 7373, 8058–8060, 8128, 12037, 14510–14513, and 15654–15656 (cf. these positions with figs. 1 and 2 in Anderson et al. 1982).

Since the intergenic DNA has no known function except perhaps for acting as spacers between the informative genomic components, it is very unlikely that the nucleotide composition of this DNA is subject to selection. Consequently, an analysis of the intergenic DNA provides the best opportunity for gaining an insight into which mutational forces determine the evolution of metazoan mtDNA.

Data Analysis

The asymmetrical directional mutation pressure exerted on a mitochondrial genome is defined as the A+C content of its intergenic DNA. Deviation from 0.5 (the unbiased state) is evaluated using a standard chi-square test.

The analyses below contain multiple comparisons, and statistical significance may therefore be obtained by chance (Sokal and Rohlf 1981, pp. 242–262). We use the sequential Bonferroni technique (Rice 1989) to control for these groupwide Type I errors.

Results and Discussion

Size Variation of the Intergenic DNA

The number of intergenic sites varies substantially among the 24 metazoan species (table 1). The mitochondrial genome with the largest number of intergenic sites is that of *Apis mellifera* (620 bp), whereas those with the smallest number of intergenic sites are from *Balaenoptera physalus, B. musculus, Phoca vitulina, Halichoerus grypus,* and *Bos taurus* (25 bp each).

Since metazoan mtDNA, with one known exception (Wolstenholme 1992), lacks introns (Crozier 1990; Wolstenholme 1992), the above result supports the notion that metazoan mtDNA is extremely economical with respect to the number of redundant sites (e.g., Attardi 1985; Andersson and Kurland 1991).

Asymmetrical Directional Mutation Pressure

The intergenic DNA is significantly A+C or G+T biased in only four species, and among these, three species are A+C biased (*Homo sapiens, Gallus gallus,* and *Asterina pectinifera*) and one species is G+T biased (*Ascaris suum*) (table 1). Several species have the same degree of bias as these four species (e.g., *B. taurus* and *B. musculus*), but their bias is not significant because the sample sizes are too small.

Table 1
Analysis of the Asymmetrical Directional Mutation Pressure Exerted on the Intergenic DNA of 24 Completely Sequenced Metazoan Mitochondrial Genomes

Latin Name	Common Name	GenBank	*N*	A+C content ± SD
Homo sapiens	Human	J01415	56	0.804 ± 0.053***
Mus musculus	Mouse	J01420	32	0.406 ± 0.087
Rattus norvegicus	Rat	X14848	28	0.607 ± 0.092
Balaenoptera physalus	Fin whale	X61145	25	0.680 ± 0.093
Balaenoptera musculus	Blue whale	X72204	25	0.800 ± 0.080
Phoca vitulina	Harbor seal	X63726	25	0.560 ± 0.096
Halichoerus grypus	Grey seal	X72004	25	0.600 ± 0.098
Bos taurus	Cow	J01394	25	0.720 ± 0.090
Equus caballus	Horse	X79547	40	0.550 ± 0.079
Didelphis virginiana	Opossum	Z29573	43	0.558 ± 0.076
Gallus gallus	Chicken	X52392	45	0.756 ± 0.064*
Xenopus laevis	Toad	M10217	50	0.520 ± 0.071
Cyprinus carpio	Carp	X61010	39	0.692 ± 0.074
Crossostoma lacustre[a]	Loach	M91245	40	0.625 ± 0.077
Ascaris suum[a]	Pig gut nematode	X54253	89	0.169 ± 0.040***
Caenorhabditis elegans[a]	Soil nematode	X54252	38	0.500 ± 0.018
Apis mellifera[a]	Honey bee	L06178	620	0.537 ± 0.020
Drosophila yakuba	Fruit fly	X03240	181	0.492 ± 0.037
Anopheles gambiae	Mosquito	L20934	43	0.605 + 0.075
Anopheles quadrimaculatus	Mosquito	L04272	57	0.596 ± 0.065
Artemia franciscana	Brine shrimp	X69067	153	0.510 ± 0.040
Asterina pectinifera	Starfish	D16387	172	0.640 ± 0.037**
Paracentrotus lividus	Sea urchin	J04815	78	0.474 ± 0.054
Strongylocentrotus purpuratus	Sea urchin	X12631	88	0.443 ± 0.053

NOTE.—The standard deviation is given as $\sqrt{(1 - M) \times M/N}$, where M is equal to the A+C content. The tests of asymmetrical directional mutation pressure (H_0: A+C content = 0.5; H_1: A+C content $\neq$ 0.5) were subjected to a sequential Bonferroni correction (*, significant at a groupwide 5% level; **, significant at a groupwide 1% level; ***, significant at a groupwide 0.1% level).

[a] A putative light-strand replication origin is not reported for *Crossostoma lacustre* (Tzeng et al. 1992). However, the noncoding mtDNA sequences between the transfer RNAs for cysteine and asparagine are considered putative light-strand replication origins in this study because those found in the carp, the toad, and the eutherian mammals are in the same relative position. Likewise, the long, noncoding mtDNA sequences between the cytochrome oxidase subunit I and II genes in *Apis mellifera* (Crozier et al. 1989) and between the NADH dehydrogenase subunit 4 and cytochrome oxidase subunit I genes in *Ascaris suum* and *Caenorhabditis elegans* may be putative origins of light-strand replication (Cornuet et al. 1991; Okimoto et al. 1992).

Since the intergenic DNA can be considered selectively neutral with respect to its nucleotide content, the result supports previous suggestions of strand-specific directional mutation pressure being exerted on the mitochondrial genome of some metazoan species (Andersson and Kurland 1991; Asakawa et al. 1991, 1995; Osawa et al. 1992). However, the result does not support the notion that echinoderms and vertebrates are unanimously subject to A+C pressure (Andersson and Kurland 1991; Asakawa et al. 1991), since several species are far from being significantly biased (e.g., *Mus musculus, Phoca vitulina, Didelphis virginiana, Xenopus lividus, Paracentrotus lividus,* and *Strongylocentrotus purpuratus*) and some are in fact slightly, although not significantly, G+T biased (e.g., *M. musculus, P. lividus,* and *S. purpuratus*).

The discrepancy between the present conclusion and those by Andersson and Kurland (1991), Asakawa et al. (1991, 1995), and Osawa et al. (1992) is most likely due to translational selection being exerted on the protein-coding mtDNA. Although Asakawa et al. (1991) argued that translational selection is unlikely in mtDNA, a recent statistical analysis of the nucleotide content of protein-coding mtDNA (Jermiin 1994) has shown that the G+C content at third-codon positions differs significantly among codon families in 23 of the 24 species analyzed. Since the G+C content is a strand-nonspecific measurement, the result can only be takes as evidence of translational selection (Jermiin 1994). This finding, and the additional discovery of heterogeneity among genes in their composition of codon families (Jermiin 1994), can easily explain the findings of Andersson and Kurland (1991) and Asakawa et al. (1991)—the genes on the H-strand must comprise a surplus of codon families that are translated by tRNAs with either T or G at the wobble position.

Further Implications on the Evolution of mtDNA

The result from our analysis is important because it partially supports conclusions by previous researchers (Andersson and Kurland 1991; Asakawa et al. 1991, 1995; Osawa et al. 1992) and because it implies that asymmetrical directional mutation pressure is responsible to some extent for the biased nucleotide composition that has been observed in the mtDNA of many species (see, e.g., Brown 1985; Clary and Wolstenholme 1985; Jukes and Bhushan 1986; Crozier et al. 1989; Crozier 1990; Irwin et al. 1991; Liu and Beckenbach 1992; Okimoto et al. 1992; Crozier and Crozier 1992, 1993; Beard et al. 1993; Mitchell et al. 1993).

The existence of asymmetrical directional mutation pressure is difficult to explain, but it is believed that the asymmetrical replication of mtDNA, which is reported to occur in many species (Clayton 1992), leaves the H-strand exposed as a single strand for much longer than the L-strand and prevents double-stranded DNA editing of the former (Asakawa et al. 1991; Osawa et al. 1992). This probably facilitates the directional mutation pressure. Alternatively, a defective mtDNA γ-polymerase may explain the directional mutation pressure. Misincorporation by this enzyme has been reported (Kunkel 1985), but whether or not it can systematically change the A+C content of mtDNAs over long periods of time is not known.

The fact that metazoan mtDNA appears to be subject to directional mutation pressure and that the directional mutation pressure seems to have created the biased nucleotide content reported here and elsewhere (e.g., Brown 1985; Clary and Wolstenholme 1985; Jukes and Bhushan 1986; Crozier et al. 1989; Crozier 1990; Irwin et al. 1991; Liu and Beckenbach 1992; Okimoto et al. 1992; Crozier and Crozier 1992, 1993; Beard et al. 1993; Mitchell et al. 1993) may imply that the one- (Jukes and Cantor 1969), two- (Kimura 1980), and three-parameter (Kimura 1981) models, which describe patterns of nucleotide substitution in terms of either one, two, or three mutation rates, are unsuitable for estimating mtDNA sequence divergence. These models assume that the nucleotide composition is unbiased and they are thus not strictly applicable to analyses of mtDNA. However, we stress that it is unknown whether or not biased base composition seriously biases estimates of DNA sequence divergence, but there is certainly reason to believe that the forces biasing base composition can invalidate methods of phylogenetic inference (e.g., Saccone et al. 1990; Lockhart et al. 1994).

Acknowledgments

This article constitutes a part of the research done by L.S.J. in partial fulfillment of a Ph.D. degree at La Trobe University. We thank Ú. Árnason, A. Janke, and K. Watanabe for providing DNA sequences (*Equus caballus, Didelphis virginiana,* and *Asterina pectinifera,* respectively) and manuscripts before they were published, and B. J. Crespi, J.-M. Cornuet, P. Pamilo, and T. H. Jukes for constructive comments on a previous version of the manuscript. This research was financially supported by the Danish Research Academy (L.S.J.), La Trobe University's Postgraduate Scholarship (L.S.J.), the Australian Government's Overseas Postgraduate Research Scholarship (L.S.J.), a CRA–La Trobe University Distinguished Visitor Fellowship (D.G.), the Australian Research Council (R.H.C.), La Trobe University (R.H.C.), and the Ian Potter Foundation (R.H.C.).

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RODNEY L. HONEYCUTT, reviewing editor

Received May 24, 1994

Accepted February 14, 1995