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Cloning and Characterization of the Platypus Mitochondrial Genome

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Abstract. The vertebrate mitochondrial genome is highly conserved in size and gene content. Among the chordates there appears to be one basic gene arrangement, but rearrangements in the mitochondrial gene order of the avian lineages have indicated that the mitochondrial genome may be more variable than once thought. Different gene orders in marsupials and eutherian mammals leave the ancestral mammalian order in some doubt. We have investigated the mitochondrial gene order in the platypus (*Ornithorhynchus anatinus*), a representative of the third major group of mammals, to determine which mitochondrial gene arrangement is ancestral in mammals. We have found that the platypus mtDNA conforms to the basic chordate gene arrangement, common to fish, amphibians, and eutherian mammals, indicating that this arrangement was the original mammalian arrangement, and that the unusual rearrangements observed in the avians and marsupials are probably lineage-specific.

Key words: Evolution — Monotreme — Platypus — mtDNA — tRNA

Introduction

The mitochondrial genome (mtDNA) of all higher organisms consists of a closed circular DNA molecule which encodes 13 subunits of the inner-membrane respiratory complexes and a complete set of transfer RNAs (tRNAs). It is highly conserved in size, being about 15,000–17,000 base pairs in most vertebrates examined. Gene content is also invariant, although gene order may differ within phyla (Cantatore and Saccone 1987).

Among the chordate mtDNAs examined so far there appears to be one basic gene arrangement shared by fish, amphibians, and eutherian ("placental") mammals (Anderson *et al.* 1981, 1982; Bibb *et al.* 1981; Gadaleta *et al.* 1989; Roe *et al.* 1985; Johansen *et al.* 1990). However, there are several exceptions to this general conservation—notably, the rearrangements of NADH dehydrogenase subunit 6 (ND6) and cytochrome b (Cytb) in birds (Desjardins and Morias 1990, 1991), gene duplications in amphibians (Yoneyama 1987) and reptiles (Moritz and Brown 1986, 1987), and tRNA gene rearrangements in the marsupials (Pääbo *et al.* 1991), which indicate that the vertebrate mitochondrial genome may be more variable than was previously thought. In particular, the different arrangements in eutherian and marsupial mammals have left the ancestral mammalian gene order in some doubt. Whereas eutherians have the basic chordate gene arrangement, the transfer RNA genes surrounding the light-strand origin of replication are arranged differently in the marsupials (Pääbo *et al.* 1991). This makes it particularly interesting to extend the examination of the vertebrate mitochondrial genome to a study of mitochondrial gene order in the third major group of mammals, the monotremes. The monotremes should allow us to test the hypothesis that the mtDNA of the ancestral mammal conformed to the basic chordate gene arrangement and to confirm that the variant arrangements observed in the marsupials and avians arose independently in those lineages.

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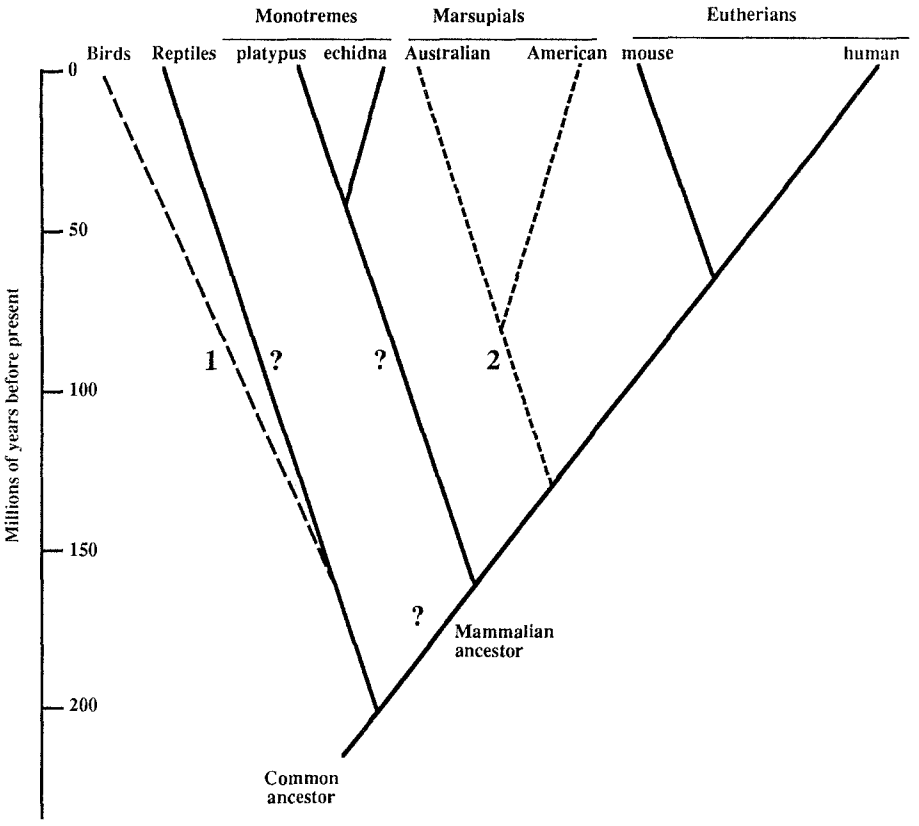

Fig. 1. Diagramatic representation of the phylogenetic relationships of the mammals. Mammalian phylogeny, showing the approximate divergence dates of the eutherian, marsupial, and monotreme mammals. Mitochondrial DNA gene rearrangements are indicated as follows: **1** ND6/Cytb rearrangement in the avian lineage. **2** Transfer RNA rearrangements around the light-strand origin of replication in the marsupial lineage. **?** Gene arrangement unknown in these lineages. Figure modified from Graves JAM and Watson JM (1991) *Chromosoma* 101:63–68.

Marsupials and eutherians represent two infraclasses of therian mammals which diverged ~130 mya (reviewed Hope *et al.* 1990). The egg-laying monotremes (subclass Prototheria) are an even more distantly related group, having diverged about 170 mya from the therian (marsupial-eutherian) line of descent (Kemp 1982) (Fig. 1). The platypus (*Ornithorhynchus anatinus*) is one of only three extant monotreme species.

To investigate the characteristics of the platypus mitochondrial genome, we isolated a clone representing most of the platypus mtDNA. This clone, as well as purified platypus mtDNA samples, was restriction mapped, and gene domains were localized to the resulting composite restriction map by Southern analysis with heterologous PCR probes, enabling us quickly to determine gene order in the platypus mitochondrial genome.

Materials and Methods

Animals. The platypus is a protected species, and obtaining tissue of any kind, particularly the fresh tissue optimal for mtDNA extractions, is extremely difficult. The tissues obtained for this study and their sources were as follows. A fresh heart from an adult male platypus was supplied by Dr. Ian McDonald, University of Melbourne, Australia while six platypus livers (three of each sex) stored in 70% ethanol were supplied by Dr. Paul Manger, University of Queensland, Australia.

Isolation of Mitochondria. Mitochondria were isolated from fresh heart muscle and platypus livers using an adaption of the method of Lansman *et al.* (1981). The heart was macerated and homogenized in 20 ml of MSB-Ca^{+2} buffer (0.21 M mannitol, 0.07 M sucrose, 3 mM $CaCl_2$ and 0.05 M Tris-HCl, pH 7.5). Nuclei and debris were removed from the homogenate by centrifugation at 700*g* for 5 min in a swinging bucket rotor. The supernatant was then layered over a step gradient consisting of 5 ml of 1.5 M sucrose and 10 ml of 1.0 M sucrose and centrifuged at 90,000*g* for 30 min. The mitochondrial band was recovered from the 1.0–1.5 M sucrose interface, recovered, diluted three times with MSB-EDTA (0.21 M mannitol, 0.07 M sucrose, 0.01 M EDTA, 0.05 M Tris-HCl, pH 7.5), and pelleted by centrifugation at 20,000*g* for 20 min.

Purification of mtDNA. The purification of mtDNA was accomplished using the method of Dowling et al. (1990). The DNA was extracted from the mitochondria by lysis with 50 ml of 20% SDS and purified in a 1.40 *g*/ml CsCl gradient containing 230 mg/ml propridium iodide (PI). The fraction containing the supercoiled mtDNA, approximately 2–6 mm below the main DNA band, was extracted three times with CsCl-saturated butanol to remove the propridium iodide and then dialyzed against T.E for 24 h. The purified mtDNA was stored at −20°C.

Cloning of the mtDNA. A size-selected library, enriched for mitochondrial DNA, was constructed in the vector EMBL 3A (Sambrook *et al.* 1989). DNA, extracted from liver, was partially digested with Sau3A and size selected on a glycerol gradient. The fractions containing DNA fragments of 15–23 kb were pooled and ligated into *Bam*H1-cut EMBL 3A arms (Stratagene). Following ligation the

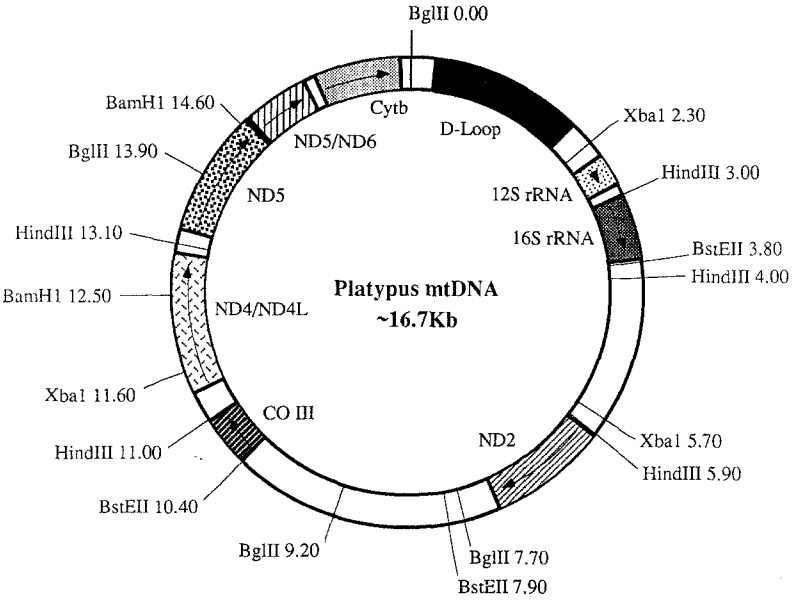

Fig. 2. Composite restriction map of the platypus mitochondrial genome. Composite restriction map constructed from cloned and purified platypus mtDNAs using six restriction enzymes. The position of restriction sites and gene localizations are indicated on the map. The map origin has arbitrarily been set at a *Bg*lII site 5′ to the D-loop region. The total genome size is calculated to be 16.7 kb.

phage constructs were packaged using Gigapack II packaging extract (Stratagene) and plated with the bacterial strain KW251 (Promega). This library was screened with a *Sminthopsis crassicaudata* mtDNA probe supplied by Dr. R. Hope (University of Adelaide) in order to identify clones containing mtDNA inserts.

Sequence Analysis of the OriL. From an mtDNA-positive EMBL3A clone (pPmt 1, see below) we subcloned a 1.9-kb *Hin*dIII/*Bgl*II fragment, containing the light-strand origin of replication and flanking transfer RNAs, into pBluescript SK$^+$ (Stratagene). Both strands of this insert were sequenced with universal primers and insert specific primers, using the dideoxy chain termination technique (Sanger *et al.* 1977). A list of primers used for sequencing is available from A.J. and this sequence has been deposited in the Genbank repository.

Results

Initial restriction analysis of purified platypus mtDNA with *Eco*R1, *Hin*dIII, and *Bam*H1, followed by Southern analysis using the *S. crassicaudata* mtDNA clone, to identify potential cloning sites and provide a preliminary restriction map, indicated that the molecule was a closed circular DNA of about 16–18 kb.

Screening ~120,000 platypus DNA clones with the *S. crassicaudata* probe yielded 12 positive clones. Analysis of these clones with *Bam*H1, *Hin*dIII, and *Sal*1 (which excised the cloned insert DNA from the phage arms) identified eight clones (three of which were unique, the others being duplicate but independent isolates of these clones) that had restriction patterns similar to those observed in our pilot studies. The largest clone (designated pPmt) contained a 16.5-kb insert that overlapped all but a small portion (<1 kb) of the other two mitochondrial clones and was found to represent ~97% of the platypus mitochondrial genome after comparison to purified mtDNA. pPmt was therefore the only clone used in the subsequent restriction-mapping analysis.

Restriction maps of the cloned and purified mtDNAs were constructed using agarose gel electrophoresis of single and double digests with the restriction enzymes *Bam*H1, *Bgl*II, *Bst*EII, *Hin*dIII, *Sal*1, and *Xba*1. Using the double-digestion procedure with these six restriction enzymes on purified mtDNAs and the clone pPmt, 18 restriction sites have been orientated, allowing physical maps for the platypus mtDNA to be assembled. A consensus restriction map (Fig. 2) was produced by comparing the restriction maps generated. The total size of the platypus mitochondrial genome, calculated by summing fragment sizes from our mapping analyses of purified mtDNA, was estimated to be approximately 16,700 bp. However, four different length variants (±200 bp) were identified in the seven animals examined, suggesting that length variation is frequent. This variation was confined to fragments which hybridized with the displacement loop (D-loop) PCR product, suggesting that this length variation is generated within the D-loop (or control) region.

Southern analysis (Sambrook *et al.* 1989) of the mapping gels used in the construction of the restriction maps, using homologous or heterologous PCR products (Table 1), has allowed the assignment of gene localizations to the restriction maps. To date we have mapped 10 PCR products, representing 10 different genes, to our platypus mtDNA restriction map (Fig. 2). The genes/regions mapped so far are cytochrome b (Cyt b); cytochrome oxidase II (Co II); cytochrome oxidase III (Co III); NADH dehydrogenase (ND) subunits 2, 4, 5, 6; the 12S and 16S rRNAs; and the displacement loop (D-loop).

While our mapping strategy was sufficient to determine gross gene order, it was not adequate to examine the arrangement of the small, tightly clustered tRNA genes surrounding the light-strand origin of replication (OriL). In order to address this question we have se-

Table 1. Amplified gene regions used as probes in southern analysis[a]

Gene region	Nucleotide position relative to human sequence	Primers	Product size (kb)	Reference
Cytochrome b	14,841–15,915	L14841 H15915	1.1	Irwin *et al.* (1991)
D-loop	15,926–00651	L15926 H00651	1.8	Kocher *et al.* (1989)
12S rRNA	1091–1478	L1091 H1478	0.39	Kocher *et al.* (1989)
16S rRNA	1581–2906	A_2F_1 H_{290}	1.3	Marzuki *et al.* (1991)
ND2	4476–5482	B_2F_1 B_H	1.0	Marzuki *et al.* (1991)
CoIII	9380–10,182	DF1 DR2	0.8	Marzuki *et al.* (1991)
ND4L/ND4	10515–11,942	DF3 D_H	1.4	Marzuki *et al.* (1991)
ND5	12367–13,928	F_L F_1R_1	1.5	Marzuki *et al.* (1991)
ND5/ND6	13,848–14,873	L13848 F_2R_2	1.1	Marzuki *et al.* (1991)

[a] DNA probes PCR amplified using platypus DNA as the template are highlighted in bold. All other probes were amplified from human DNA. The PCR amplification conditions, for each of the gene regions used in this analysis, were as detailed in the cited reference
The primer sequences for each region are as follows:
L14841 5′-AAAAAGCTTCCATCCATCCAACATCTCAGCATGATGAAA-3′
H15915 5′-AACTGCAGTCATCTCCGGTTTACAAGAC-3′
L15926 5′-TCAAAGCTTACACCAGTCTTGTAAACC-3′
H00651 5′-TAACTGCAGAAGGCTAGGACCAAACCT-3′
L1091 5′-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3′
H1478 5′-TGACTGCAGAGGGTGACGGGCGGTGTGT-3′
A_2F_1 5′-GGAAAGTGCACTTGGACGAACCAG-3′
H_{290} 5′-GTTATTGGATCAATTGAGTATAGT-3′
B_2F_1 5′-CCCCTGGCCCAACCCGTCATCTAC-3′
B_H 5′-GGTAGGAGTAGCGTGGTAAGGGCG-3′
DF1 5′-GCGCGATGTAACACGAGAAAGCAC-3′
DR2 5′-CGAAGCCGCACTCGTAAGGGGTGG-3′
DF3 5′-CTTCTAGGAATACTAGTATATCGC-3′
D_H 5′-GTAGGAGAGTGATATTTGATCAGG-3′
F_L 5′-ACCCTGACTTCCCTAATTCCCCCC-3′
F_1R_1 5′-CTAGGGTAGAATCCGAGTATGTTG-3′
L13848 5′-CAACTACCTAACCAACAAACTTAAA-3′
F_2R_1 5′-GGATCAGGCAGGCGCCAAGGAGTG-3′

quenced a 1,959-bp *Hin*dIII/*Bg*1II fragment (see Fig. 2), which includes the OriL and the flanking tRNA genes of the platypus mitochondrial genome. The location and identity of the transfer RNA genes around the OriL were identified by comparison to published vertebrate sequences, and the platypus tRNA gene arrangement has been compared to those of eutherian and marsupial mammals, as well as to chicken and cod (Fig. 3).

Discussion

The average size of the platypus mitochondrial genome is approximately 16.7 kb, well within the 15–17-kb range of the highly conserved vertebrate mtDNA. However, despite this overall conservation in the platypus mitochondrial genome size, we did observe length variation between animals, which was confined to the control region of the mitochondrial genome. While length variation in the control region has now been reported in a growing number of species (see references in La Roche *et al.* 1990 and Rand 1993), the molecular mechanisms that generate this variation are poorly understood, and more detailed intraspecific analyses are therefore being undertaken to investigate this phenomena in the platypus. The platypus mtDNA gene arrangement (Fig. 2) is identical to the conserved order common to amphibians (Roe *et al.* 1985), fishes (Johansen *et al.* 1990), and placental mammals (Anderson *et al.* 1981, 1982). This finding suggests that the ancestral mammalian mtDNA arrangement is the same as that of fish and amphibia, confirming that it is ancestral to all vertebrates.

The conclusion that the ancestral mammalian mtDNA gene arrangement is the same as that in most vertebrates indicates that the rearrangement observed in the birds is probably lineage specific. However; it is possible that the avian rearrangement may also be observed in some reptile species, and it now seems crucial to our understanding of vertebrate mtDNA evolution that the complete sequence of a reptilian mitochondrial genome be added to the data based.

Since the tRNA gene arrangement around the origin of light-strand replication in the platypus is the same as in eutherian mammals, birds, and amphibians, the rearrangement of these genes in marsupials (Pääbo *et al.* 1991) is specific to the marsupial line. Furthermore, since that rearrangement is conserved among all mar-

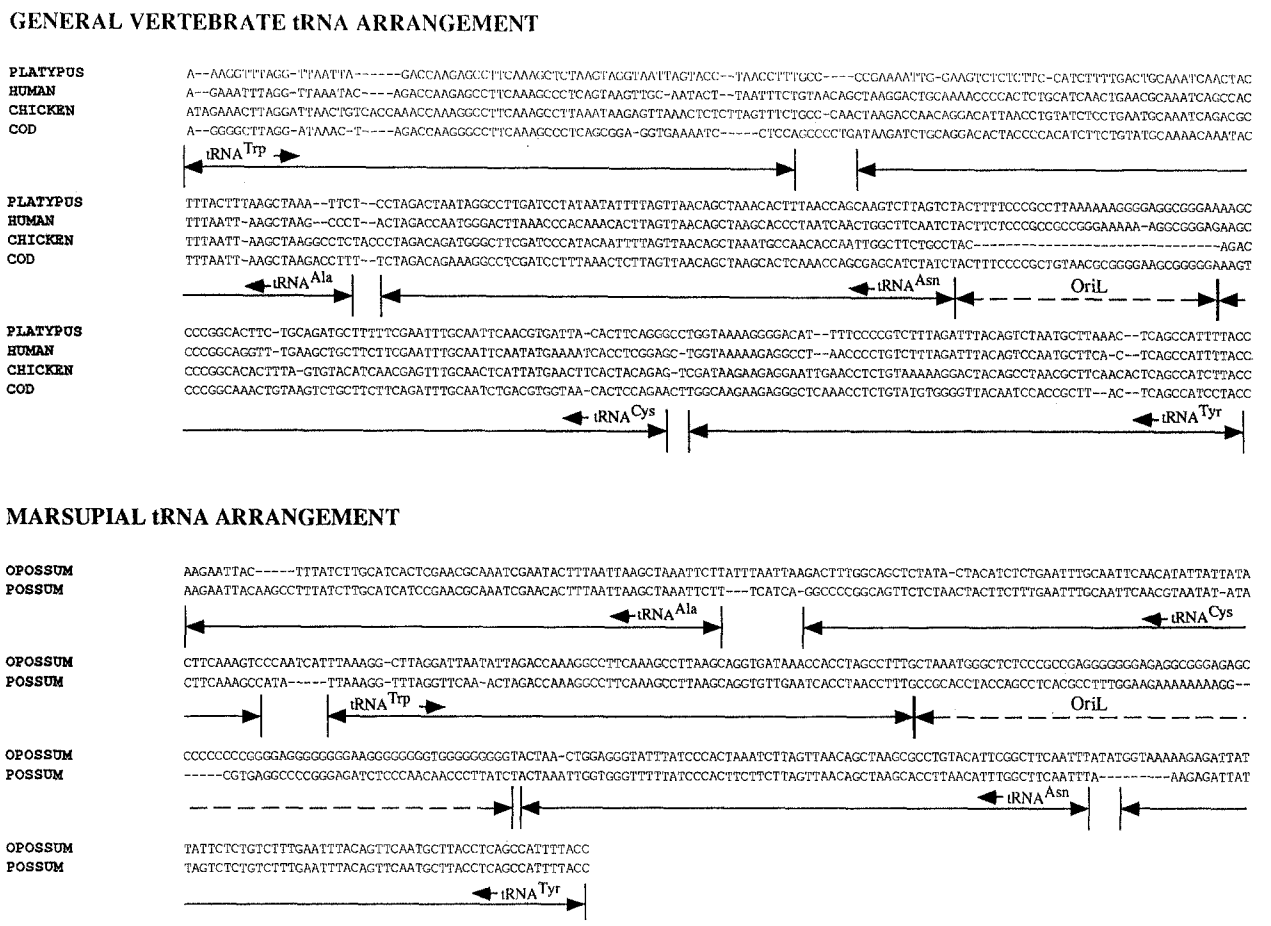

Fig. 3. Comparative alignment of OriL and adjacent tRNA genes. Sequences of the mitochondrial light-strand origin of replication and flanking tRNA genes corresponding to positions 5512–5892 in the human mitochondrial DNA sequence (Anderson *et al.* 1981). The species used and appropriate references are: human (Anderson *et al.* 1981), chicken (Desjardins and Morais 1990), cod (Johansen *et al.* 1990), opossum (*Didelphis virginiana*, Janke *et al.* 1994) and possum (*Pilander opossum*, Pääbo *et al.* 1991). Note that there are two tRNA gene arrangements, the arrangement observed in the marsupials and the general arrangement observed in the other vertebrates.

supials tested to date, it appears to be a unique event that happened in an early marsupial ancestor.

The evolutionary significance of mitochondrial gene rearrangements is currently unknown. Some, such as the marsupial rearrangement, are conserved within taxa indicating that the rearrangement occurred early in the history of the lineage and has been retained. Given that it is extremely unlikely that such complex rearrangements could occur twice independently, or revert back to the original state once they have occurred, rearrangements such as that observed in the marsupials may prove to be useful markers for elucidating the ancestral relationships of the modern vertebrates.

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