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Phylogenetic Analysis of 18S rRNA and the Mitochondrial Genomes of the Wombat, *Vombatus ursinus,* and the Spiny Anteater, *Tachyglossus aculeatus:* Increased Support for the Marsupionta Hypothesis

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Abstract. The monotremes, the duck-billed platypus and the echidnas, are characterized by a number of unique morphological characteristics, which have led to the common belief that they represent the living survivors of an ancestral stock of mammals. Analysis of new data from the complete mitochondrial (mt) genomes of a second monotreme, the spiny anteater, and another marsupial, the wombat, yielded clear support for the Marsupionta hypothesis. According to this hypothesis marsupials are more closely related to monotremes than to eutherians, consistent with a basal split between eutherians and marsupials/monotremes among extant mammals. This finding was also supported by analysis of new sequences from a nuclear gene—18S rRNA. The mt genome of the wombat shares some unique features with previously described marsupial mtDNAs (tRNA rearrangement, a missing $tRNA^{Lys}$, and evidence for RNA editing of the $tRNA^{Asp}$). Molecular estimates of genetic divergence suggest that the divergence between the platypus and the spiny anteater took place ≈34 million years before present (MYBP), and that between South American and Australian marsupials ≈72 MYBP.

Key words: Phylogenetics — Marsupionta — Theria — Spiny anteater — Wombat — Mitochondrial DNA — 18S rDNA

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Introduction

Monotremes are a unique group of mammals. As "egg-laying" mammals and with a number of apparently plesiomorphic anatomical characters, they play a crucial role in the study of mammalian evolution. A clearer understanding of monotreme relationships vis-à-vis other mammals would render invaluable insight into the origin of modern mammals in general. Unfortunately there are only three extant and highly specialized monotreme species living in Australia and New Guinea, though previously they had a wider distribution. Thus Cretaceous monotreme fossils have been found not only in Australia (Musser 1999) but also in South America (Pascual et al. 1992). However, the generally poor fossil record of Monotremes (Carroll 1988) essentially limits analysis of their phylogeny to recent species. Even the relationships between, and time of origin of the two extant monotreme families remains somewhat enigmatic, despite new fossils findings from extinct monotreme families having been described (Flannery et al. 1995; Musser 1999).

Paleontology of Early Cretaceous mammals has been limited to a few fragmentary findings, mostly teeth, but recently nearly complete skeletons have been discovered (Hu et al. 1997; Rich et al. 1997; Qiang et al. 1999). The mosaic assembly of ancestral and modern characters in *Jeholodens* (Qiang et al. 1999) demonstrates that homoplasies in skeleton characters are common in the morphology of early mammals (Qiang et al. 1999; Zimmer 1999; Rowe 1999). Despite the relatively poor early mammalian fossil record, homoplasies, and the highly

specialized morphology of today's living monotremes, the basic relationship among the monotremes, marsupials, and eutherians appeared to be well understood on the basis of numerous other characters (Marshall 1979). Traditionally there has been a general consensus that monotremes represent the earliest branch among mammals and that marsupials and eutherians are sister groups (Gill 1872; Simpson 1945; McKenna 1975; Novacek 1992). Only two morphological studies questioned this understanding.

Based on his palimpsest theory Gregory (1947) concluded that monotremes are closely related to a particular group of marsupials, the Diprotodontia. This would make the marsupials paraphyletic. Based on the apparently identical tooth development, Kühne (1973) concluded that monotremes are sistergroup to all marsupials (Kühne 1973, 1975, 1977). This generalized Marsupionta hypothesis resulted in a short revival of the idea of a monotreme/marsupial relationship. Kühne's interpretation of tooth development, however, has been questioned following a more detailed histological study (Lucket and Zeller 1989).

Support for the more generalized Marsupionta hypothesis in which monotremes are the sister group to all marsupials, based on molecular sequence data, was first formalized by Janke et al. (1996). Phylogenetic analysis of 12 concatenated mitochondrial (mt) protein-coding genes showed strong support for a monotreme/marsupial clade. This relationship remained well supported even after the addition of more out- and in-group taxa as well as using different analytical methods (Janke et al. 1997; Janke and Arnason 1997; Cao et al. 1998; Zardoya and Meyer 1998; Kumazawa and Nishida 1999). However, to date the phylogenetic branch leading to the monotremes has been represented by only a single species, the platypus, which might affect analytical outcomes.

Some analysis of nuclear DNA sequence and comprehensive DNA–DNA hybridization studies also support the Marsupionta hypothesis (Toyosawa et al. 1998; Kirsch and Mayer 1998). Kirsch and Mayer (1998) reviewed the morphological and molecular evidence for the Marsupionta hypothesis and concluded that it could not be ruled out by morphological data but that its support from molecular data might be an artifact, e.g., because of limited taxon sampling.

As monotremes lack a comprehensive fossil record, little is known about the origin of the two extant families, the Tachyglossidae (echidnas) and Ornithorhynchidae (platypus). Phylogenetic analysis of globin genes suggest a divergence time at about 50 million years before present (MYBP) (28–73 MYBP) depending on the precise data set used for this estimate (Hope et al. 1990). Later studies on the basis of α-lactalbumin (Messer et al. 1998), DNA–DNA hybridization (Westerman and Edwards 1991a), protamine P1 (Retief et al. 1993), and mt 12S rDNA (Gemmell and Westerman 1994) resulted in the same wide range of estimates for divergence times of the two monotreme families. These large differences in estimating the divergence time within monotremes are probably the results of stochastic fluctuations, which might be overcome by longer-sequence data sets.

To investigate the Marsupionta hypothesis in further detail, we have extended the existing data set by sequencing the complete mtDNAs of the common echidna and of the wombat. As a member of a different Australian marsupial suborder (Vombatiformes), the wombat not only increases the number of marsupialian species in the analysis but also splits the long branch leading to the wallaroo. In addition to these two species, the data set included a large number of taxa representing all vertebrate classes. Furthermore, new 18S rDNA sequences from eutherians, marsupials, and a monotreme are reported and analyzed.

Materials and Methods

Whole cellular DNA was purified (Sambrook et al. 1989) from liver tissue of both species. Initially two regions of the mtDNA were amplified using conserved primers designed to fit various tetrapods (12SL, 5′-aactgggattagataccccact-3′; tMetH, 5′-gtatgggcccgatagctt-3′; tLeuL, 5′-aagtcttccattgaccttag-3′; tThrH, 5′-tttttggyttacaagacc-3′). Sequences from these PCR products were used to design species-specific primers for PCR amplification of the remaining regions.

The PCR products were twice precipitated with ethanol and sequenced directly by cycle sequencing (Thermo Sequinase fluorescent-labeled primer cycle sequencing kit deaza-dGTP; Amersham) using fluorescent dye-labeled primers (IRD-800; MWG Biotech) and analyzed on a LICOR 4000L (AH Diagnostics/MWG Biotech).

In addition to the echidna and wombat, the phylogenetic analyses included the following species: common dogfish, *Scyliorhinus canicula* (Delarbre et al. 1998); star spotted dogfish, *Mustelus manazo* (Cao et al. 1998); spiny dogfish, *Squalus acanthias* (Rasmussen and Arnason 1999); starry skate, *Raja radiata* (Rasmussen and Arnason 1999); loach, *Crossostoma lacustre* (Tzeng et al. 1992); carp, *Cyprinus carpio* (Chang et al. 1994); cod, *Gadus morhua* (Johansen and Bakke 1996); trout, *Onchorynchus mykiss* (Zardoya et al. 1995); flounder, *Paralichthys olivaceus* (Saitoh et al. 2000); African clawed frog, *Xenopus laevis* (Roe at al. 1985); mole skink, *Eumeces egregius,* and green turtle, *Chelonia mydas* (Kuniazawa and Nishida 1999); painted turtle, *Chrysemys picta* (Mindell et al. 1999); alligator, *Alligator mississippiensis* (Janke and Arnason 1997); rook, *Corvus frugilegus* (Härlid and Arnason 1998); indigo bird, *Vidua chalybeata,* broadbill, *Smithornis sharpei,* and falcon, *Falco peregrinus* (Mindell et al. 1999); rhea, *Rhea americana* (Härlid et al. 1998); ostrich, *Struthio camelus* (Härlid et al. 1997); chicken, *Gallus gallus* (Desjardins and Morais 1990); platypus, *Ornithorhynchus anatinus* (Janke et al. 1996); opossum, *Didelphis virginiana* (Janke et al. 1994); wallaroo, *Macropus robustus* (Janke et al. 1997); mouse, *Mus musculus* (Bibb et al. 1981); rat, *Rattus norvegicus* (Gadaleta et al. 1989); human, *Homo sapiens,* and chimpanzee, *Pan troglodytes* (Arnason et al. 1996a); gibbon, *Hylobates lar* (Arnason et al. 1996b); African elephant, *Loxodonta africana* (Hauf et al., 2000); aardvark, *Orycteropus afer* (Arnason et al. 1999); armadillo, *Dasypus novemcinctus* (Arnason et al. 1997); bat, *Artibeus jamaicensis* (Pumo et al. 1998); mole, *Talpa europea* (Mouchaty et al. 2000); dog, *Canis familiaris* (Kim et al. 1998); cat, *Felix catus* (Lopez et al. 1996); harbor seal, *Phoca vitulina* (Arnason and Johnsson 1992); donkey, *Equus asinus* (Xu et al. 1996a); horse, *Equus caballus* (Xu and Arnason 1994); Indian rhinoceros, *Rhinoceros unicornis* (Xu et al. 1996b); white rhinoceros, *Ceratotherium simum* (Xu and Arnason 1997); cow,

Bos taurus (Anderson et al. 1982); sheep, *Ovis aries* (Hiendleder et al. 1998); and fin whale, *Balaenoptera physalus* (Arnason et al. 1991), and blue whale, *Balaenoptera musculus* (Arnason and Gullberg 1993). Thus the alignment included the main eutherian lineages and a variety of unambiguous outgroups.

Phylogenetic analyses were performed on the concatenated sequences of 12 protein-coding genes. The ND6 gene has been eliminated from the analysis due to its deviation in amino acid (aa) composition from the remaining protein coding genes as tested by a χ^2 test. The data set was analyzed using maximum parsimony (MP) (Fitch 1971), neighbor joining (NJ) (Saitou and Nei 1987), the Fitch–Margoliash method (FITCH) (Fitch and Margoliash 1967), maximum likelihood (ML) (Felsenstein 1981), and bootstrapping as implemented in the PHYLIP program package (Felsenstein 1991). Each of these methods has its own specific strengths and pitfalls. Congruence of the results would lead to higher confidence in the reconstructed tree. The Templeton (1983) test, as implemented in the PHYLIP program package, was used to evaluate differences in the number of substitutions and their standard deviation for different topologies relative to the MP tree. The MOLPHY package (Adachi and Hasegawa 1996b) was used to calculate local bootstrap probabilities (LBP) and bootstrap probabilities according to the RELL method (Hasegawa and Kishino 1994). Standard errors of the log-likelihood difference were estimated according to Kishino and Hasegawa's (1989) formula. The PUZZLE version 4.0 package (Strimmer and von Haeseler 1996) calculated quartet puzzling (QP) support values (Strimmer and von Haeseler 1996). The function Likelihood Mapping (Strimmer and von Haeseler 1997) of the PUZZLE package was used to investigate and visualize the support for different hypotheses. The PAUP* program (Swofford 1998) was used to make NJ analyses based on paralinear distances (Lake 1994), because this method is robust against violation of the assumption of compositional homogeneity generally made by phylogenetic reconstruction methods. Congruence of the reconstructed trees can be taken as an indication that compositional bias is not effecting the topology. If not stated otherwise, the mtREV24 (Adachi and Hasegawa 1996a) matrix of aa evolution was used for the analysis of aa sequence data. The mtREV24 is based on mt protein-coding genes and fits the data better than other matrices based on nuclear-encoded genes. The TN model of nucleotide (nt) evolution (Tamura and Nei 1993) was used for the analysis of nt sequence data.

18S rDNA sequences were PCR amplified from whole-genomic DNA preparations of the horse (*Equus caballus*), armadillo (*Dasypus novemcinctus*), hedgehog (*Erinaceus europaeus*), opossum (*Didelphis virginiana*), wombat (*Vombatus ursinus*), gray short-tailed opossum (*Monodelphis domestica*), platypus (*Ornithorhynchus anatinus*), and Nile crocodile (*Crocodylus niloticus*) using a conserved primer pair (p18S3 5′-ctggttgatcctgccagt-3′, p18S5 5′-taatgatccttccgcaggt-3′) that amplifies the 18S rDNA sequences from most vertebrates at annealing temperatures of 45–55°C. The PCR product was twice ethanol precipitated and sequenced from both strands using conserved IRD-800 labeled sequencing primers (MWG Biotech) and analyzed on a LICOR-4000L (AH Diagnostics) according to the manufacturer's protocol. In addition to the above species, the 18S rDNA sequences from the human (*Homo sapiens;* M10098), mouse (*Mus musculus;* X00686), rat (*Rattus norvegicus;* M11188), chicken (*Gallus gallus;* AF173612), Andalusian buttonquail (*Turnix sylvatica;* AF173631), sandhill crane (*Grus canadensis;* AF173632), and alligator (*Alligator mississippiensis;* AF173605) were obtained from the EMBL database. The sequences were aligned by ClustalW (Thompson et al. 1994) and inspected by eye. The phylogenetic analysis was performed by the methods and programs as described above using the Tamura–Nei (TN) model of nt sequence evolution. Additional MP analysis was performed using PAUP*, coding gaps as new character states.

The complete mtDNA sequences of the echidna and the wombat have been deposited at the EMBL database with accession numbers AJ303116 and AJ304826, respectively. The corresponding numbers for the 18S rDNA are AJ311672–AJ311679. The alignments are available from the corresponding author upon request.

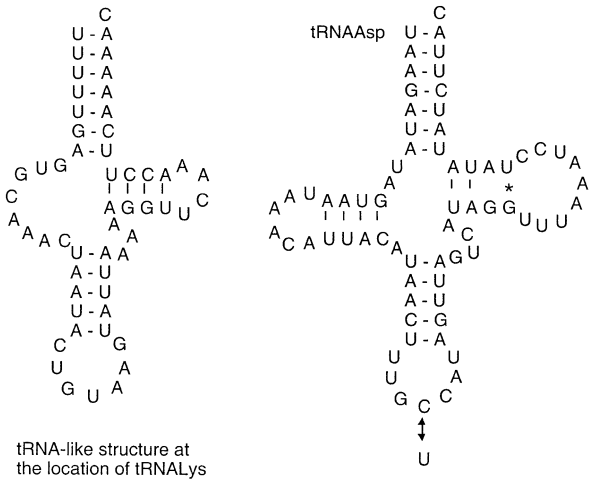

Fig. 1. Inferred secondary structures of two unusual tRNAs of the wombat mt genome.

Results

The PCR amplifications yielded discrete products in all cases. The mtDNA fragments ranging in size from 2.5 to 6 kbp were substantially (500–1000 nt) overlapping with their neighbors. Sequence differences in the overlapping regions were not observed, ensuring that possible nuclear copies of the mt genome were not amplified and sequenced.

The length of the mtDNA of the echidna is 16,630 nt. This is not an absolute value, as different numbers of repetitive elements in the control region can cause length differences. The molecule encodes the expected 37 genes, which are organized as in most other vertebrate groups. Many of the protein coding genes encode only a partial stop codon, which is then completed by polyadenylation on the RNA level (Ojala et al. 1981). All tRNA sequences fold into the expected secondary structure shown by other vertebrates.

An extra 84-nt-long conserved intergenic sequence is found between the $tRNA^{Leu}$ and the gene for the NADH1 subunit. This sequence is well conserved in all monotremes (Janke et al. 1996), although its function remains enigmatic. A BLAST search at the National Center for Biotechnology Information (NCBI) against the whole database did not reveal any similarity to other sequences, nor can this sequence be folded into a stable secondary structure. As in the platypus, it may be transcribed specifically from the H-strand (Janke et al. 1996).

The mitochondrial genome of the wombat is 16,985 nt long and shows the same arrangement of genes as that of marsupials (Janke et al. 1997, 1994). The middle anticodon of the $tRNA^{Asp}$ is encoded as cytosine (C) instead of the expected thymine (T) as shown in Fig. 1. Consequently the anticodon GCC would recognize glycine codons, while only a GUC anticodon would recognize aspartic acid codons. It has been demonstrated that RNA editing modifies the middle position to the cognate anticodon (Janke and Pääbo 1993; Mörl et al. 1995).

In the wombat mt genome no cognate gene for a $tRNA^{Lys}$ was found. At the common site for the $tRNA^{Lys}$ a surprisingly tRNA-like structure was identified (Fig. 1), which, if functional, would encode a tRNA for the aa tyrosine. However, such a tRNA gene with its usual features is located at the expected position. A sequence search of the tRNA-like structure at the NCBI database did not reveal specific similarity to other published sequences.

After alignment, gaps and ambiguous sites around them were removed until all species showed the same aa before and after the gap. This approach minimizes the risk of analyzing nonhomologous positions, which are generally and unavoidably produced when introducing gaps to equalize length differences of the sequences in an alignment. It, furthermore, eliminates the most highly variable regions of protein-coding genes and thus reduces the overall rate heterogeneity. The NADH6 sequence was excluded from analyses because of its deviation in both nt and aa composition and its nontrivial alignment problems among distantly related taxa. The final data set thus had a length of 9456 nt (=3152 aa). Analysis of nt sequence was of nonsynonymous changes at the first codon position plus all changes at the second codon position.

A χ^2 analysis of the aa compositions as implemented in the PUZZLE program package does not reject the assumptions of compositional homogeneity among the species that are involved in the final analysis, except for the teleost exemplars. Excluding these did not affect the results and they were therefore left in the analysis. The χ^2 test rejects compositional homogeneity for nt sequences for some of the vertebrate species. However, the phylogenetic analysis was also performed on nt sequences to get a more complete picture of the results. Tree reconstruction based on paralinear distances resulted in the same topology as those based on the TN model, but with higher support values for the Marsupionta.

Initially a heuristic search using ML and the alignment of aa data was performed. On the basis of the best ML tree, 10 indisputable operational taxonomic units were formed to constrain the data set, which enabled us then to make an exhaustive search among the 2,027,025 possible topologies. The best among these conformed to the best of the initial heuristic search. Figure 2 shows this ML tree based on aa sequences. The phylogenetic positions of the turtle and the alligator have been left unresolved, as their positions could not be conclusively determined by all methods or data sets. A more detailed analysis of the relationship among the Diapsia has been published elsewhere (Kumazawa and Nishida 1999; Mindell et al. 1999; Janke et al. 2000) and is therefore not repeated here.

The rate heterogeneity among aa sites is not pronounced. Assuming one class of constant and four

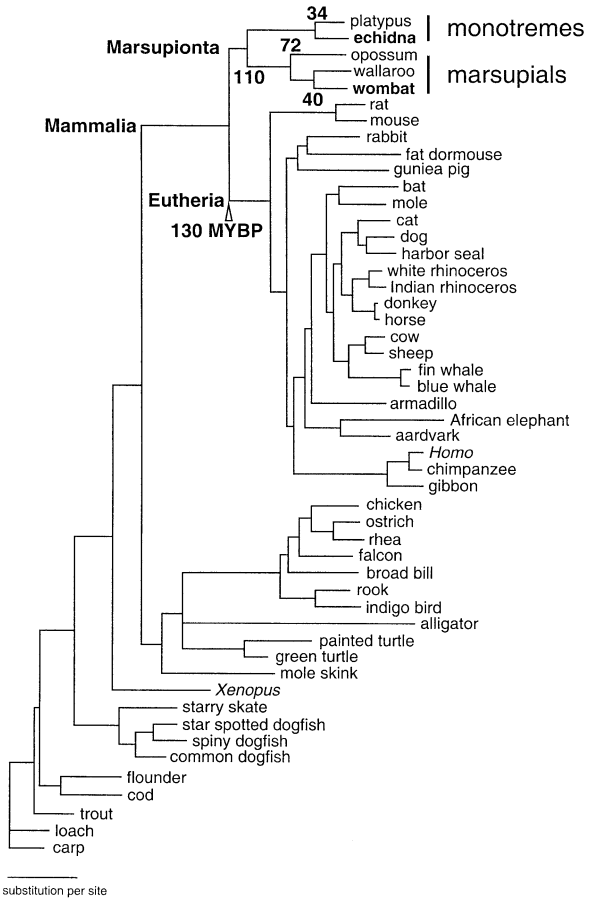

Fig. 2. Maximum likelihood tree reconstructed from aa sequence data and estimated divergence times on the branches leading to the marsupionts.

classes of variable sites, PUZZLE estimated an α value of 0.68 and a 35-fold rate difference between the slowest- and the fastest-evolving sites. This can be considered moderate, as the fraction of slow-evolving sites includes the majority of constant positions.

Table 1 shows the bootstrap and quartet puzzling support values for the three branches that are essential for the definition of the monotreme position in the phylogenetic tree. The Marsupionta hypothesis is supported by >90% in most of the resampling analyses. The Kishino–Hasegawa and Templeton tests (Table 2) also have the Marsupionta hypothesis as the best supported. The orthodox Theria hypothesis receives the least support and can even be rejected above 2 SE assuming rate homogeneity and 1.6 SE assuming rate heterogeneity.

The marsupial–monotreme relationship remained the best-supported grouping even when applying simpler models of sequence evolution (not shown). Using aa transition matrices from nuclear data such as the JTT matrix, the more limited Dayhoff matrix, or the simplest of all, a Poisson model, resulted in all cases in the reconstruction of the Marsupionta relationship. This demonstrated the robustness of the results even against severe violations of the evolutionary model by the simpler models.

Table 1. Bootstrap and reliability values for selected branches[a]

	Marsupionta	Eutheria	Mammalia
QP/LBP			
aa	100/80	99/100	99/100
12	99/74	100/100	99/100
NJ/FITCH			
aa	100/100	100/100	100/100
12	93/91	83/75	78/74
MP			
aa	76/59	100/100	100/100
12	51	100	100

[a] The first value for the MP aa analysis was calculated by the PHYLIP package, weighting according to number of reconstructed nt changes, while the second value was calculated by PAUP*, assigning equal weight for each aa substitution. The bootstrap analysis was based on 200 replicates for aa sequences and 1000 replicates for nt data.

Table 2. Phylogenetic analysis of aa sequences for the basic three mammalian lineages[a]

Topology	ΔlnL	SE	p_{boot}	ΔlnL_{RH}	SE	Steps	SD
Marsupionta	⟨−94,919⟩		0.775	⟨−80,892⟩		⟨16,638⟩	
Unnamed	−17.2	±21.0	0.220	−4.6	±12.7	+11	±10
Theria	−38.4	±18.6	0.005	−15.7	± 9.8	+13	± 9

[a] The value in angle braces indicates the log-likelihood and MP value of the best tree. The differences of the log-likelihood values of the alternative trees from that of the best tree, ΔlnL, are shown with their standard errors and bootstrap probabilities (p_{boot}). The likelihood analysis was done assuming rate homogeneity (lnL) or rate heterogeneity (lnL_{RH}) among sites. The likelihood analysis was complemented by the Templeton test as implemented in the PHYLIP program package and shows the difference in substitutions (steps) and their standard deviations relative to the number of inferred nucleotide substitutions of the MP tree.

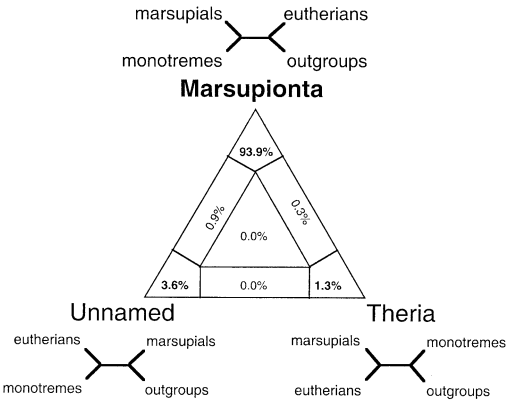

Fig. 3. Likelihood mapping triangle showing the percentage of quartets supporting the relationships of the three main mammalian groups.

Likelihood mapping (Strimmer and von Haeseler 1997), a ML analysis of all possible quartets including eutherians, marsupials, monotremes, and outgroups in four respective clusters, was used to investigate further the support for the Marsupionta hypothesis and the amount of phylogenetic signal of the data set (Fig. 3). All data points were in the extreme corners or along the edges and are therefore not depictable. Of the 3024 possible quartets, 2840 (93.9%) found significant support for the Marsupionta hypothesis, 110 (3.6%) found a sister-group relationship of monotremes and eutherians, and 39 (1.3%) reconstructed the traditional Theria hypothesis. The remaining 35 (1.3%) quartets did not find significant support for a single tree, but 27 (0.9%) of these significantly rejected the Theria hypothesis. None of the quartets remained fully unresolved.

Divergence times were calculated using the ML branch lengths and assuming a last common ancestor to all recent mammals at 130 MYBP (Fig. 2). As estimated from the branch lengths, differences in evolutionary rates were not significant and therefore a global clock was assumed for the marsupiont branches. Improved estimates of evolutionary rates would require a more extensive taxon sampling and a more extensive palaeontological record (Arnason et al. 2000).

The 18S rDNA nuclear sequences are highly conserved within each of the mammalian groups. However, insertion and deletion (indel) events that are characteristic for eutherians, marsupials, and the monotreme occur. Figure 4 shows an extensive indel region in the first third of the partial 18S rDNA sequence, providing unequivocal support for the Marsupionta hypothesis. The insertions of 20–22 nt and later two additional 2-nt insertions are synapomorphic traits for marsupials and monotremes. Except for MP, there are no methods or models available for inclusion of indels in a phylogenetic analysis. Therefore all gaps were removed, leaving 1694 nt for the NJ and ML analysis. Of these 1694 sites, 99 (6.8%) were variable. Despite the limited sequence variation, NJ and ML identified the Marsupionta relationship (Fig. 5). The bootstrap/QP support was moderate (70–80%), however, due to the few differences between the mammalian groups. MP identified 19 equally parsimonious trees, of which 90% showed the Marsupionta relationship. Including gaps and coding gaps as fifth character states in the MP analysis resulted in significant support for the Marsupionta hypothesis. This result was insensitive to the coding of gaps as several individual events or a single event regardless of the gap length.

Discussion

The two new mt genomes show the expected features generally found in marsupials and monotremes, respectively. The marsupial mt genomes have the most deviant characteristics found among mammals. The rearrangement of tRNA (Pääbo et al. 1991; Janke et al. 1994) is the least surprising trait, as gene rearrangement is a common feature in mt genomes (Desjardins 1990; Janke et al. 1994; Janke and Arnason 1997; Boore 1999). RNA ed-

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alligator          GCGCTGACCT----------CC----------------------GGGGATGCGTGCATTTATCAGACCAAAACCAACCCGG---GCTCGCC-----------------------------------------CGGCCGCTTTGGT
crocodile          GCGCCGACCT----------CC----------------------GGGGACGCGTGCATTTATCAGACCAAAACCAACCCGG---GCCCGCC-----------------------------------------CGGCCGCTTTGGT
chicken            GCGCCGACCT----------CC----------------------GGGGACGCGTGCATTTATCAGACCAAAACCAACCCGG---GCTCGCC-----------------------------------------CGGCGGCTTTGGT
buttonquail        GCGCCGACCT----------CC----------------------GGGGACGCGTGCATTTATCAGACCAAAACCAACCCGG---GCCCGCC-----------------------------------------CGGCAGCTTTGGT
sandhill crane     GCGCCGACCT----------CC----------------------GGGGACGCGTGCATTTATCAGACCAAAACCAACCCGG---GCCCGCC-----------------------------------------CGGCAGCTTTGGT
Homo               GCGCTGACCC----------CCTTCGCGGG----------GGGGATGCGTGCATTTATCAGATCAAAACCAACCCGGTCAGCCCCTCTCCGGCCCC--GGCCGGGGGGCGGGGCCGC--GG-CGGCTTTGGT
rat                GCGCTGACCC----------CCCTTCCCGTGGG--------GGGGACGCGTGCATTTATCAGATCAAAACCAACCCGGTCAGCCCCCTCCCGGCTCC--GGCCGGGGGTCGGGCGCC--GGACGGCTTTGGT
mouse              GCGCTGACCC----------CCCTTCCCGGGG----------GGGGATGCGTGCATTTATCAGATCAAAACCAACCCGGTGAGCTCCCTCCCGGCTCC--GGCCGGGGGTCGGGCGCC--GG-CGGCTT-GGT
horse              GCGCTGACCC----------CCTTCGCGGG----------GGGGATGCGTGCATTTATCAGATCAAAACCAACCCGGTCAGCCTCCTNCCGGCCCC--GGCCGGGGGG-CGGCGCC--CGGCGGCTTTGGT
armadillo          GCGCTGACCC----------CCTTCGCGGG----------GGGGATGCGTGCATTTATCAGATCAAAACCAACCCGGTCAGCTCCCTCCCGGCCCC--GGCCGGGGGG-CGGCGCC--CGGCGGCTTTGGT
hedgehog           GCGCTGACCC----------CCTTCGCGGG----------GGGGATGCGTGCATTTATCAGATCAAAACCAACCCGGTCAGCCTCCCTCCGGCTCC--GGCCGGGGGGCGGGCGC--CGGCGGCTTTGGT
s.-tailed opossum  GCGCTGACCCGGGCCCCCTTCCCCCCGCGGGTGGGGGCCCCCGGGGATGCGTGCATTTATCAGACCAAAACCAACCCGG-CGGCTTCCCCCCAACCCCCCAACCAGGGGCGGGGGTGCCCCGGCCGCTTTGGT
virginia opossum   GCGCTGACCCGGGCCCCCTTCCCCCCGCGGGTGGGGGCCCCCGGGGATGCGTGCATTTATCAGACCAAAACCAACCCGG-CGGCTTCCCCCCAACCCCCCAACCAGGGGCGGGGGTGCCCCGGCCGCTTTGGT
wombat             GCGCTGACCCGGGCCTCCTTCCCCCC-GCGGGTGGGGGCCCC-GGGGATGCGTGCATTTATCAGACCAAAACCAACCCGG-CGGTTTCCCCCCAACCCCCCAACCAGGGGCGGGGGTGCCCCGGCCGCTTTGGT
platypus           GCGCTGACCCGGGCCCCTTCCCCCCGTGGGTGGGGGCTCC-GGGGATGCGTGCATTTATCAGACCAAAACCAACCCGG-CGGTTTCCCCCCAACCCCCCAACCAGGGGCGGGGGTGCCCCGGCCGCTTTGGT
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Fig. 4. Indels at the 3′ region of the 18S rDNA.

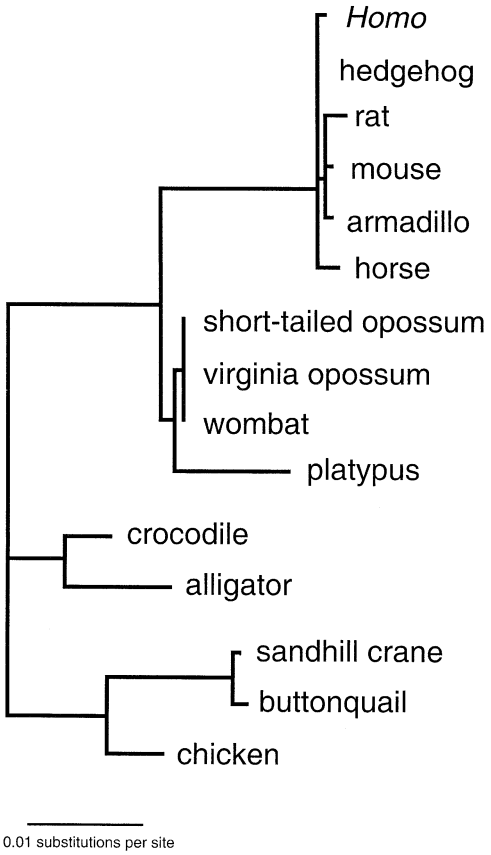

Fig. 5. ML/QP tree from 18S rDNA sequences.

iting, a postranscriptional alteration of genetic information, and RNA import into vertebrate mitochondria are more unusual and were first detected in marsupials. It has been shown that in marsupials an RNA editing mechanism converts the anticodon of the $tRNA^{Asp}$ into its cognate sequence (Janke and Paabo 1993; Mörl et al. 1995). The GCC anticodon sequence of the wombat $tRNA^{Asp}$ indicates that, in wombats too, RNA editing modifies the anticodon of this tRNA.

None of the marsupial mt genomes analyzed to date encodes a functional $tRNA^{Lys}$. For a mt protein translation machinery to work, a $tRNA^{Lys}$ must therefore be imported from the nucleus as discussed previously for the nonfunctional $tRNA^{Lys}$ of the wallaroo and opossum (Janke et al. 1997). Other mechanisms such as RNA editing which could aid in reconstructing a cognate $tRNA^{Lys}$ seem unlikely. So, each marsupial species would need its own specific mechanism, because as yet no common sequence features at the usual lysyl tRNA location of other vertebrates can be identified in the marsupial sequences.

The mt genomes of the two monotremes lack all of the above-mentioned features but have an unusual conserved sequence with a still unknown function (Janke et al. 1996). The presence of this same feature in two widely divergent monotremes indicates some kind of function since the high evolutionary rate of change in noncon-

straint regions of the mt genome would be expected rapidly to erode away nonfunctional sequences.

The divergence times between the South American and the Australian marsupials, calculated at ≈75 MYBP, and for the origin of the marsupionts, at ≈110 MYBP, are in agreement with former published data (Janke et al. 1997). As expected, due to the reduced stochastic effects of the 12 mt protein-coding genes that were analyzed, the estimated divergence time between the platypus and the echidna, at ≈34 MYBP, is almost an average of previously published data based on single genes.

The accumulation of sequence data from mt genomes has now made it possible to investigate the basal and controversial phylogenetic relationships of mammals in considerable detail. At least one representative of each major tetrapod class has been included as outgroups. Even though increasing numbers of nuclear genes have been used to investigate eutherian relationships, very few of these can currently be used to examine basal mammalian divergences, as data from appropriate nonmammalian outgroups for rooting such trees are usually unavailable.

The long and isolated branch for monotremes, formerly represented only by the platypus, has now been split by the addition of the echidna sequence. Splitting the monotreme branch not too close to its extreme ends aims at reducing the problems potentially associated with long branch attraction (LBA) (Felsenstein 1978; Hillis 1996; Graybeal 1998). Furthermore, long sequences may provide a better approach to overcome LBA than increasing the number of taxa sampled (Poe and Swofford 1999). In the present study we have considered both approaches by (i) increasing the taxon sampling and (ii) using extensive sequence data, to investigate the relationship among monotremes, marsupials, and eutherians. As the analyses showed, the Marsupionta hypothesis remained the best-supported hypothesis for the relationship of the three primary mammalian groups.

As the two living echidna species, *Tachyglossus* and *Zaglossus,* are genetically similar (Westerman and Edwards 1991b), the monotreme branch cannot be shortened further into useful segments. In the case of marsupials, the split between American and Australian lineages, usually regarded as the basal marsupial divergence, is represented by the opossum on one side and the wallaroo and wombat on the other. All splits along the marsupial branch are not at extreme ends but lie at the basal lower and upper third (wombat/wallaroo) of the marsupial branch. These positions are well suited for splitting the branches and to avoid LBA (Graybeal 1998). Even though the terminal branches of the South American and Australian marsupials can be shortened further in the future, this may have only a small impact on the marsupial–monotreme relationship. It will therefore be essential in future studies to analyze more nuclear data, since adding more taxa to the mitochondrial alignment is unlikely to have a significant impact on the support for the alternative hypothesis of basal mammalian relationships.

Support for the traditional Theria hypothesis has been claimed in a recent study of sequences from the mannose 6-phosphate/insulin-like growth factor II receptor (Killian et al. 2001). This conclusion may need further substantiation, however, as this gene seems to have different functions in monotremes, marsupials, and eutherians. It is, e.g., only imprinted in marsupials and eutherians, but not in monotremes, and in monotremes it lacks the IGF binding receptor (Killian et al. 2000), making the homology of this gene among mammals and outgroups questionable. Previously published globin data remain ambiguous in their support for any of the three possible topologies (Janke et al. 1997). Two studies on nuclear sequences did not support the Marsupionta hypothesis. These studies, however, were based on the inclusion of gaps or short sequences (Retief et al. 1995) or on a rooting with a nonhomologous gene (Messer et al. 1998). We note, in addition, that both studies observed extremely high distance values that were close to or exceeding unity, suggesting an average of one change per amino acid site and thus a high degree of randomization in the data. Other studies of more suitable nuclear data (Toyosawa et al. 1998; Kirsch and Mayer 1998) support the mt results. With the addition of the newly sequenced 18S rDNA sequences, the currently unambiguous support for the Marsupionta hypothesis from molecular data can no longer be ignored.

The recently discovered triconodont fossil (Qiang et al. 1999) bears a mosaic of advanced and ancestral mammalian skeletal characters, notably a therian shoulder girdle. Mosaic traits have also been observed in other groups of early mammals, e.g., the multituberculates. On the basis of the girdle characters, multituberculates have been placed with the traditionally recognized Theria (Sereno and McKenna 1995), despite the presence of several other characters that are believed to be ancestral to therians. The formation of the side wall of the brain case and the dentition would make multituberculates a sister group to the monotremes (Carroll 1988; Benton 1990), which are traditionally held to be the most primitive group of living mammals. Thus the characters that support the traditional Theria hypothesis, such as the shoulder girdle, dentition, and formation of the side wall of the brain case, seem to be inconsistent and at least partially homoplasious.

Recently it has also been recognized that monotremes have tribosphenic teeth (Luo et al. 2001), thus confirming a much earlier observation of this trait (Archer et al. 1985). This settles a controversy about the presence of tribosphenic teeth in monotremes, which was believed to be a characteristic of only marsupials and eutherians and, thus, support for the traditional Theria hypothesis (Marshall 1979). Luo et al. (2001) interpret their findings as

evidence for a rare diphyletic origin of this character. The Marsupionta hypothesis, however, would also explain the evolution of the tribosphenic teeth but without the assumption of convergent evolution of this complex structure.

It is remarkable to observe the ease with which some morphological characters appear to be accepted as evolving convergently or not, as long as the traditional Theria tree is not called into question. However, more and more cornerstones for the Theria hypothesis weaken or disappear, and accumulating molecular data from mt and nuclear sequences support the Marsupionta hypothesis. Thus, 5 years after the initial molecular support for the Marsupionta hypothesis (Janke et al. 1996), it now appears reasonable to seek a consensus between the molecular results and traditional morphological studies, rather than simply referring to the molecular findings as peculiar.

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