Mitochondrial genomes of a bandicoot and a brushtail possum confirm the monophyly of australidelphian marsupials

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Recent molecular analyses suggest that the position of bandicoots is the major difficulty in determining the root of the tree of extant marsupials. To resolve this, we analyse mitochondrial genome sequences of a bandicoot (*Isodon macrourus*) and a brushtail possum (*Trichosurus vulpecula*) together with the previously available marsupial mitochondrial genomes, the Virginia opossum (*Didelphis virginiana*) and the wallaroo (*Macropus robustus*). Analyses of mitochondrial protein-coding and RNA genes strongly support the bandicoot as sister to the wallaroo and the brushtail possum. This result, combined with other recent molecular analyses, confirms the monophyly of Australidelphia (Australasian marsupials plus *Dromiciops* from South America). Further, RY coding was found to nullify AGCT coding nucleotide composition bias.

Keywords: Australidelphia; marsupial; bandicoot; *Isodon*; *Trichosurus*; nucleotide composition bias

1. INTRODUCTION

Aplin & Archer (1987) recognize seven well-defined extant marsupial orders. Out of these, Didelphimorphia (opossums), Paucituberculata (shrew opossums, including caenolestids) and the monotypic Microbiotheria (*Dromiciops gliroides*) have an American distribution, while Diprotodontia (kangaroos, possums, koalas etc.), Dasyuromorphia (marsupial mice/cats/wolves, numbat), Peramelemorphia (bandicoots) and Notoryctemorphia (marsupial moles) have an Australasian distribution. Morphological studies (e.g. Ride 1962; Szalay 1993) have offered little consistent resolution of affinities among these seven orders. Nevertheless, epididymal sperm pairing (Temple-Smith 1987) unites didelphimorphians and paucituberculates as the cohort Ameridelphia, and a continuous lower ankle joint (as opposed to separate) unites the remaining taxa (*Dromiciops* and the Australasian marsupials) as the cohort Australidelphia (Szalay 1982). Unfortunately, recent marsupial fossil finds from early Tertiary sites in South America, Antarctica and Australia (see Woodburne & Case 1996; Godthelp *et al.* 1999; Goin *et al.* 1999) have provided little information to confirm this null hypothesis of a basic split among marsupials. This paper focuses on the monophyly of the Australidelphia.

Our mitochondrial genomes for the northern brown bandicoot (*Isodon macrourus*) and the common brushtail possum (*Trichosurus vulpecula*), from the diprotodontian superfamily Phalangerioidea, supplement those for the didelphid *Didelphis virginiana* (Janke *et al.* 1994) and the wallaroo *Macropus robustus* (Janke *et al.* 1997), from the diprotodontian superfamily Macropodoidea. Several molecular studies (e.g. Retief *et al.* 1995; Kirsch *et al.* 1997) have singled out bandicoot affinities as the key to resolving the issue of australidelphian monophyly. Using DNA-hybridization distances for the (taxonomically) largest molecular study yet of marsupial relationships, Kirsch *et al.* (1997) found essentially an unresolved polytomy of

didelphimorphians, caenolestids, bandicoots and the remaining australasian taxa (plus *Dromiciops*). Mitochondrial DNA sequences (Springer *et al.* 1994; Burk *et al.* 1999; Palma & Spotorno 1999) have tended to favour bandicoot associations with one or both ameridelphian groups or, otherwise, as the marsupial root. However, nuclear genes (protamine P1 (Retief *et al.* 1995), interphotoreceptor retinoid-binding protein (Springer *et al.* 1997) and phosphoglycerate kinase (Colgan 1999)) have tended to favour a monophyletic Australidelphia. Springer *et al.* (1998) merge nuclear interphotoreceptor retinoid-binding protein with the mitochondrial genes 12S rRNA, tRNA valine, 16S rRNA and cytochrome *b*, with analyses favouring Australidelphia with 62%–84% bootstrap support.

Apart from the position of bandicoots, almost all recent molecular and morphological studies are consistent with australidelphian monophyly. Aside from ankle morphology, only a few controversial characters, such as reduced lower-incisor number, have been proposed as australidelphian synapomorphies (see Marshall *et al.* (1990) and Godthelp *et al.* (1999) for discussions of these characters). Bandicoot affinities tested in this study are: first, an association with diprotodontians, essentially confirming Australidelphia; second, bandicoots as sister to all other extant marsupials ('Aplacentalia'), a group characterized by the loss of several characters bandicoots share with placentals (these include a rudimentary chorio-allantoic placenta with umbilicus (Hughes 1974), an extended corpus luteum life and progesterone-secretion period (Gemmell 1995) and a robust patella (Szalay 1993)); and third, opossum–bandicoot (*Didelphis* with *Isodon*). Few, if any, derived morphological characters support bandicoots being associated with ameridelphians, though, as noted above, some molecular studies favour this relationship.

2. METHODS

Fresh liver samples from a northern brown bandicoot (*I. macrourus*) and a common brushtail possum (*T. vulpecula*) were

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respectively donated by Dr Robert Gemmell (University of Queensland, Australia) and Dr Phil Cowan (Massey University, New Zealand). Mitochondrial-DNA enriched extracts were obtained by preceding standard phenol–chloroform methodology with two initial 1 min spins ($400 \times g$ then $1000 \times g$). Roche Molecular Biochemicals (Switzerland) Expand Long Template Polymerase Chain Reaction System was used to amplify fragments of 3–6 kb, which overlapped identically, confirming complete mitochondrial genomes. Polymerase chain reaction (PCR) products were directly sequenced on an Applied Biosystems (USA) 377 ABI automated DNA sequencer. (See <http://imbs.massey.ac.nz/research/molevol/farside/welcome.htm> for primer details, PCR conditions and aligned datasets.) Sequences were deposited in GenBank under accession numbers AF358864 (bandicoot) and AF357238 (brushtail possum).

The Virginia opossum (Z29573) and wallaroo (Y10524) along with our bandicoot and brushtail possum sequences form the marsupial ingroup in our analyses. Outgroup taxa include *Ornithorhynchus anatinus* (platypus; X83427), two afrotherians, *Loxodonta africana* (African elephant; AJ224821) and *Orycteropus avar* (aardvark; Y18475), the xenarthran *Dasypus novemcinctus* (nine-banded armadillo; Y11832) and three laurasiatherians, *Balaenoptera physalus* (fin whale; X61145), *Hippopotamus amphibius* (hippopotamus; AJ010957) and *Talpa europaea* (European mole; Y19192).

Taxa such as the hedgehog, primates and rodents were excluded because they inflate both of the following undesirable qualities. First, the number of phylogenetic unknowns: excluding taxa that have possibly erroneous associations limits their influence on estimating parameters and transformations. Second, relative loss of ancestral signal: in general, the further a taxon is from internal nodes (due to a fast or unusual substitution regime), the less information on ancestry is retained and the more opportunity there is for substitution bias to accumulate.

Sequences were aligned manually within Se-Al v. 1.0a1 (Rambaut 1996). Ribosomal-RNA (12S and 16S) structure was based on the models of Gutell *et al.* (1993) and Springer & Douzery (1996). We designed putative tRNA secondary structures for each taxon. With gaps and ambiguous sites removed, concatenated datasets for the 11 mammals contain 14 365 nucleotide sites, with the 13 protein-coding genes contributing 11 010 sites and RNA genes contributing 3355 sites. Unlike the other proteins, NADH6 is not H-strand coded and differs in nucleotide composition. Other than providing extra data, inclusion of an L-strand gene should not affect maximum-parsimony analyses (which treat sites independently). Further, as NADH6 exclusion alters bootstrap support for our maximum-likelihood analyses by only a few percent (less than 1% among marsupials), the data shown include NADH6, reflecting our intention to be data inclusive rather than exclusive. The RNA concatenation included 12S and 16S rRNA and 21 out of the 22 tRNA genes. In agreement with Janke *et al.* (1994, 1997), tRNA-Lys is apparently a pseudogene in marsupials and is too variable to align confidently. We refer to the concatenated protein-coding sequences as PTNaa, for the amino-acid translation, and PTN123, PTN12 or PTN3, for the nucleotide sequences, with the numbers defining the codon positions included. The concatenation that includes each rRNA and the 21 tRNA genes is referred to as RNArt. RY coding pools both purines (adenine and guanine: R) and both pyrimidines (cytosine and thymine: Y) into two-state categories (R, Y).

Many phylogenetic analyses of mitochondrial genomes (including Janke *et al.* 1994, 1997) prefer to exclude PTN3 due to

Table 1. Stemminess and composition homogeneity.

(PAUP* homogeneity χ^2 p -values for the 11 mammals and for the four marsupials alone and stemminess values were calculated from variable sites only, for PTN3, PTN12, PTNaa and RNArt, each coded as standard nucleotides, RY or amino acids. χ^2 $p > 0.05$ and stemminess > 0.20 are in bold.)

	frequency χ^2		stemminess
	mammals	marsupials	mammals
PTN3 AGCT	< 0.00001	< 0.00001	0.0392
PTN3 RY	< 0.00001	< 0.00001	0.0742
PTN12 AGCT	< 0.00001	< 0.00001	0.1284
PTN12 RY	0.1424	0.99092	0.2143
PTNaa	0.30287	0.20936	0.1383
RNArt AGCT	< 0.00001	0.00012	0.1436
RNArt RY	0.87878	0.49718	0.2171

near substitution saturation. Exclusion of PTN3 from phylogenetic analyses here is warranted by their highly significant base composition heterogeneity and low signal-to-noise ratio (inferred from stemminess). ‘Stemminess’ is a relative measure of phylogenetic structure (Lanyon 1988), defined here as the percentage of uncorrected neighbour-joining total tree distance attributed to internal branches (see table 1). Phylogenetic analyses were performed on individual genes and on PTN12 and RNArt, either separately or together. Maximum-likelihood analysis of PTNaa used ProtML within MOLPHY 2.3 (Adachi & Hasegawa 1996). All maximum-parsimony (M.P.), neighbour-joining (N.J.), minimum- evolution (M.E.), maximum-likelihood (M.L.) and partition-homogeneity (P.H.) (5000 replicates) analyses of nucleotide sequences were performed within PAUP* v. 4.0b3a (Swofford 1998). Bootstrap percentage values associated with all trees were calculated from 1000 resampled heuristic searches. Maximum-likelihood and maximum-parsimony Kishino–Hasegawa (Kishino & Hasegawa 1989) tests were performed within PAUP* v. 4.0b3a (Swofford 1998).

3. RESULTS

(a) Analysis of composition heterogeneity

Quantitative analysis revealed differences in nucleotide composition between taxa. χ^2 -Tests for standard nucleotide (AGCT) base frequency revealed highly significant heterogeneity, even for PTN12 and RNArt (table 1). Conversely, for RY-coding, composition bias was not significant for either PTN12 or RNArt. RY coding also increased the stemminess of both the protein-coding and the RNArt data (table 1). Furthermore, the partition-homogeneity test for the 16 partitions (13 PTN12 genes, 12S rRNA, 16S rRNA and tRNA concatenate) revealed highly significant ($p=0.0148$) incongruence with the nucleotide (AGCT) coding, while RY congruence could not be rejected even at the 10% significance level ($p=0.1114$).

Highly significant nucleotide composition bias, yet low RY composition bias, implies significant bias among purines and/or among pyrimidines. The relative contributions of these purine and pyrimidine biases are indicated by standard deviations among the 11 taxa for relative frequencies of adenine minus guanine (A–G) and thymine minus cytosine (T–C), which are 205.9 and

Figure 1. Among-pyrimidine bias measured as relative frequency difference between thymine and cytosine (T–C) for RNArt, PTN12 and PTN3.

546.4, respectively, for PTN123 + RNArt (14 365 sites). Bias among pyrimidines is extreme for marsupials, for the PTN123 + RNArt dataset, with relatively low T:C ratios for the wallaroo (1.1095) and the brushtail possum (1.1075) and high T:C ratios for the bandicoot (1.4757) and the opossum (1.4965). This T:C bias among marsupials is greatest for PTN3, but prominent for PTN12 and RNArt (figure 1). Correlation (r^2) coefficients for T–C bias among taxa for PTN3 against PTN12 and PTN3 against RNArt are 0.8630 and 0.7786, respectively, while these coefficients for A–G are 0.2939 and 0.2629, respectively, and net transversions (Y–R) are 0.2529 and 0.0433, respectively. Hence, the main effect is from T–C bias.

(b) Phylogenetic analysis

Maximum-likelihood analysis of the PTN12 + RNArt RY-coded data, incorporating PAUP* estimates for invariable sites and gamma distribution (figure 2 and table 2), favours Australidelphia and Epitheria, and, as expected, Diprotodontia, Cetartiodactyla, Laurasiatheria and Afrotheria. This tree is also found with maximum likelihood without assuming any rate heterogeneity across sites, and with maximum parsimony. Except for Epitheria, for this selection of taxa, the same tree is found for all datasets, analytical methods and optimality criteria (figure 2). When Epitheria was not favoured, the eutherian root variously associated with Afrotheria or Atlantogenata (grouping Afrotheria and Xenarthra).

For RY-coded data, table 3 shows 13 out of the 16 gene partitions favouring Australidelphia, with the most significant support tending to be conferred by the genes with the largest number of variable sites. Across genes there is a 60% correlation (r^2) between the number of variable sites and $-\ln L(\text{Australidelphia–Aplacentalia}) + -\ln L(\text{Australidelphia–opossum/bandicoot})$. Three genes did not favour Australidelphia (NADH3, cytochrome oxidase I and Cytb), though in every case the $-\ln L$ for Australidelphia differed by less than one standard deviation. Upon concatenation of the 16 partitions, Kishino–Hasegawa tests for maximum parsimony

Figure 2. Maximum-likelihood phylogeny for RY-coded PTN12 + RNArt, with PAUP* estimates of invariant sites ($I = 0.5052$) and gamma distribution ($\alpha = 0.4106$). Table 2 contains bootstrap results for nodes a–e, which represent Australidelphia, Diprotodontia, Epitheria, Laurasiatheria and Afrotheria, respectively. Unmarked nodes gained 100% bootstrap support in all analyses.

and maximum likelihood (with and without invariant sites and gamma distribution estimates) found significant support ($p < 0.05$) for Australidelphia. Epitheria was favoured in each case, though not significantly at $p \leq 0.05$.

4. DISCUSSION

Transversions tend to code for more conservative amino-acid substitutions than do transitions (Zhang 2000), and RY-coding eliminates signal from transitions producing 'semi-stable' G–T RNA-stem pairs (Patel *et al.* 1984). These phenomena may partly explain why RY-coded PTN12 and RNArt had more than 50% greater stemminess than AGCT-coded or amino-acid translated equivalents. This implies that RY-coded data possess higher signal-to-noise ratios and, therefore, are less susceptible to phylogenetic signal erosion and systematic biases. Certainly, in this dataset, RY coding of nucleotides greatly reduced composition bias (table 1) and incongruence among gene partitions. Simply eliminating transitions may be less effective than RY coding, as unobserved (multiple) transitions may bias the distribution of the four transversion categories. Thus, RY coding appears to be the most appropriate treatment of this data.

The maximum-likelihood tree (figure 2) incorporating RY coding and estimates for an invariant-sites-plus-gamma-distribution model represents our best estimate of the phylogeny. This tree favours Australidelphia and indicates overall substitution-rate homogeneity among marsupials. Kishino–Hasegawa tests for maximum likelihood (table 3) reject Aplacentalia and an opossum–bandicoot grouping for RY-coded PTN12 + RNArt, even with the increased variance-to-signal ratio that Sullivan *et al.* (1999) suggest is imposed by including invariant sites and gamma distribution estimates. Australidelphia is confirmed for each maximum-likelihood model and

Table 2. Bootstrap support (1000 replications) for the nodes a (Australidelphia), b (Diprotodontia), c (Epitheria), d (Laurasiatheria) and e (Afrotheria) in figure 2.

(Maximum-parsimony analyses for PTN12 + RNArt (AGCT and RY) and the translated protein-coding concatenation (PTNaa) are unweighted. Maximum-likelihood models include F81 and HKY85 with a 2:1 transition-to-transversion ratio for nucleotides, Cavender–Farris for RY coding and mtREV-24F for PTNaa. Minimum-evolution (M.E.) distances are uncorrected, GTR corrected or LogDet corrected. Bold indicates Epitheria.)

	(Australidelphia)	(Diprotodontia)	(Epitheria)	(Laurasiatheria)	(Afrotheria)
maximum parsimony (PTNaa)	100	99	3 ^a	100	47
maximum-parsimony (AGCT)	100	100	43 ^b	99	91
maximum-parsimony (RY)	100	100	92	99	98
maximum-likelihood (PTNaa) mtREV-24F	100	100	3 ^a	100	53
maximum-likelihood (AGCT) F81	99	100	31 ^b	100	96
maximum-likelihood (AGCT) HKY85	100	100	49	100	97
maximum-likelihood (RY)	100	100	82	100	99
M.E. (PTNaa) uncorrected	100	100	1 ^a	100	83
M.E. (AGCT) uncorrected	100	100	1 ^a	100	96
M.E. (AGCT) GTR	100	100	3 ^a	100	97
M.E. (AGCT) LogDet	100	100	3 ^a	100	97
M.E. (RY) uncorrected	100	100	18 ^a	100	99
M.E. (RY) LogDet	100	100	19 ^a	100	98

^a Afrotheria as the basal placental clade.

^b Atlantogenta as the basal placental clade.

maximum parsimony, with two-tailed significance values well below $p = 0.05$. These results combined with bootstrap resampling support of 99% or greater, regardless of coding (nucleotide, RY, amino acid), substitution model and optimization criterion (maximum likelihood, maximum parsimony, minimum-evolution) for PTN12 + RNArt (table 2), must be considered as convincing evidence for Australidelphian monophyly.

Nucleotide composition heterogeneity is highly significant across taxa (table 1), even with PTN3 excluded, warning against the use of standard nucleotide coding for the analysis of mitochondrial sequences. We suspect that nucleotide composition heterogeneity is more common than reported for mitochondrial data, as many studies fail to exclude constant sites from χ^2 base-frequency tests, thus diluting nucleotide composition variability between taxa. With RY composition for PTN12 and RNArt being robust to bias (table 1), highly significant nucleotide composition bias implies considerable bias among purines and/or among pyrimidines. A considerably greater standard deviation between taxa for T–C (546.4) than for A–G (205.9) indicates that pyrimidines make the greatest contribution to nucleotide composition bias. Greater PTN3 substitution synonymy (including all PTN3 transitions) probably explains why the T–C variation is far greater for PTN3 than for PTN12 or RNArt (figure 1). Synonymous substitutions are expected to be neutral (Maynard Smith 1994) and reflect underlying mutation biases. However, mutation biases that are small compared to the constraints of non-synonymous substitutions and RNA folding will explain little of the variation across taxa for PTN12 and RNArt. As such, the potential phylogenetic influence of T–C mutation bias for this dataset is illustrated by the high correlation (r^2) of T–C variation among taxa for PTN3 with that of PTN12 and RNA (86% and 78%, respectively).

The bandicoot and opossum have a greater thymine minus cytosine (T–C) than the diprotodontians and

outgroup taxa for PTN3, PTN12 and RNArt (figure 1). This T–C bias will tend to act as a non-independent across-sites ‘synapomorphy’ for opossum–bandicoot. However, australidelphian signal strength is such that it receives at least 99% bootstrap support even for standard nucleotide coding (AGCT) analyses (table 2). Contrary to this, depending on coding and optimality criteria, Afrotheria, Atlantogenta (Xenarthra, Afrotheria) and Xenarthra are variously favoured as basal among the eutherians (figure 2 and table 2). Previous mitochondrial genome studies that included laurasiatherians, afrotherians and the armadillo have tended to associate the armadillo with laurasiatherian taxa (Arnason *et al.* 1999; Mouchaty *et al.* 2000). However, morphological studies almost uniformly support Epitheria, finding Xenarthra (or Edentata, depending on pangolin affinities) basal among extant eutherians (e.g. Rose & Emry 1993; Shoshani & McKenna 1998). As such it is interesting that maximum likelihood and maximum parsimony favoured Epitheria in every ‘phylogenetics friendly’ RY-coded analysis of PTN12 + RNArt (table 2). Inclusion of primate, rodent, lagomorph and erinacid sequences is important for testing Epitheria. However, additional sequences from these groups, and Xenarthra and Afrotheria, are required to maintain the sampling strategy of this study (to minimize the number of phylogenetic unknowns and to minimize the loss of ancestral signal). Increased sampling will help discriminate phylogenetic signal from systematic biases, reduce variance and, hence, allow eutherian rooting to be amenable to more sophisticated methods, such as data partitioning.

One obvious drawback of pooling adenine and guanine as R and thymine and cytosine as Y is that it reduces overall genetic variability and hence increases the susceptibility to sampling error. This is not a problem for PTN12 + RNArt, as table 2 shows that RY coding provides equal or better support than does nucleotide-coding for ‘known’ relationships such as

Table 3. Log-likelihood differences between alternative marsupial roots for the 16 protein-coding and RNA gene partitions.

(For each gene partition and hypothesis of bandicoot relationships, the difference in RY-coded $-\ln$ likelihood in terms of the number of standard deviations (Kishino–Hasegawa (K.H.) test) below the favoured hypothesis (✓) is indicated as *(> 1 s.d.); **(> 1.5 s.d.); ***(> 2 s.d.). The number of RY variable sites among the 11 taxa for each partition is given in brackets. *Isodon* and *Trichosurus* protein-coding and RNA-coding genes conform to the standard marsupial order (Janke *et al.* 1994, 1997). K.H. test p -values for maximum parsimony (M.P.), maximum likelihood (M.L.) and maximum likelihood plus invariant sites plus gamma model (M.L. + I. + Γ) are given for PTN12 + RNArt.)

	Aus. ^a	Apl. ^b	o./b. ^c
NADH1 (105)	✓	10.20*	13.37**
NADH2 (227)	✓	7.47*	7.43*
COI (46)	3.53	✓	3.53
COII (48)	✓	3.42	3.53
ATP8 (46)	✓	0.96	0.96
ATP6 (87)	✓	1.63	1.63
COIII (61)	✓	4.85*	4.85*
NADH3 (46)	3.01	1.20	✓
NADH4L (54)	✓	2.77	2.30
NADH4 (245)	✓	11.00**	11.71**
NADH5 (315)	✓	17.13***	13.65**
NADH6 (108)	✓	0.23	2.19
Cytb (99)	1.83	4.51*	✓
12S (158)	✓	4.63	5.32*
16S (246)	✓	2.81	7.78*
tRNA (191)	✓	6.56*	3.91
PTN12 + RNArt K.H. test p -values			
M.P.	✓	< 0.0001	< 0.0001
M.L.	✓	< 0.0001	< 0.0001
M.L. + I. + Γ	✓	0.0256	0.0310

^a Australidelphia.

^b Aplacentalia.

^c Opossum/bandicoot.

Diprotodontia, Laurasiatheria and Afrotheria. Cao *et al.* (1998) showed that, owing to stochastic variation and systematic differences between individual genes, support for certain affinities only emerges upon summation or concatenation of mitochondrial genes. However, given 100% bootstrap support from the concatenated sequence and relative congruence (suggested by the homogeneity-partitioning test), it is not surprising that for the RY-coded data, 13 out of the 16 gene partitions favour Australidelphia (table 3). As alternative hypotheses favoured by individual genes (opossum–bandicoot by NADH3 and Cytb or Aplacentalia by COI) differ in $-\ln$ likelihood from Australidelphia by less than one standard deviation, they cannot be said to conflict with Australidelphia. Notably, these three genes are below the 16-partition average for variable site number, and correlation (r^2) suggests that the number of variable sites describes 60% of the variation in australidelphian signal strength among the gene partitions. As such, a greater relative importance of stochastic variation in small datasets, combined with a T–C bias among marsupials, may explain the alternative relation-

ships offered by studies of one or a few mitochondrial genes (e.g. Springer *et al.* 1994; Cao *et al.* 1998; Burk *et al.* 1999; Palma & Spotorno 1999). With many of the more variable gene partitions favouring Australidelphia over one or both alternatives by one or even two standard deviations (table 3), the individual genes taken as a whole offer overwhelming support for Australidelphia. This corroborates the mostly nuclear molecular studies (Retief *et al.* 1995; Springer *et al.* 1997, 1998; Colgan 1999) tentatively favouring Australidelphia.

Australidelphian monophyly has important biogeographical implications. Unlike Aplacentalia or opossum–bandicoot, Australidelphia does not require a complex pattern of trans-Antarctic dispersals and/or continental extinctions of various marsupial orders. Microbiotheriids were present in the Antarctic Eocene (Goin & Carlini 1995). Determining whether they are sister to or nested within the australasian marsupials will provide another major constraint for testing hypotheses of marsupial biogeography. However, in the absence of molecular-clock and reference-date combinations with satisfactory accuracy for examining just the last few million years of the Cretaceous and the first few million years of the Tertiary, respectively, biogeographical inference from molecular sequences is limited. Providing a solid phylogeny is perhaps the most important role for this and other molecular studies in understanding marsupial biogeography.

Considering the difficulties of placing bandicoots with respect to the marsupial root in previous studies, the clear australidelphian affinities shown here with both protein-coding and RNA genes hint that whole-mitochondrial-genome analysis should fully resolve marsupial interordinal relationships. For palaeontology, which can pinpoint taxa in space and time, this would provide a reference independent of morphology for determining the reliability of specific character transformations. For example, our results increase the diagnostic weight of a continuous lower ankle joint as an australidelphian synapomorphy, while implying that the robust patella and placental-like bandicoot reproductive traits are convergent with eutherians, or were lost more than once by marsupials.

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