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Molecular phylogenetics of the Diprotodontia (kangaroos, wombats, koala, possums, and allies)

M.J. Osborne,[a,b,*] L. Christidis,[a] and J.A. Norman[a]

[a] *Sciences Department, Museum Victoria, P.O. Box 666E, Melbourne 3001, Australia*
[b] *Department of Genetics, La Trobe University, Bundoora 3083, Australia*

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Abstract

Mitochondrial ND2 sequences were used to investigate the phylogenetic relationships amongst 31 diprotodontid marsupials (kangaroos, wombats, koala, possums, and allies). ND2 sequences were analyzed separately and in conjunction with available 12S rDNA sequences for 22 diprotodontid taxa. Phylogenetic analyses consistently identified monophyly for the Burramyoidea, Phalangeroidea, Petauroidea, Tarsipedoidea, Macropodoidea, and the Vombatiformes. Like previous molecular and morphological studies, relationships between the super-families were less well resolved. Inconsistency between taxonomic rank and genetic distance was identified amongst the diprotodontids.

1. Introduction

The Diprotodontia is the largest and most diverse of the marsupial orders comprising approximately 131 species of kangaroos, wombats, koalas, possums, and allies (Nowak, 1999). The order is divided into the suborders Vombatiformes and Phalangerida (Aplin and Archer, 1987). Vombatiformes comprises two super-families, Vombatoidea (wombats) and Phascolarctoidea (koala) while Phalangerida is more diverse, with five super-families, Macropodoidea (kangaroos, rat-kangaroos, and wallabies), Tarsipedoidea (honey possum and feather-tail possums), Phalangeroidea (brushtails and cuscuses), Petauroidea (gliders, striped possums, and ringtails), and Burramyoidea (pygmy possums). The last four super-families are often grouped as 'possums.' The lineages comprising the Macropodoidea (Macropodidae and Hypsiprymnodontidae) and Vombatiformes (Vombatoidea and Phascolarctoidea) have each been consistently recognised as monophyletic by morphological characters (e.g., Aplin and Archer, 1987; Flannery, 1987; Marshall et al., 1990) and molecular data (e.g., Kirsch, 1977; Springer and Kirsch, 1991). In contrast, monophyly of the possums is not easily demonstrated by either morphological traits or by molecular evidence (e.g., Aplin and Archer, 1987; Baverstock et al., 1990; Springer and Kirsch, 1991; Springer et al., 1999; Springer et al., 1994).

A close evolutionary connection between the possums and kangaroos has been suggested by numerous authors (e.g., Aplin and Archer, 1987; Bensley, 1903; Flannery, 1987; Kirsch, 1977; Szalay, 1994). Flannery (1987) discussed two hypotheses regarding the relationships of the kangaroos to the possums. The first hypothesis (and previously suggested by Archer, 1984) was that a sister relationship existed between the Macropodoidea and Phalangeroidea, and that this group was then the sister lineage of the remaining possums. Flannery (1987) identified four synapomorphies in support of this association. The alternative hypothesis that the kangaroos were the sister group to all possums was based on a single synapomorphy. Nevertheless, Flannery (1987) preferred this latter hypothesis, arguing that the synapomorphies cited in support of the first hypothesis were either independently derived in both lineages or that they were plesiomorphic for the possums and kangaroos but had been lost in all species except for the Phalangeroidea and the Macropodoidea.

* Corresponding author. Present address: 81 Rowe St, Nth. Fitzroy, Vic. 3068, Australia. Fax: +61-3-83417442.
E-mail addresses: megozzz@yahoo.com.au, m.j.osborne@latrobe.edu.au (M.J. Osborne), .

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A sister relationship between kangaroos and possums is supported by DNA–DNA hybridization data (Kirsch et al., 1997), whereas micro-complement fixation of albumin data (Maxson et al., 1975) indicated that the kangaroos are highly divergent from the other diprotodontids, whilst a more comprehensive study united *Trichosurus* (Phalangeroidea) and *Macropus* (Macropodoidea) (Baverstock et al., 1990). Micro-complement fixation of albumin data also indicated that Phascolarctidae (koala) and Vombatidae (wombat) fell within the cluster of possum families (Baverstock et al., 1990). Springer et al. (1994) using 12S rDNA sequences also found some evidence for a sister relationship between the Vombatidae and the Phalangeridae. However, this study also failed to confirm the monophyly of Vombatiformes and of other recognised groups such as the Petauridae and Pseudocheiridae.

Further debate on the systematics of the diprotodontids centres on the affinities of *Acrobates pygmeaus* (feather-tail glider), *Distoechurus pennatus* (feather-tail possum) and *Tarsipes rostratus* (honey possum). The inclusion of *Tarsipes* within the possums was based on morphological characters (Aplin and Archer, 1987) and micro-complement fixation of albumin data (Baverstock et al., 1990) which linked *Tarsipes* with *Acrobates*. Baverstock et al. (1990) showed that *Acrobates* and *Distoechurus* were sister taxa. Furthermore, micro-complement fixation of albumin data suggested that *Acrobates*, *Distoechurus*, and *Tarsipes* were linked to the Petauroidea (Baverstock et al., 1990). This relationship is also supported by DNA–DNA hybridization data (Edwards and Westerman, 1995; Kirsch et al., 1997) and 12S rDNA sequences (Springer et al., 1994). An alternative arrangement proposed by Kirsch (1977), and later supported by Szalay (1994) suggested that the Acrobatidae were, instead, linked to the Burramyidae.

The affinities of the Burramyidae have also been debated with Szalay (1994) including this group within the Petauridae on the basis of pedal anatomy. Alternatively, the DNA–DNA hybridization study of Kirsch et al. (1997) indicated that the Burramyidae was linked to the Phalangeridae. Aplin and Archer (1987) suggested Burramyidae may be basal to the remainder of the possums.

Here we use DNA sequence data from the mitochondrial nicotinamide dehydrogense sub-unit two gene (ND2) from 31 taxa (representing all ten families within the diprotodontids), in combination with available 12S rDNA sequences for 22 species to investigate relationships between the seven super-families of Diprotodontia. In particular we will be investigating whether: (1) the Vombatiformes and Phalangerida are each monophyletic assemblages; and (2) the four "possum" superfamilies form a monophyletic group to the exclusion of the Vombatiformes and the Macropodoidea.

2. Methods

2.1. Taxon sampling

Specimens examined, source of sample, collection localities and GenBank accession numbers are given in Table 1. Genomic DNA was extracted from tissue samples following the procedures of Gemmell and Akiyama (1996) for representatives of the following seven species: *Tarsipes rostratus* ($n = 2$), *Acrobates pygmaeus* ($n = 2$), *Phascolarctos cinereus*, *Hypsiprymnodon moschatus*, *Macropus agilis*, *Potorous longipes*, *Isoodon macrourus*, and *Antechinus flavipes*. Sequence data previously obtained from 25 species: *Burramys parvus Cercartetus caudatus*, *C. concinnus*, *C. lepidus*, *C. nanus* (Osborne and Christidis, 2002a), *Dactylopsila trivirgata*, *Dactylonax palpator*, *Petaurus breviceps*, *P. norfolcensis*, *Pseudocheirus peregrinus* ($n = 4$), *Petauroides volans*, *Gymnobelideus leadbeateri* (Osborne and Christidis, 2001), *Trichosurus vulpecula*, *T. caninus*, *Wyulda squamicaudata*, *Phalanger gymnotis*, *P. lullulae*, *P. seri-*

Table 1
Species sampled, voucher specimen location, locality information and GenBank Accession Nos.

Species	Common name	Voucher	Locality	GenBank Accession Nos.
Acrobates pygmaeus	Feather-tail glider	M16432 (ANWC)	Mongarlowe River, NSW	AF25986
**Acrobates pygmaeus*		AP2 (MU)	Unknown	–
Tarsipes rostratus	Honey possum	TR1 (LU)	Unknown	AF25979
**Tarsipes rostratus*		M55442 (WAM)	Camel Lake Nature Reserve, WA	–
Potorous longipes	Long-footed potoroo	DNA (LU)	Unknown	AF25980
Macropus agilis	Agile wallaby	M16443 (ANWC)	Shoalwater Bay, NSW	AF25981
Hypsiprymnodon mochatus	Musky rat-kangaroo	ABTC27634 (SAM)	Severin Forest, Nth. QLD	AF25982
Phascolarctos cinereus	Koala	M16990 (ANWC)	Nullica State Forest, NSW	AF25985
Antechinus flavipes	Yellow-footed antechinus	M16325 (ANWC)	NE NSW (Nth of Coffs Harbour)	AF25984
Isoodon macrourus	Northern brown bandicoot	M16374 (ANWC)	Shoalwater Bay, NSW	AF25983

Asterisks indicate individuals for which double stranded sequence was not obtained. ANWC, Australian National Wildlife Collection; MU, Melbourne University; LU, La Trobe University; WAM, Western Australian Museum; MV, Museum Victoria; SAM, South Australian Museum; WA, Western Australia; NSW, New South Wales; QLD, Queensland.

cus, *P. vestitus*, *P. orientalis*, *P. carmelitae*, *Spilocuscus maculatus*, *S. rufoniger*, *Vombatus ursinus* (Osborne and Christidis, 2002b), and *Macropus robustus* (GenBank Accession No. Y10524) were included. *Isoodon macrourus* (Order Peramelemorphia, Family Peramelidae) and *Antechinus flavipes* (Order Dasyuromorphia, Family Dasyuridae), in addition to *Didelphis virginiana* (Order Didelphimorphia, Family Didelphidae) (Janke et al., 1994) are included as outgroups. Sister group status of the Peramelemorphia to the other diprotodontids has been suggested by Bensley (1903) and Ride (1959, 1964) based on the shared character of syndactyly. Aplin and Archer (1987) also considered that the Diprotodontia was the likely sister lineage of the Australian Polyprotodontia (comprising Dasyuromorphia–Peramelemorphia–Notoryctemorphia).

Sequence was also available for 395 base pairs of the mitochondrial 12S rDNA (Burk et al., 1998; Hamilton and Springer, 1999; Springer et al., 1994) for 22 species. This data was analyzed in conjunction with the ND2 sequences (reduced to include the same taxa). *Isoodon macrourus* (Springer et al., 1994), *Antechinus flavipes* (Armstrong et al., 1998) and *Didelphis virginiana* (Janke et al., 1994) were again used as the outgroups.

2.2. PCR amplification and sequencing

The ND2 gene was amplified and sequenced using the primers and methods described in Osborne and Christidis (2001). Double stranded sequence was obtained from a representative of each taxon.

2.3. Analyses

ND2 sequences were aligned by visual inspection. Amino acid translations were obtained using MEGA (Molecular Evolutionary Genetic Analysis) Version 2.1 (Kumar et al., 2001).

Parsimony analyses were conducted using PAUP 4.0b4a (Swofford, 2000). Minimum length trees were found using the heuristic search option (random addition, 10 replicates). Parsimony uninformative characters were excluded. Data partitions were used to exclude more rapidly evolving characters and included excluding third position transitions, weighting transversions twice that of transitions. Consensus trees (50% majority rule) were computed if more than one equally parsimonious tree was found.

Modeltest Version 3.06 (Posada and Crandall, 1998) was used to determine the most appropriate distance model for maximum likelihood analyses. The PAUP block (modelblock3) provided with Modeltest was used to compare 56 different models of DNA substitution, the program interprets the resulting *P* values and selects the model that best describes the data. For both the ND2 and the combined data the general time reversible model (GTR) (Rodriguez et al., 1990) with an unequal distribution of nucleotides, a proportion of invariant sites and among site rate variation (gamma distribution, discrete approximation) was found to be the most applicable model. Hence the maximum likelihood analyses were conducted using this model with empirical base frequencies using the heuristic search option (with 10 random additions). For the ND2 data the assumed proportion of invariant sites was 0.2627 and the shape parameter of the gamma distribution was 0.5378. For the combined data the assumed proportion of invariant sites was 0.1720 and the shape parameter of the gamma distribution was 0.2859.

Neighbor-joining analyses (Saitou and Nei, 1987) were conducted using the General Time Reversible (Rodriguez et al., 1990) and HKY85 (Hasegawa et al., 1985) distance options in PAUP 4.0b4a (Swofford, 2000). Neighbor-joining analyses was also conducted using amino acid sequences (total character change).

Branch support was evaluated using the bootstrap approach (Felsenstein, 1985). One thousand replications were conducted for the Neighbor-joining and parsimony analyses. One hundred replications were conducted for the maximum likelihood tree for the combined data set. For the maximum likelihood tree of the ND2 data, bootstrapping was not conducted because of computational constraints given the large number of taxa considered. Branch support in parsimony trees was also estimated by the decay index value (d) (Bremer, 1988) as calculated using the program Auto Decay 4.02 (Eriksson, 1998). In addition, Kishino–Hasegawa tests (KH) (Kishino and Hasegawa, 1989) were conducted (using PAUP Version 4.0b10) to test for significant differences between constrained trees and the best trees found by maximum parsimony and maximum likelihood methods.

Relative rate tests were conducted using the program RRTree (Robinson-Rechavi and Huchon, 2000). RRTree allows comparisons to be made between two lineages (containing any number of DNA sequences) and an outgroup. This program permits different types of substitution to be compared, for example synonymous or nonsynonymous substitutions. As saturation is likely to be evident for synonymous substitutions between the families being compared, only K_a (number of non-synonymous per non-synonymous site) was computed. K_a was estimated using the method of Li (1993). Single representatives of all taxa were used.

3. Results

3.1. Sequence analyses

Comparisons of 1040 base pairs of ND2 between the 31 ingroup taxa identified 643 variable sites with 556 informative for parsimony analyses. The majority of

informative changes were at the third codon position (309), with 168 at the first codon position and 79 at the second codon position.

There was a deficiency of guanine residues in ND2, which were present at an average frequency of 0.08. Adenines occurred the most frequently (0.35), followed by cytosine residues (0.30). The paucity of guanines (0.02) and the excess of adenines (0.47) was most extreme at the third position. The bias in favor of pyrimidines was the most extreme at the second position, with a frequency of 0.77.

Sequence divergences ranged from 23.00% to 37.83% for comparisons between the super-families Burramyoidea, Petauroidea, Phalangeroidea, Tarsipedoidea, Macropodoidea, Vombatoidea, and Phascolarctoidea. The minimum value was observed between *Potorous longipes* (Macropodoidea) and *Phalanger lullulae* (Phalangeroidea) whilst the maximum value was between *Phascolarctos cinereus* (Phascolarctoidea) and *Acrobates pygmaeus* (Tarsipedoidea). Divergences between families (within super-families) ranged from 23.04% to 33.58%. There was 23.38% divergence between *Vombatus* (wombat) and *Phascolarctos* (koala) and 12.70% to 25.65% between the kangaroos *Macropus*, *Potorous*, and *Hypsiprymnodon*.

3.2. Phylogenetic analyses

The following monophyletic groups were consistently identified by ND2 and combined sequences (ND2 and 12S rDNA): Phalangeroidea (comprising *Phalanger*, *Spilocuscus*, *Wyulda*, and *Trichosurus*), Macropodoidea (comprising Macropodidae and Hypsiprymnodontidae) and Vombatiformes (comprising Vombatoidea and Phascolarctoidea). Monophyly for the lineages comprising Burramyoidea (*Burramys* and *Cercartetus*), Petauroidea (Petauridae and Pseudocheiridae), and Tarsipedoidea (comprising *Acrobates* and *Tarsipes*) (12S rDNA sequence was not available for *Acrobates*) were recognised in the majority of analyses. Within the Macropodoidea, *Hypsiprymnodon* was basal and *Potorous* was identified as the sister taxon of *Macropus*.

Identical topologies were obtained when GTR or HKY85 distances were used to derive Neighbor-joining trees (Fig. 1). ND2 and combined sequences identified the Burramyoidea as basal within the diprotodontids and identified a clade comprising Macropodoidea, Phalangeroidea, and Vombatiformes. The latter two super-families were identified as sister lineages (67–70% bootstrap support) (Table 2). Combined sequences identified *Tarsipes* as the sister taxon to the Petauroidea.

Neighbor-joining analyses of the amino acid sequences linked Phalangeroidea, Macropodoidea and Vombatiformes however there was less than 50% nodal support (Table 2). Burramyoidea was identified as the sister lineage to this group. *Acrobates* fell within the Petauroidea and *Tarsipes* was identified as basal within the Diprotodontia.

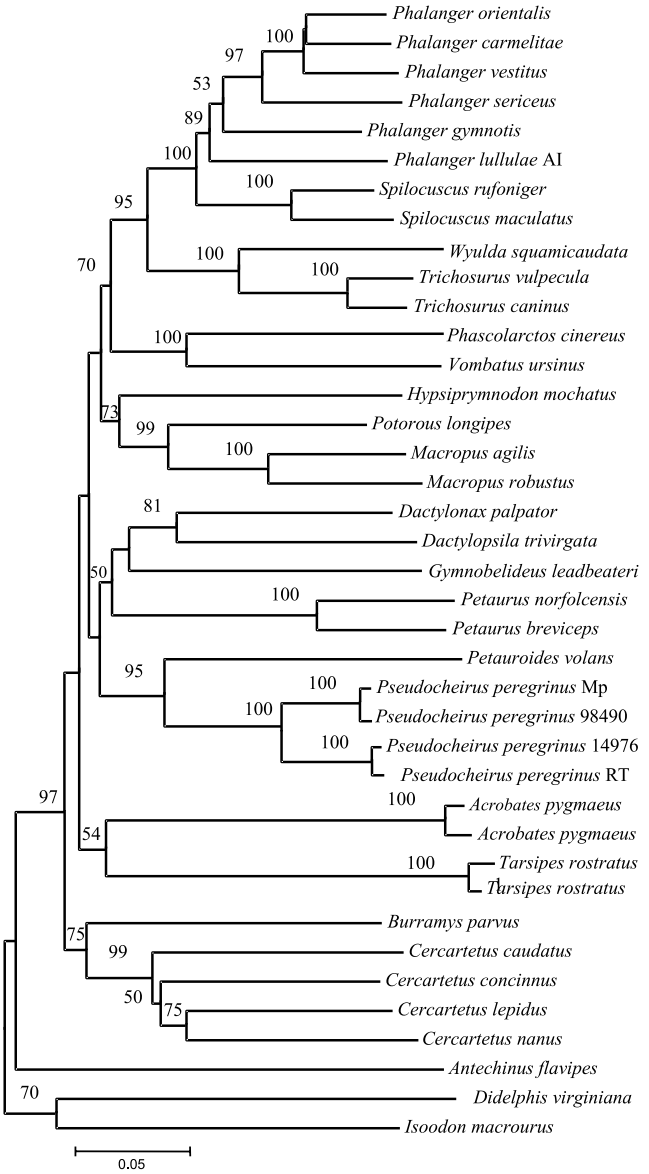

Fig. 1. Neighbor-joining tree (using GTR distances) for ND2 sequences. Bootstrap values are given above branches (bootstrap values below 50% are not shown). The outgroups are *Antechinus flavipes*, *Didelphis virginiana*, and *Isoodon macrourus*.

Maximum likelihood analyses of ND2 ($-\mathrm{Ln} = 16675.82$) failed to identify monophyly for the Diprotodontia, however each of the families comprising this group were recognised as monophyletic (with the exception of the Tarsipedoidea). *Tarsipes* was identified as the sister taxon to the Burramyoidea, whilst *Acrobates* fell within the Petauroidea. The Macropodoidea was recognised as the sister lineage to the Petauroidea. The combined sequences ($-\mathrm{Ln} = 14848.26$) produced a tree in which the Burramyoidea again occupied the basal position within the diprotodontids (Fig. 2). The Pha-

Table 2
Bootstrap support values for diprotodontid lineages for Neighbor-joining (NJ, GTR method; AA, amino acid sequences for ND2), maximum likelihood (ML) and parsimony analyses (Bremer support values are given after the bootstrap values) for ND2 and combined ND2 and 12S rDNA sequences (Com)

Analyses	ML	NJ			MP		1, 0		2, 1	
Gene	Com	ND2	AA	Com	ND2	Com	ND2	Com	ND2	Com
Phalangeroidea	100	95	99	100	93, 7	82, 2	95, 9	84, 4	85, 13	83, 3
Petauroidea	50	50	*	60	*, 3	*, 2	*, 1	*, 1	*, 2	*, 2
Burramyoidea	80	75	97	72	–	–	56, 0	–	–	–
Tarsipedoidea	n.e	54	–	n.e	*, 8	n.e	*, 0	n.e	*, 6	n.e
Macropodoidea	84	73	91	97	*, 4	87, 7	51, 2	72, 6	*, 3	89, 7
Vombatiformes	96	100	73	100	99, 22	99, 18	99, 16	97, 19	99, 26	98, 20

Nodes with less than 50% bootstrap support are denoted with an asterisk. MP, maximum parsimony; 1, 0—excluding third position transitions; and 2, 1—weighting transversions twice that of transitions.

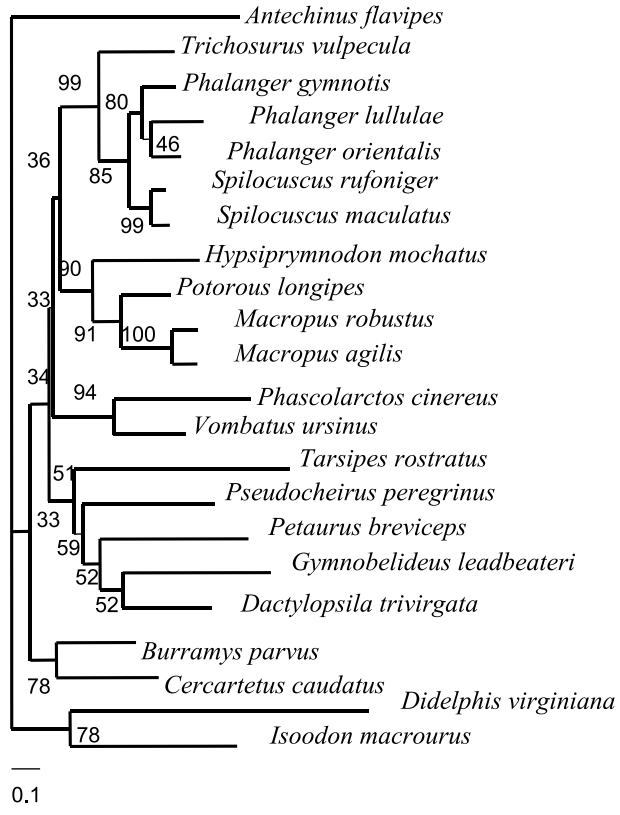

Fig. 2. Maximum likelihood tree for combined ND2 and 12S rDNA sequences. Bootstrap values are given above branches. The outgroups are *Antechinus flavipes*, *Didelphis virginiana*, and *Isoodon macrourus*.

langeroidea and Macropodoidea were linked, with Vombatiformes identified as the sister lineage to this assemblage. *Tarsipes* was the sister taxon of the Petauroidea. For both the ND2 and combined sequences KH tests did not reveal any significant differences between the maximum likelihood tree and the alternative constrained trees (Table 3).

Unweighted parsimony analyses of the ND2 sequences revealed four trees of equal length (length = 4010, consistency index = 0.263, retention index = 0.106, rescaled consistency index = 0.106) (Table 2). The majority rule consensus tree identified an assemblage containing Petauroidea, Vombatiformes, Phalangeroidea, and Macropodoidea. The latter two groups were identified as sister lineages. The Tarsipedoidea was basal to this entire assemblage whilst *Burramys* and *Cercartetus* were basal within the diprotodontids but were not identified as monophyletic. KH tests revealed no significant differences between the parsimony tree and constrained trees (Table 3).

Excluding third position transitions produced three trees in which the Vombatiformes were basal within the diprotodontids (length = 2505, consistency index = 0.233, retention index = 0.481, rescaled consistency index = 0.128). In the majority rule consensus tree Macropodoidea, Petauridae, and Pseudocheiridae were linked and Acrobates fell within the latter group (Table 2). Burramyoidea was identified as the sister lineage to this group, whilst Phalangeroidea was basal to this entire assemblage. The Vombatiformes were basal to all diprotodontids (with the exception of *Tarsipes* which fell outside this group). Weighing transversions twice that of transitions produced a single tree in which Burramyoidea and Phalangeroidea where sister lineages whilst the Tarsipedoidea was the sister lineage of the Petauroidea (length = 5547, consistency index = 0.258, retention index = 0.443, rescaled consistency index = 0.114) (Fig. 3). The Macropodoidea was the sister lineage to the Petauroidea–Tarsipedoidea assemblage. The Vombatiformes occupied the basal position within the Diprotodontia. The constrained trees were not significantly worse than the weighted trees as shown by the KH tests. The only exception to this was when third position transitions were excluded, where the constrained tree: Phalangeroidea and Vombatiformes was significantly worse than the best tree found by parsimony analyses (Table 3).

For the combined sequences, unweighted parsimony analysis (length = 3217, consistency index = 0.340, retention index = 0.338, rescaled consistency index = 0.115) and weighting transversions twice that of transitions (length = 3307, consistency index = 0.346, retention

Table 3
Significance levels for Kishino–Hasegawa tests

Constraint (versus best)	ND2	Combined	ND2			Combined		
	Maximum likelihood		MP	1/0	2/1	MP	1/0	2/1
1. Mono possums	0.309	0.057	0.701	0.213	0.175	0.005*	0.458	0.016*
2. Phalangeroidea–Macropodiformes	0.542	0.056	0.941	0.211	2.667	0.017*	0.778	<0.001*
3. Phalanegroidea–Vombatiformes	0.744	0.071	0.945	0.663	0.738	0.003*	0.461	0.002*

MP, maximum parsimony; 1, 0—excluding third position transitions and 2, 1—weighting transversions twice that of transitions. The following constrained trees were compared to the minimum length trees found by parsimony analyses, and the best trees found by maximum likelihood analyses: (1) Monophyly of 'possums,' (2) monophyly of Phangeroidea and Macropodoidea (as suggested by Flannery, 1987), (3) monophyly of Phalangeroidea and Vombatiformes. Significance level at $P < 0.05$ are denoted with an asterisk.

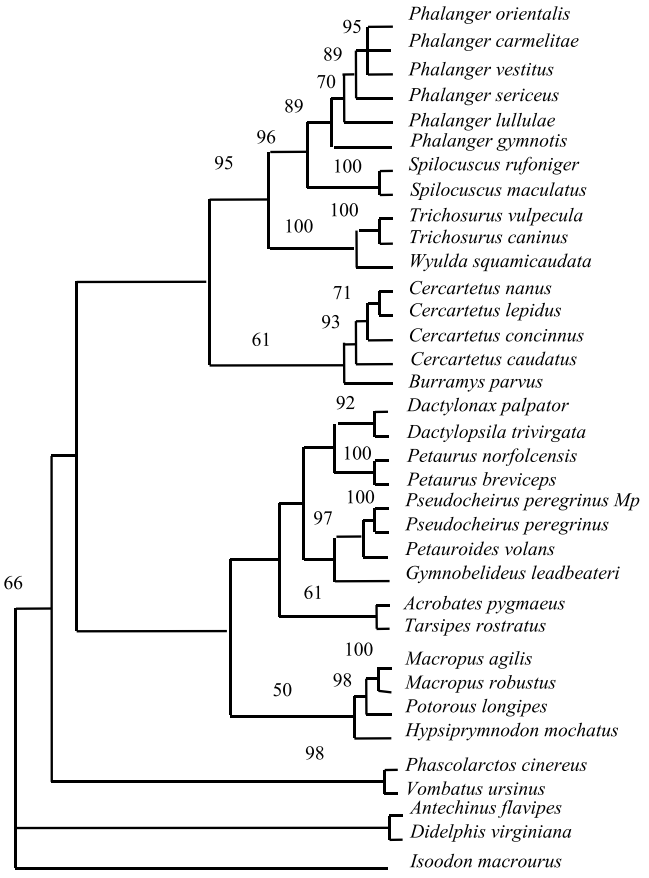

Fig. 3. Parsimony tree (weighting transversions twice that of transitions) for ND2 sequences. The outgroups are *Antechinus flavipes*, *Didelphis virginiana*, and *Isoodon macrourus*.

index = 0.338, rescaled consistency index = 0.117) produced essentially the same topology as the NJ tree. *Burramys* and *Cercartetus* were basal but not monophyletic, *Tarsipes* was linked to *Petaurus*, and *Pseudocheirus* was linked to *Gymnobelideus–Dactylopsila*. For unweighted parsimony and weighting transversions twice that of transitions KH tests indicated that the alternative topologies were not supported by the combined sequences (Table 3). Excluding third position transitions also produced a similar topology (length = 3043, consistency index = 0.342, retention index = 0.340, rescaled consistency index = 0.117) however, the Vombatiformes, Macropodiformes, and Phalangeroidea formed an unresolved trichotomy. *Tarsipes* again grouped with *Petaurus*, and *Burramys* and *Cercartetus* were not recognised as monophyletic. *Cercartetus* occupied the basal position within the Diprotodontia. This tree was not significantly better than the constrained trees (Table 3).

3.3. Relative rate test

Significant differences in the rates of nonsynonymous substitutions were observed for six comparisons: Macropodoidea and Petauroidea; Macropodoidea and *Tarsipes*; *Acrobates* and *Trichosurus–Wyulda*; *Acrobates* and *Phalanger–Spilocuscus*; *Acrobates* and Macropodoidea; and *Acrobates* and Burramyoidea. In the four comparisons involving *Acrobates* a faster rate of evolution was observed in this taxon. In the two comparisons involving Macropodoidea, Petauroidea, and *Tarsipes* had the faster rates.

4. Discussion

4.1. Phylogenetic relationships within super-families

Analysis of the total ND2 data consistently identified the following diprotodontid lineages as monophyletic: Vombatiformes (*Vombatus*, *Phascolarctos*), Macropodoidea (*Potorous*, *Macropus*, *Hypsiprymnodon*), Phalangeroidea (*Trichosurus*, *Wyulda*, *Spilocuscus*, *Phalanger*), Petauroidea (Petauridae, Pseudocheiridae), and Tarsipedoidea (*Tarsipes*, *Acrobates*). Both NJ and ML trees identified a monophyletic Burramyoidea (*Burramys*, *Cercartetus*) but this was not obtained in the MP trees. The ability of ND2 sequences to identify these well-established groups indicates that although the sequences are very divergent and in some cases saturated, phylogenetic signal is evident at this level (see also Osborne and Christidis, 2001).

Relationships within the Petauroidea, Phalangeroidea, and Burramyoidea have been discussed in Osborne

and Christidis (2001) and Osborne and Christidis (2002a,b).

Monophyly of the Vombatiformes is consistent with morphological characters (Marshall et al., 1990), serology (Kirsch, 1977) and DNA–DNA hybridization data (Springer and Kirsch, 1991). The serological evidence found these groups to be quite distinct (Kirsch, 1977) which is compatible with the levels of ND2 sequence divergence (ca. 23%) between these lineages. Nevertheless, the level of divergence between *Vombatus* and *Phascolarctos* is less than that observed between genera within the families Burramyidae and Petauridae. This suggests that the recognition of separate infra-orders or super-families for *Vombatus* and *Phascolarctos* may not be warranted. Kirsch et al. (1997) did not use the infra-ordinal separation between these lineages with only relatively short DNA–DNA hybridization distances recorded between them.

Within the Macropodoidea, *Hypsiprymnodon* was identified as the basal lineage; a result which is concordant with 12S rDNA sequences (Burk et al., 1998) but contradictory to micro-complement fixation of albumin data, which suggested a closer relationships between *Potorous* and *Hypsiprymnodon* (Baverstock et al., 1989; Baverstock et al., 1990). Traditionally, *Hypsiprymnodon* has been placed in the family Potoroinae on the basis of apparent shared derived morphological characters (Archer, 1984; Flannery, 1988). Burk et al. (1998) however, identified several morphological characters that occurred in all macropodoids with the exception of *Hypsiprymnodon* and also noted that several characters were most plesiomorphic in this species.

Most analyses identified a monophyletic relationship between *Burramys* and *Cercartetus*, which is consistent with dental and morphological characters (Archer, 1984) and other molecular evidence (Baverstock et al., 1990; Kirsch, 1977). The level of sequence divergence between *Cercartetus* and *Burramys* indicates that they are distantly related, in agreement with micro-complement fixation studies (Baverstock et al., 1990).

Monophyly of *Acrobates* and *Tarsipes* as identified by the ND2 data confirms a relationship previously indicated by micro-complement fixation of albumin data (Baverstock, 1984; Baverstock et al., 1987). Morphological and dental characters (Aplin and Archer, 1987) and features of reproductive anatomy (Renfree, 1980; Ward and Renfree, 1986) have also been cited to support the link between *Acrobates* and *Tarsipes*. Nevertheless, the high level of sequence divergence between *Acrobates* and *Tarsipes* (ca. 33%) suggests that although they constitute sister taxa, they are not particularly closely related and family level separation is appropriate.

Several examples of inconsistency between taxonomic rank and genetic distance are apparent within the diprotodontids. For example, the 23% sequence divergence distinguishing the two infra-orders/super-families within the Vombatiformes is similar to that observed within the genus *Cercartetus*. This particular disparity is not a product of differences in rates of molecular evolution as faster rates were only apparent for the Tarsipedoidea and Petauroidea. In a recent consideration of cytochrome-*b* sequences from mammalian lineages, a similar discrepancy was also noted and a correlation was observed between body size and the degree of taxonomic partitioning with assemblages (Castresana, 2001). There was a tendency toward over-splitting large bodied taxa, whilst those of small body size tended to be over-lumped. Castresana (2001) suggested that this could be a consequence of the difficulty in identifying diagnostic characters in small animals. A similar trend is apparent for the diprotodontids; lower levels of genetic divergence between taxonomic rank are evident in the larger bodied taxa such as Vombatiformes and Phalangeroidea, while higher levels of genetic divergence characterise equivalent taxonomic ranks in smaller bodied taxa such as Burramyoidea and Petauroidea. Taxonomic divisions within the diprotodontids may need to be revised to more accurately reflect the levels of genetic divergence between taxa.

4.2. Phylogenetic relationships between super-families

Studies of relationships within the diprotodontid marsupials have failed to provide robust resolution of the branching pattern at the base of the tree (e.g., Baverstock et al., 1990; Springer and Kirsch, 1991; Springer et al., 1994). The present study is no different in this respect with few of the deeper nodes involving relationships between super-families being supported by significant bootstrap values (Table 2). The ability to resolve the deeper nodes was enhanced to some extent by the inclusion of 12S rDNA sequences. The present study has identified some novel relationships within the diprotodontids which were consistently evident in analyses of the ND2 and combined (ND2 and 12S rDNA) data sets. However, in some cases KH tests did not reveal significant differences between the best topologies and alternative constrained trees.

The major division of the Diprotodontia into Phalangerida and Vombatiformes (Aplin and Archer, 1987) was identified when only the more slowly evolving characters were considered (e.g., transversions only, amino acid sequences). A basal position for the Vombatiformes in the diprotodontids is supported by DNA–DNA hybridization data (Kirsch et al., 1997) and morphological characters (Aplin and Archer, 1987). However, a division into a 'possum' and a 'kangaroo' assemblage (Phalangeriformes and Macropodiformes) as suggested by Flannery (1987) and Kirsch et al. (1997) was not supported in the present study. The failure to obtain monophyly for the possums

is consistent with Aplin and Archer's (1987) inability to identify any morphological characters that were shared by all possum lineages. Nevertheless, Aplin and Archer (1987) did advocate monophyly of the 'typical' possums (Phalangeridae, Burramyidae, Petauridae, and Pseudocheiridae).

Neither ND2 nor combined sequences supported the suggested links between the Burramyidae and the Acrobatidae (Kirsch, 1977). ND2 sequences only provided weak support (when transversion were weighted twice that of transitions) for links between the Burramyidae and Phalangeroidea. Support for this association also comes from DNA–DNA hybridization data (Harding, 1987; Kirsch et al., 1997).

Alternatively, amino acid sequences and ML analyses of combined sequences indicated that Phalangeroidea had links with the Macropodiformes. An association between these two super-families has been suggested previously (Archer, 1984; Flannery, 1987). Flannery (1987) identified several synapomorphic dental features in support of a sister relationship between Macropodoidea and Phalangeroidea. In a study of higher order marsupial relationships using micro-complement fixation of albumin, *Trichosurus* (Phalangeroidea) and *Macropus* (Macropodoidea) were united (Baverstock et al., 1990) whilst the Vombatiformes were identified as the sister lineage to a group comprising Macropodoidea, Phalangeroidea, Petauroidea, and Tarsipedoidea. There was also some evidence from serological results for links between the Phalangeroidea and the Macropodoidea (Kirsch, 1977) whilst 12S rDNA sequences linked the Phalangeroidea with the Vombatiformes and placed the Burramyoidea as basal to this assemblage (Springer et al., 1994).

The ND2 sequences were equivocal on the placement of the Tarsipedoidea within the diprotodontids. Tarsipedoidea was either basal to an assemblage comprising Petauroidea–Vombatiformes, Phalangeroidea, and Macropodoidea, or alternatively was the sister lineage of the Petauroidea. Combined ND2 and 12S rDNA analyses not only identified a link between Tarsipedoidea (only *Tarsipes* was represented) and Petauroidea but in some trees, *Tarsipes* nested within this group. Micro-complement fixation (Baverstock et al., 1989; Baverstock et al., 1990) and DNA–DNA hybridization (Edwards and Westerman, 1995; Kirsch et al., 1997) data are indicative of a sister relationship between the Tarsipedoidea and Petauridae. However, there is a lack of identified morphological traits to link these lineages (Aplin and Archer, 1987).

4.3. Relative rates of DNA evolution

Within the diprotodontids several cases of unequal evolutionary rates of molecular evolution have been reported (e.g., Baverstock et al., 1987; Baverstock et al., 1989; Baverstock et al., 1990; Springer and Kirsch, 1989). Rates of albumin (Baverstock et al., 1990) and single copy DNA (Springer and Kirsch, 1989) evolution are reported to be slower in the branches leading to the Burramyidae and Phalangeridae, but no such rate deceleration in the mitochondrial ND2 gene was observed for these lineages. In contrast, relative rate tests for the ND2 gene identified faster rates of DNA evolution in the branches leading to *Acrobates*, *Tarsipes*, and the Petauroidea. Accelerated evolutionary rates have also been observed for the Petauroidea in single copy DNA (Springer and Kirsch, 1989; Springer et al., 1992) and in albumin for the Pseudocheiridae (Baverstock et al., 1990).

Several underlying causes of disparate rates of molecular evolution have been suggested including differences in metabolic rate, generation time, rate of germ cell division, body temperature, DNA repair efficiency and clade sizes (reviewed in Martin and Palumbi, 1993; Rand, 1994). An inverse correlation has been observed between the evolutionary rate of mitochondrial DNA and body size (Martin and Palumbi, 1993 but see Slowinski and Arbogast, 1999). In this regard it is worth noting that two of the taxa (*Acrobates pygmaeus* and *Tarsipes rostratus*) for which a faster rate of evolution was evident, are the smallest of the diprotodontids (with the exception of *Cercartetus lepidus*). The Burramyidae are an apparent exception to the correlation between small body size and accelerated evolutionary rate with no evidence for a faster rate of mitochondrial DNA evolution in this lineage. This contrasts with Springer and Kirsch's (1989) finding of a slow down in single copy nuclear DNA evolution in the Burramyidae (represented by *Cercartetus* in their study).

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