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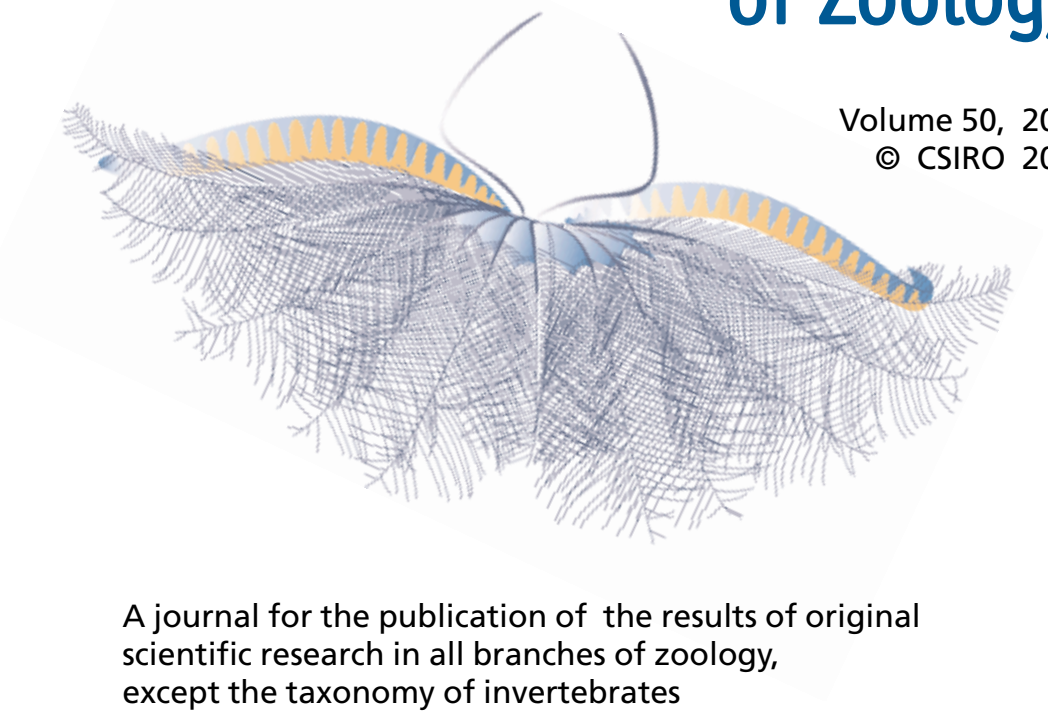

Volume 50, 2002

A journal for the publication of the results of original scientific research in all branches of zoology, except the taxonomy of invertebrates

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Australian Journal of Zoology, 2002, **50**, 135–149

Molecular relationships of the cuscuses, brushtail and scaly-tailed possums (Phalangerinae)

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Abstract

DNA sequence data (1040 base pairs) from the mitochondrial nicotinamide dehydrogenase subunit 2 gene (ND2) was used to elucidate species relationships within the Phalangerinae (cuscuses, brushtail and scaly-tailed possums). Phylogenetic analyses revealed three lineages within this family (*Spilocuscus*, *Phalanger* and *Trichosurus–Wyulda*), which is congruent with the results of other molecular studies. Sequence data also demonstrate that *P. orientalis*, *P. carmelitae*, *P. vestitus*, and *P. sericeus* are closely related and indicates that *P. gymnotis* is the sister lineage to the genus *Phalanger*. Divergence time estimates indicate that the radiation of *Phalanger* and *Spilocuscus* occurred during the middle Miocene to Pliocene periods.

Introduction

The Phalangeridae (Superfamily Phalangeroidea) is the most diverse and widely distributed of the possum families (Fig. 1). There are approximately 20 species of cuscus (*Ailurops*, *Phalanger*, *Spilocuscus*, *Strigocuscus*) occurring from northern Australia to New Guinea and surrounding islands, with two species reaching Sulawesi, the only marsupials to do so. Also included in the Phalangeridae are the brushtail (*Trichosurus*) and scaly-tailed (*Wyulda*) possums, which are restricted to Australia.

The composition of the genera *Trichosurus*, *Wyulda*, *Spilocuscus* and *Ailurops* is relatively stable. This contrasts dramatically with that of *Phalanger* and *Strigocuscus*, the composition and affinities of which are debated (e.g. Flannery *et al.* 1987*a*; George 1987). Much of the dispute has centered on the taxonomic significance of the various morphological characters examined (e.g. Menzies and Pernetta 1986; Flannery *et al.* 1987*a*; George 1987) but some has also been a consequence of erroneous molecular results (Baverstock 1984, corrected in Baverstock *et al.* 1990).

According to the most recent classification (Flannery 1994), the Phalangeridae comprises two subfamilies: Ailuropinae (*Ailurops*) and Phalangerinae (remaining genera). Flannery (1994) split the latter subfamily into two tribes: Trichosurini, comprising *Wyulda* (monotypic), *Trichosurus* (four species) and *Strigocuscus* (two species), and Phalangerini, encompassing the remaining cuscus genera: *Spilocuscus* (four species) and *Phalanger* (12 species). Flannery's (1994) current view differs from that previously presented by him, in which *Strigocuscus* was expanded to include *mimicus*, *gymnotis* and *ornatus* (Flannery *et al.* 1987*a*). Flannery *et al.* (1987*a*) suggested that this genus was closer to *Trichosurus–Wyulda* than to *Phalanger* and *Spilocuscus*, a view that is reflected in the current taxonomic arrangement. The removal of *gymnotis* (and subsequently *mimicus* and *ornatus*) from *Strigocuscus* (Flannery and Boeadi 1995) came in light of micro-complement fixation of albumin (Baverstock *et al.* 1990) and DNA–DNA hybridisation data that indicated that *gymnotis* was a *Phalanger* (Springer *et al.* 1990). DNA–DNA hybridisation data did not resolve the particular affinities of *P. gymnotis* within *Phalanger*, and the strict consensus jackknife tree only linked *P. vestitus* and *P. carmelitae* (with the position of *P. gymnotis*, *P. sericeus* and *P. orientalis* unresolved). For 12S rDNA sequences obtaining monophyly

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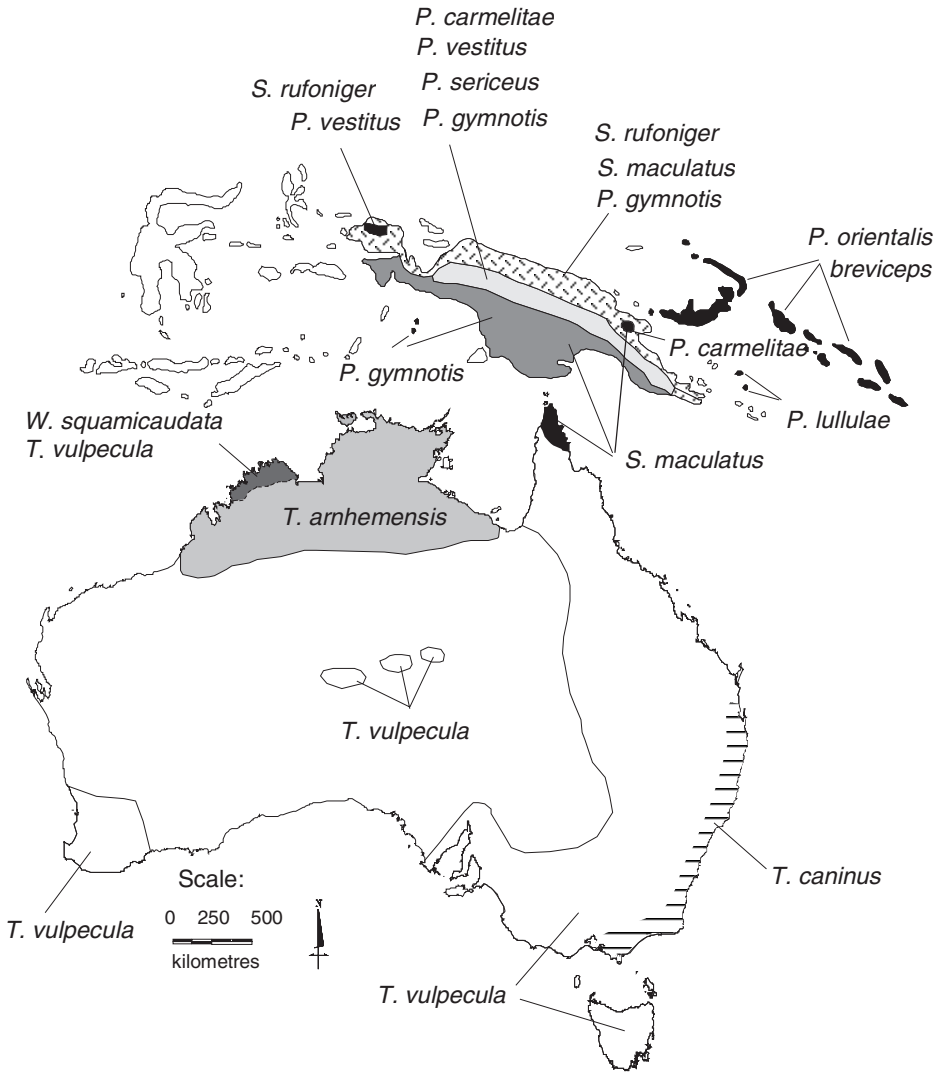

Fig. 1. Map of Australia and New Guinea region indicating the distributions for the species examined (from Flannery 1994). Note that for *P. orientalis* only the distribution for the subspecies *P. orientalis breviceps* is shown.

for the phalangerids was dependent on the weighting scheme that was used, and monophyly was not obtained in unweighted parsimony analysis (Hamilton and Springer 1999).

Flannery's (1994) current arrangement for the Phalangeridae essentially follows that presented by George (1987). George (1987) recognised four cuscus genera: *Ailurops*, *Spilocuscus*, *Phalanger* and *Strigocuscus* (including only *S. celebensis*). On the basis of dental characters, George (1987) also suggested that *Strigocuscus* had stronger links with *Trichosurus–Wyulda* than with the other cuscuses. Norris (1994) examined periotic bones and found that *Strigocuscus celebensis* shared characters with *Trichosurus–Wyulda*, whilst the other cuscuses grouped together, with the exception of *Ailurops*, which represented a distinct lineage.

In contrast to George (1987), Flannery *et al.* (1987*a*) and Norris (1994), Menzies and Pernetta (1986) considered that the taxa comprising *Strigocuscus* belonged within *Phalanger*. Menzies and Pernetta (1986) considered there to be five groupings within the *Phalanger* '*orientalis* group' (*sensu* Tate 1945) on the basis of skull, dental and pelage characters: *orientalis*, *gymnotis*, a montane group (comprising *vestitus*, *sericeus*, and *carmelitae*), *permixtio*, and finally the '*celebensis* group' (comprising *ornatus*, *rothschildi*, *lullulae* and *celebensis*). The '*celebensis* group' was regarded as the most distinct lineage within *Phalanger*. Menzies and Pernetta (1986) found that *P. gymnotis* shared some characters with the '*celebensis* group' and others with the '*orientalis* group' and were therefore unable to determine its particular affinities.

The other cuscus lineages, *Spilocuscus* and *Ailurops*, have consistently been recognised as distinct on the basis of morphological characters (e.g. Tate 1945; George 1987). *Spilocuscus* has been identified as the sister lineage of *Phalanger* by DNA–DNA hybridisation (Springer *et al.* 1990) and mitochondrial DNA sequence data (Hamilton and Springer 1999). Several studies have indicated close links between *Wyulda* and *Trichosurus* including serology (Kirsch 1977), morphology (Flannery *et al.* 1987a), allozymes (Kerle *et al.* 1991) and micro-complement fixation of albumin (Baverstock *et al.* 1990).

DNA sequence data from the mitochondrial nicotinamide dehydrogenase subunit 2 gene (ND2) and in combination with the previously published mitochondrial DNA sequence data (Hamilton and Springer 1999) was used to determine species relationships within the genus *Phalanger* (particularly the affinities of *P. gymnotis* and *P. lullulae*) and to assess the links between the major lineages within the Phalangerinae: *Spilocuscus*, *Phalanger*, and *Trichosurus–Wyulda*.

Methods

Samples

Specimens examined, collection localities and GenBank accession numbers are given in Table 1. Genomic DNA was extracted from tissue samples following the procedure of Gemmell and Akiyama (1996). Wallaroo, *Macropus robustus* (Macropodidae) (GenBank Accession Y10524), and North American opossum, *Didelphis virginiana* (Didelphidae) (Janke *et al.* 1994) sequences were included as outgroup taxa. In addition, sequences from the mountain pygmy-possum, *Burramys parvus* (Burramyidae) (Osborne *et al.* 2000), and the common wombat, *Vombatus ursinus* (Vombatidae), were obtained in order to match the outgroups used by Hamilton and Springer (1999). The $tRNA^{phe}$, 12S ribosomal RNA, $tRNA^{val}$ and partial 16S ribosomal DNA sequence (referred to as 12S–16S hereafter) alignments used by Hamilton and Springer (1999) were kindly made available by A. Hamilton.

PCR amplification and sequencing

The ND2 gene (1040 base pairs) 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 species.

Phylogenetic analyses

ND2 sequences were aligned by visual inspection. Transition : transversion ratios were assessed using MEGA (Molecular Evolutionary Genetic Analyses) Ver. 2.1 (Kumar *et al.* 2001). Phylogenetic analyses of ND2 data was conducted using different outgroup combinations to assess the effect of outgroup selection on the topologies obtained.

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. To alleviate potential effects of saturation in ND2, various partitioning schemes were used: exclusion of third positions, exclusion of third-position transitions, and exclusion of all transitions. Consensus trees (50% majority rule) were computed if more than one equally parsimonious tree was found. Branch support was estimated by the decay index value (d) (Bremer 1988), as calculated using

Table 1. Sample information and GenBank Accession numbers

Double-stranded ND2 sequence was obtained from all individuals except for EBU9564. Voucher locations: AM, Australian Museum; MV, Museum Victoria; ANWC, Australian National Wildlife Collection; SAM, South Australia Museum. Collection localities: NG, New Guinea; NSW, New South Wales; NT, Northern Territory; Vic., Victoria; WA, Western Australia

Species	Common name	Voucher	Collection locality	GenBank Acc. No.
Phalanger orientalis breviceps	Northern common cuscus	M23014 (AM)	Choiseul I., Solomon Is, NG	AF343881
Phalanger lullulae	Woodlark cuscus	EBU9533 (AM)	Woodlark I., NG	AF343882
Phalanger lullulae	Woodlark cuscus	EBU9564 (AM)	Alcester I., NG	AF343883
Phalanger sericeus	Silky cuscus	ABTC42510 (SAM)	Kosipe Mission, NG	
Phalanger vestitus	Stein's cuscus	EBU26417 (AM)	West Sepik Province, NG	AF343885
Phalanger gymnotis	Ground cuscus	ABTC46665F (SAM)	Namosado, NG	AF343886
Phalanger carmelitae	Mountain cuscus	M19104 (AM)	Arwoma Village, NG	AF343887
Spilocuscus rufoniger	Black spotted cuscus	ABTC42634 (SAM)	Kobobip, NG	AF343888
Spilocuscus maculatus	Spotted cuscus	AMM29482 (AM)	Makalu Province, NG	
Trichosurus vulpecula	Common brushtail possum	C30954 (MV)	Richmond, Vic., Australia	AF300999
Trichosurus arnhemensis	Northern brushtail possum	ABTC29990F (SAM)	Melville I., NT, Australia	AF343890
Trichosurus caninus	Mountain brushtail possum	M16324 (ANWC)	Coffs Harbour, NSW, Australia	AF343891
Wyulda squamicaudata	Scaly-tail possum	ABTC7727F (SAM)	Mitchell Plateau, WA, Australia	AF343892
Vombatus ursinus	Common wombat	W1 (MV, unreg)	Buxton, Vic., Australia	AF343893

the program Auto Decay 4.02 (Eriksson 1998) and with the bootstrap approach (Felsenstein 1985) with 1000 replications.

Modeltest Ver. 3.06 (Posada and Crandall 1998) was used to determine the most appropriate distance method for maximum-likelihood analyses. Modeltest compares different nested models of DNA substitution and calculates the likelihood ratio score $\delta = -2(\log \Lambda)$ and the associated *P*-value with a χ^2 distribution (and *q* degrees of freedom) (Posada and Crandall 1998). This allows the null hypotheses about the process of DNA substitution to be accepted or rejected. The PAUP block (modelblock3) provided with Modeltest was used to compare 56 different models of DNA substitution. Modeltest interprets the resulting *P*-values and chooses the model that best describes the data.

The General Time Reversible model (GTR) (Rodriguez *et al.* 1990) with unequal distribution of nucleotides, a proportion of invariant sites and among-site rate variation (gamma distribution, discrete approximation) was found to best fit the ND2 and the combined data. Thus, 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 0.4197 and the shape parameter of the gamma distribution was 1.8727. For the combined data these parameters were 0.4481 (proportion of invariant sites) and 0.8555 (the shape parameter of the gamma distribution).

Neighbour-joining analyses (Saitou and Nei 1987) were conducted using the GTR (Rodriguez *et al.* 1990), and HKY85 (Hasegawa *et al.* 1985) distance options in PAUP 4.0b4a (Swofford 2000). Branch support was evaluated using the bootstrap approach (Felsenstein 1985). One thousand replications were conducted for the neighbour-joining and 100 replications for maximum-likelihood analyses.

The 12S–16S rDNA sequences (1185 base pairs) were combined with the ND2 data (reduced to include the same taxa) and analysed in the same manner as ND2. Ambiguous sites were excluded, as in Hamilton and Springer (1999).

Spectral analysis (Hendy and Penny 1993) was conducted using the programme Spectrum 2.0 (Charleston and Page 1997; Charleston 1998) using a distance matrix (Kimura two-parameter method) (Kimura 1980).

Results

Sequence analyses

Sequence for 1040 base pairs of ND2 was obtained for all 14 individuals examined. Double-stranded sequence was obtained for each species. Among the 13 ingroup taxa there were 403 variable sites; of these, 296 were parsimony informative. The majority of informative sites (193) occurred at the third codon and the fewest informative changes (32) were at the second position. In the combined data set of ND2 and 12S–16S rDNA (2225 base pairs) there were 516 variable sites, of which 445 were parsimony informative.

The transition : transversion ratios for ND2 were biased in favor of transitions for all ingroup comparisons (Table 2). Ratios ranged from 1.49 to 17.00 for intra-generic comparisons and between 1.20 and 5.83 for inter-generic comparisons. Comparisons between the Phalangerinae and the outgroups *Macropus* and *Didelphis* were biased toward transversions.

Sequence divergences ranged from 3.61 to 16.26% for intra-generic comparisons and between 14.01 and 25.87% for inter-generic comparisons (Table 2). For ND2 the lowest level of divergence was between *Trichosurus vulpecula* and *T. arnhemensis* (3.61%). This was less than the divergence observed between individuals of *P. lullulae* from Woodlark and Alcester Islands (4.75%). Among the Phalangerinae the highest inter-generic levels of divergence were those between *Wyulda* and *Phalanger–Spilocuscus* (23.38–25.87%), which were similar to those observed between the outgroup *Macropus robustus* (23.85–32.40%) and the ingroup taxa.

Phylogenetic analyses

Parsimony, maximum-likelihood and neighbour-joining analyses (using both GTR and HKY85 distances) produced trees with similar topologies. *Trichosurus* and *Wyulda* were

Table 2. Percentage sequence divergence for ND2 pairwise comparisons corrected using Kimura two-parameter method (above diagonal) and transition : transversion ratios for ND2 (below the diagonal)

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *F. orientalis*	–	15.49	14.89	12.51	7.78	13.73	0.73	14.44	15.90	18.70	19.45	19.20	24.78	27.28	37.77
2. *F. lullulae* (9533)	2.11	–	4.75	14.93	16.26	14.25	15.66	16.16	16.50	23.39	23.86	23.41	24.45	28.31	35.86
3. *F. lullulae* (9564)	1.72	4.22	–	15.34	15.01	15.09	14.81	16.68	17.01	23.77	24.52	23.35	24.99	27.73	38.12
4. *F. sericeus*	2.74	1.65	1.49	–	11.80	13.52	11.45	15.59	17.32	19.97	20.64	21.34	23.60	27.15	37.32
5. *F. vestitus*	3.56	2.15	1.69	2.58	–	14.21	7.90	15.03	17.39	19.47	19.10	18.44	23.38	27.03	38.94
6. *F. gymnotis*	3.03	2.00	2.09	2.00	2.97	–	13.25	14.01	16.34	19.43	20.64	19.18	23.67	23.85	35.63
7. *P. carmelitae*	4.67	2.33	2.00	2.48	3.81	2.97	–	15.42	16.64	20.85	20.33	21.01	24.62	26.52	36.49
8. *S. rufoniger*	1.93	1.64	1.53	1.57	1.86	2.10	2.04	–	7.95	19.07	19.07	19.62	25.87	25.97	37.40
9. *S. maculatus*	1.94	1.44	1.34	1.63	2.00	2.24	2.00	8.63	–	20.35	22.03	21.46	25.19	25.33	37.45
10. *T. vulpecula*	1.51	1.23	1.20	1.20	1.69	1.38	1.55	1.61	1.60	–	3.61	5.45	17.54	28.08	36.08
11. *T. arnhemensis*	1.51	1.21	1.21	1.20	1.55	1.43	1.43	1.54	1.69	17.00	–	5.92	18.76	29.62	36.92
12. *T. caninus*	1.63	1.28	1.17	1.38	1.65	1.42	1.64	1.75	1.79	8.00	6.25	–	17.61	28.90	35.60
13. *W. squamicaudata*	2.30	1.44	1.47	1.75	2.27	2.17	2.21	2.49	2.22	5.83	5.68	4.85	–	32.40	37.16
14. *M. robustus*	1.10	0.96	0.91	0.86	1.07	0.96	1.00	1.25	1.09	1.13	1.13	1.16	1.29	–	39.52
15. *D. virginiana*	0.69	0.67	0.71	0.73	0.74	0.67	0.64	0.71	0.70	0.75	0.75	0.73	0.72	0.66	–

sister taxa, and *Phalanger* and *Spilocuscus* were identified as monophyletic lineages and were each other's closest relatives (Fig. 2*a–c*). *Phalanger orientalis*, *P. vestitus*, *P. carmelitae* and *P. sericeus* formed a monophyletic group relative to *P. gymnotis* and *P. lullulae*. These associations had high bootstrap support. Differences in topologies between the various analyses were restricted to relationships involving *Phalanger*. All analyses grouped *P. carmelitae* with *P. orientalis* (Fig. 2*a*), with the exception of unweighted parsimony, which identified *P. vestitus* as the sister taxon of *P. orientalis* (Fig. 2*b*). In neighbour-joining, maximum-likelihood, unweighted and transversion parsimony trees, *P. gymnotis* was basal within *Phalanger* (Fig. 2*a–b*). When third-position transitions and all third-position changes were excluded from the parsimony analyses, *P. gymnotis* and *P. lullulae* were sister taxa (Fig. 2*c*).

In all analyses of the combined sequences *Spilocuscus* was linked to *Phalanger* to the exclusion of *Trichosurus*. This association was robust (97–100% bootstrap support). Maximum-parsimony analyses found two trees of equal length (906 steps) in which the position of *P. gymnotis* varied, being either basal to *P. orientalis* and *P. lullulae* (less than 50% bootstrap support) or basal to a clade containing *Spilocuscus*, *P. orientalis* and *P. lullulae* (51% bootstrap support). In all other analyses *P. gymnotis* was linked to *P. orientalis* and *P. lullulae* (Fig. 3). This arrangement was the same as that obtained from 12S–16S rDNA sequences alone although bootstrap support was lower (51%) (Hamilton and Springer 1999).

Spectral analysis of ND2 sequences revealed three groups for which there was strong support and minimal conflict: *Trichosurus*, *Wyulda–Trichosurus*, and *Spilocuscus* (Fig. 4). The group comprising *P. orientalis*, *P. carmelitae*, *P. vestitus* and *P. sericeus* had less support but also very little conflict. There was some support for a monophyletic *Phalanger* but there was also conflict. There was no support and much conflict for the association of *P. gymnotis* with *Trichosurus–Wyulda*, and a group comprising *Phalanger* and *Spilocuscus* was not supported.

Spectral analysis of the combined sequences revealed strong support and minimal conflict for the association of *Spilocuscus maculatus* with *S. rufoniger* (Fig. 4). There was also some support for *P. orientalis*, *P. gymnotis* and *P. lullulae* grouping together. However, there was no support and significant conflict for linking *P. gymnotis* with *Trichosurus*.

Discussion

Phylogenetic relationships

Phylogenetic analyses of the ND2 sequence data identified three lineages within the Phalangerinae: *Spilocuscus*, *Phalanger*, and *Trichosurus–Wyulda*. A sister relationship between *Phalanger* and *Spilocuscus* was identified in the phylogenetic trees. Despite the high level of branch support for this association, it was not supported by spectral analyses. Other molecular data, including micro-complement fixation of albumin (Baverstock *et al.* 1990), DNA–DNA hybridisation (Springer *et al.* 1990) and 12S–16S rDNA sequences (Hamilton and Springer 1999) are congruent with the results presented here. Unlike 12S–16S rDNA sequences, monophyly for Phalangerinae is well supported by the ND2 sequences. In contrast to the DNA–DNA hybridisation data (Springer *et al.* 1990), the ND2 sequence data demonstrate that *P. sericeus*, *P. vestitus*, *P. orientalis* and *P. carmelitae* are all closely related. *P. gymnotis* and *P. lullulae* are distinct from this clade. Furthermore, *P. gymnotis* is the sister taxon to the clade comprising *Phalanger* and there is no support for an association between it and *Trichosurus–Wyulda* (*contra* Flannery *et al.* 1987*a*). This

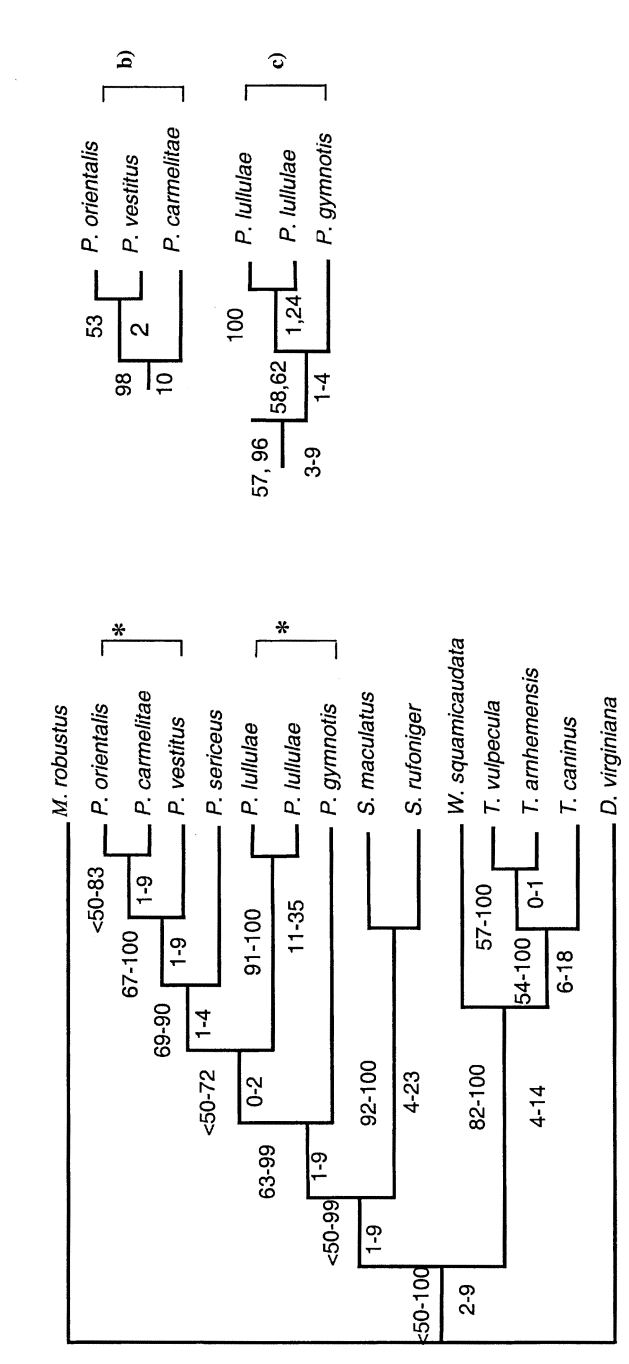

Fig. 2. Phylogenetic trees based on ND2 sequence data. Topology (*a*) produced for maximum-likelihood (–Ln = 6295.330), transversion-parsimony (length = 292, CI = 0.521, RI = 0.697, RC = 0.363) and neighbour-joining analyses, (*b*) produced for maximum-parsimony analyses (length = 1042, CI = 0.483, RI = 0.532, RC = 0.257), (*c*) produced when third-position transitions (length = 321, CI = 0.514, RI = 0.637, RC = 0.327), and all third-position transitions (length = 315, CI = 0.527, RI = 0.603, RC = 0.315) were excluded from parsimony analyses. The range of bootstrap values (above branches) and decay values (below branches) are given. *Didelphis virginiana* and *Macropus robustus* are the outgroups.

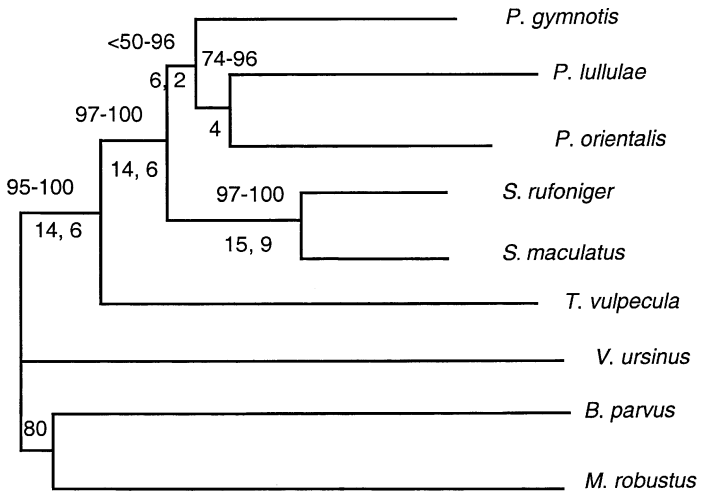

Fig. 3. Neighbour-joining tree for the combined ND2 and 12S–16S rDNA sequences. Identical topologies (except the outgroup *B. parvus* clusters with *V. ursinus*) were produced for maximum-likelihood (–Ln = 9325.099) and for parsimony analyses excluding third-position transitions (length = 685, CI = 0.603, RI = 0.464, RC = 0.279) and all third-position substitutions (length = 403, CI = 0.591, RI = 0.461, RC = 0.272) (decay values are given below branches). The range of bootstrap values are given above branches. *Burramys parvus*, *Macropus robustus* and *Vombatus ursinus* are the outgroups.

result is substantiated by the other molecular data sets (Baverstock *et al.* 1990; Springer *et al.* 1990; Hamilton and Springer 1999).

Phalanger orientalis, *P. sericeus*, *P. carmelitae* and *P. vestitus* have always been considered to be closely related (e.g. Tate 1945; Groves 1987; Colgan *et al.* 1993). On the basis of ND2 sequence divergences, *P. sericeus* is the most distinct member of this group, which is consistent with several distinguishing dental (absence of P^3, P^4 with two points, and one or two lower unicuspids) and external (naked part of the tail is entirely smooth) characters (Menzies and Pernetta 1986). In contrast DNA–DNA hybridisation results placed *P. orientalis* and not *P. sericeus* as basal within this assemblage (Springer *et al.* 1990). The discrepancy between the two data sets may be a consequence of the diversity found within *P. orientalis*.

On the basis of genetic variation at 29 protein allozyme loci, Colgan *et al.* (1993) indicated that there were three distinct geographic groups within the lineage previously referred to as *P. orientalis*: *P. orientalis breviceps* (considered in the present study) (from the Solomon Islands and Bismarck Archipelago), *P. o. orientalis* (from northern New Guinea), and *P. intercastellanus* (from eastern and southern New Guinea). Furthermore, the allozyme data indicated that traditional *P. orientalis* was paraphyletic, with *P. o. orientalis* and *P. o. breviceps* more closely related to *P. carmelitae* and *P. vestitus* than to *P. intercastellanus*. The latter species was previously included within *P. orientalis*. Hamilton and Springer (1999) refer to the individual considered in their study as *P. orientalis*. Given the locality information subsequently provided for this individual (Namosado, Central Highlands Province, New Guinea: M. Springer, personal communication) it may actually be *Phalanger intercastellanus*, according to a study of

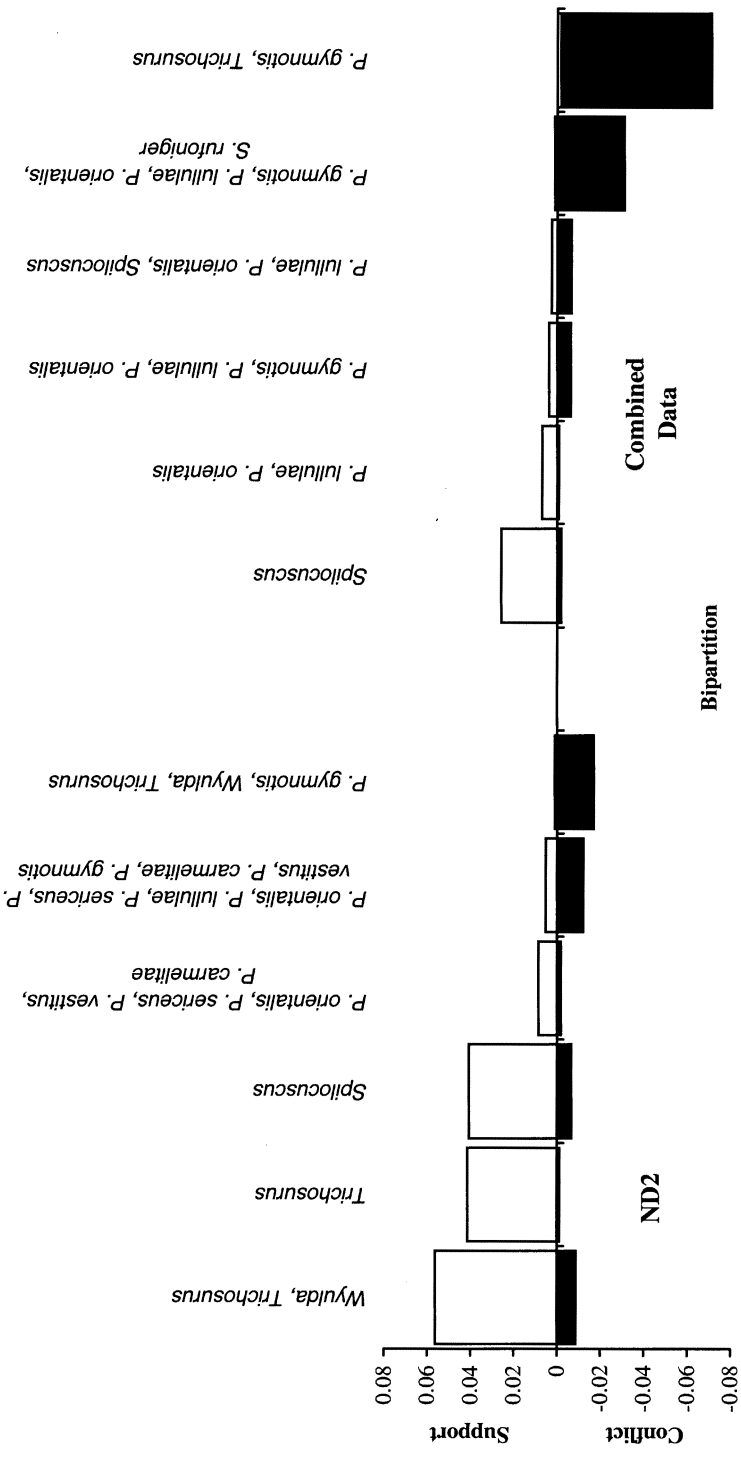

Fig. 4. Spectral analyses for the ND2 and for the combined ND2 and 12S–16S rDNA sequences. The amount of support and conflict is given for pairwise comparisions (comparisons with negative support values are not shown).

genetic and morphological variation within *P. orientalis* (Colgan *et al.* 1993). Locality data was not published for the *P. orientalis* used in Springer *et al.* (1990), which makes it difficult to compare results and therefore combining or comparing data sets may not be appropriate. This highlights the importance of providing locality data for specimens used in molecular studies (Ruedas *et al.* 2000). ND2 sequence divergence was only 7–8% between *P. orientalis breviceps*, *P. vestitus* and *P. carmelitae*, supporting the finding of Colgan *et al.* (1993) that these taxa are closely related.

Groves (1987) suggested that *P. sericeus* was not only basal to *P. orientalis*, *P. carmelitae* and *P. vestitus* but also to the insular species (*celebensis*, *ornatus*, *rothschildi*, *lullulae*). This arrangement is not supported by the present data or by other morphological studies (Flannery *et al.* 1987*a*; George 1987). Instead, the present data consistently identified *P. gymnotis* and *P. lullulae* as basal within *Phalanger.*

There was some support from ND2 sequences (when third-position transitions and all third positions were excluded) for *P. gymnotis* and *P. lullulae* representing a monophyletic group distinct from the clade comprising *P. orientalis*, *P. carmelitae*, *P. vestitus* and *P. sericeus*. Menzies and Pernetta (1986) identified cranial and dental features that were shared by *P. gymnotis* and *P. lullulae* in addition to *P. rothschildi* and *P. ornatus*, and links were particularly strong between *P. ornatus* and *P. lullulae*. George (1987) identified additional cranial similarities between *P. gymnotis* and *P. ornatus*. Furthermore, Menzies and Pernetta (1986) indicated that this assemblage was linked to *P. celebensis* (which was, however, quite distinct). Given the shared morphological characters and partitioned ND2 sequences that link two of these taxa (*gymnotis* and *lullulae*), it seems plausible that this may represent an assemblage that is distinct from other *Phalanger.* The levels of divergence, however, do not support a generic-level distinction and question the validity of *Strigocuscus*.

Although Flannery (1994) suggested links between *Strigocuscus* and *Trichosurus–Wyulda* he admitted that in terms of biogeography it was difficult to explain. Flannery (1994) suggested that *Strigocuscus* and *Ailurops* may have become isolated on Sulawesi early in the radiation of marsupials; however, in the absence of any other marsupials here, this seems unlikely. Furthermore, given the contradictory results reached by the various morphological studies, the position of *Ailurops* (in particular, whether it is basal to all of the Phalangerinae) needs to be reconsidered using molecular data.

The two individuals of *P. lullulae* examined differed from each other by 4.75%, which is similar to that obtained between species within *Phalanger* and *Trichosurus* (e.g. 5.61% between *T. caninus* and *T. vulpecula*). These two individuals came from different islands (Woodlark and Alcester), which are about 70 km apart. Although sequence was obtained only from single representatives of *P. lullulae* from the two island populations, it is improbable that such significantly divergent haplotypes would exist as polymorphisms within each of these island populations, and therefore suggests that the two populations have been disjunct since the Pliocene period. This contradicts the suggestion of Flannery (1994), that humans may have introduced the species to Alcester Island from Woodlark Island. Given that the level of divergence between populations of *P. lullulae* are similar to those seen between recognised species of *Phalanger* and *Trichosurus*, the populations may represent different subspecies. However, there are no other comparisons of subspecific or population-level differences in *Phalanger* with which to compare to the observed divergence in *P. lullulae*.

The ND2 sequences clearly identify *Wyulda* as the sister taxon of *Trichosurus*. The level of sequence divergence between *Wyulda* and *Trichosurus* (*c*. 18%) is consistent with the

generic distinction between these lineages (*contra* Flannery *et al.* 1987*a*, but in agreement with Flannery 1994). Kerle *et al.* (1991) found that there was a minimum of 31–40% fixed allozyme differences between *Wyulda* and *Trichosurus* for 21 loci. Micro-complement fixation of albumin results also indicate that *Wyulda* and *Trichosurus* are quite distinct (Baverstock *et al.* 1990).

The presence of geographically and morphologically distinct populations within *Trichosurus* has resulted in the description of several species, only two of which are consistently accepted: *T. vulpecula* and *T. caninus* (Strahan 1995). The ND2 data substantiates the species distinction of these taxa, with 5.61% divergence between them. Although Kerle *et al.* (1991) recognised these species as being distinct on the basis of external morphological characters and in the absence of hybridisation between them, they displayed no fixed allozyme differences. Flannery (1994) also recognises *T. arnhemensis* and *T. johnsonii* as additional species but these two taxa are considered by most to be subspecies within *vulpecula* (e.g. Kerle *et al.* 1991). Sequence divergence of only 3.6% between *T. arnhemensis* and *T. vulpecula* supports this notion.

Times of divergence and biogeographic considerations

Strigocuscus (*S. reidi*), *Trichosurus* (*T. dicksoni*) and *Wyulda* fossils have been recovered from the Riversleigh deposit in north-western Queensland (Flannery and Archer 1987; Archer *et al.* 1991) from an assemblage estimated to be middle Miocene in age (Archer *et al.* 1989). The cuscus fossils were described as *Strigocuscus* because of characters that were shared with *gymnotis* (Flannery and Archer 1987). Likewise, fossil material from the Pliocene Hamilton Local Fauna was also described as *Strigocuscus* (*S. notalis*) (Flannery *et al.* 1987*b*). Given substantial evidence indicating that *gymnotis* is actually a *Phalanger* (e.g. Springer *et al.* 1990; Baverstock *et al.* 1990; Flannery and Boeadi 1995; Hamilton and Springer 1999; present data), the assignment of *S. reidi* and *S. notalis* should be reconsidered (Flannery 1994; Crosby *et al.* 1999). The similarity of these taxa to *P. gymnotis* does, however, support the primitive position of this taxon within *Phalanger.* The estimated age of the older fossils (*Strigocuscus*) may predate the split of *P. gymnotis* from *P. orientalis* or the other members of *Phalanger.*

Times of divergence between phalangerine lineages were estimated using the equation derived in Osborne and Christidis (2002). Thus, *Phalanger–Spilocuscus* and *Trichosurus–Wyulda* were estimated to have diverged *c.* 25 million years ago whilst divergence between *Phalanger* and *Spilocuscus* is estimated to have occurred from *c.* 14 to *c.* 23 million years ago. Divergences between species of *Phalanger* ranged from *c.* 4 to *c.* 19 million years ago and between *Spilocuscus* species were *c.* 3 million years ago.

The divergence times obtained here for *Spilocuscus maculatus* and *S. rufoniger* are concordant with DNA–DNA hybridisation data (3.2 million years) (Kirsch *et al.* 1997) and are similar to that obtained 12S rDNA using transversion distances (2.1 million years) (Springer 1997; Hamilton and Springer 1999). DNA–DNA hybridisation data and 12S rDNA sequences estimate that the divergence of *P. gymnotis* and *P. orientalis* occurred more recently (7.4 and 4.3 million years ago, respectively) than ND2 sequences suggest (11 million years ago). This is also the case for the divergence of *Spilocuscus* and *Phalanger* (12 and 4.7 million years ago) (Kirsch *et al.* 1997; Hamilton and Springer 1999).

Using ND2 data, *Trichosurus* and *Wyulda* were estimated to have diverged approximately 8 million years ago. Micro-complement fixation of albumin data indicated that *Wyulda* and *Trichosurus* diverged 11 million years ago (Baverstock *et al.* 1990). Furthermore, fossils assigned to *Wyulda* have been recovered from the middle Miocene

(16.6 to10.4 million years ago) assemblage at Riversleigh (Archer *et al.* 1991; Crosby *et al.* 1999); this contradicts both of the estimates obtained using molecular data.

The divergence times obtained for the radiation of the *Phalanger* and *Spilocuscus* species considered here, and between these genera, correspond to the Miocene and early Pliocene periods. During this time there was extensive tectonic activity and climate change in the Australo-Papuan region. New Guinea existed as a series of islands throughout the Miocene (reviewed in Flannery 1994). There was also extensive mountain building and sea level fluctuations that caused low-lying areas to become submerged periodically (Flannery 1989). These events may have been responsible for fragmentation of populations of ancestral *Phalanger* in New Guinea, allowing speciation to occur. The islands of western New Guinea merged in the Pliocene to form the Central Cordillera (Flannery 1994). This may have re-connected populations of now divergent species. Interestingly, in parts of the Central Cordillera up to five species of cuscus co-exist including *P. sericeus*, *P. vestitus*, *P. carmelitae*, *P. gymnotis*, and *P. matanim* (Flannery 1994) which may be explained by such an event.

Acknowledgments

Our most sincere thanks are extended to those people who donated tissue samples, including D. O'Meally (Australian Museum), J. Wombey (Australian National Wildlife Collection, CSIRO), L. Frigo (Museum Victoria), S. Donnellan (South Australia Museum) and also to T. Flannery (South Australian Museum), C. Kemper (South Australian Museum) and S. Ingleby (Australian Museum) for assisting in the identification of the voucher specimens. A. Hamilton kindly made 12S–16S sequence alignments available. J. Norman provided invaluable advice during the study. Museum Victoria and La Trobe University provided funding for the project. M. Osborne was supported by a La Trobe University Postgraduate Scholarship. Two anonymous referees provided helpful comments on the manuscript.

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Manuscript received 4 October 2001; accepted 19 February 2002