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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 \textit{P. orientalis}, \textit{P. carmelitae}, \textit{P. vestitus}, and \textit{P. sericeus} are closely related and indicates that \textit{P. gymnotis} is the sister lineage to the genus \textit{Phalanger}. Divergence time estimates indicate that the radiation of \textit{Phalanger} and \textit{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 (\textit{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 (\textit{Trichosurus}) and scaly-tailed (\textit{Wyulda}) possums, which are restricted to Australia.

The composition of the genera \textit{Trichosurus, Wyulda, Spilocuscus} and \textit{Ailurops} is relatively stable. This contrasts dramatically with that of \textit{Phalanger} and \textit{Strigocuscus}, the composition and affinities of which are debated (e.g. Flannery \textit{et al.} 1987a; 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 \textit{et al.} 1987a; George 1987) but some has also been a consequence of erroneous molecular results (Baverstock 1984, corrected in Baverstock \textit{et al.} 1990).

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

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.
In contrast to George (1987), Flannery et al. (1987a) 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.

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<th>Voucher</th>
<th>Collection locality</th>
<th>GenBank Acc. No.</th>
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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 \( \Delta = -2(\log A) \) and the associated \( P \)-value with a \( \chi^2 \) 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
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sister taxa, and Phalanger and Spilocuscus were identified as monophyletic lineages and were each other’s closest relatives (Fig. 2a–c). Phalanger orientalis, P. vestitus, P. carmelitae and P. sericus 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. 2a), with the exception of unweighted parsimony, which identified P. vestitus as the sister taxon of P. orientalis (Fig. 2b). In neighbour-joining, maximum-likelihood, unweighted and transversion parsimony trees, P. gymnotis was basal within Phalanger (Fig. 2a–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. 2c).

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. sericus 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. sericus, 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. 1987a). This

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Fig. 2. Phylogenetic trees based on ND2 sequence data. Topology (a) produced for maximum-likelihood (\(-\text{Ln} = 6295.330\)), 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.
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 P1, P4 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. 3. Neighbour-joining tree for the combined ND2 and 12S–16S rDNA sequences.](image)

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.
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 comparisons (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. 1987a; George 1987). Instead, the present data consistently identified P. gymnnotis 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. gymnnotis 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. gymnnotis 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. gymnnotis 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 (gymnnotis 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. 1987a, 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. 1987b). 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.
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References


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