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Phylogenetic relationships of the cuscuses and brushtail possums (Marsupialia : Phalangeridae) using the nuclear gene *BRCA1*

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Abstract. The family Phalangeridae comprises approximately two dozen extinct and extant species that include the brushtail possums (*Trichosurus*), scaly-tailed possum (*Wyulda*) and cuscuses (*Phalanger*, *Strigocuscus*, *Spilocuscus* and *Ailurops*). Morphological studies have suggested that *Ailurops ursinus* is the sister taxon to all other phalangerids. Another species of interest is *Strigocuscus celebensis*, whose morphologically based taxonomic affinity has habitually been with trichosurins. Mitochondrial 12S rRNA results, however, found moderate support for an *Ailurops* and *Strigocuscus celebensis* clade and placed *A. ursinus* and *S. celebensis* as sister to *Phalanger* and *Spilocuscus*. This study uses nuclear sequence data from the breast cancer and ovarian cancer susceptibility gene 1 (*BRCA1*) to test previous mitochondrial DNA results and uses relaxed molecular clock methods to estimate divergence dates. The results support *Ailurops* as sister taxon to *S. celebensis* and this clade as sister to Phalangerini. Relaxed molecular-dating methods suggest a date of 23–29 million years for the split between Trichosurini and the remaining phalangerids and 19–24 million years for the split between *Ailurops* + *Strigocuscus celebensis* and Phalangerini. Several vicariant/dispersal events are necessary to explain the geographic distribution of the Phalangeridae and our estimated molecular divergence dates are congruent with previously proposed south-east Asian geological events.

Introduction

The family Phalangeridae comprises approximately two dozen extinct and extant species, including brushtailed possums (*Trichosurus*), scaly tailed possum (*Wyulda*) and cuscuses (*Phalanger*, *Strigocuscus*, *Spilocuscus* and *Ailurops*) (Springer *et al*. 1990; Flannery 1994; Hamilton and Springer 1999; Osborne and Christidis 2002). *Trichosurus* and *Wyulda* are found predominately in Australia whereas *Phalanger* and *Spilocuscus* are, for the most part, limited to New Guinea and surrounding islands. Extant species of *Strigocuscus* are found in Sulawesi and surrounding islands (Flannery 1994). *Ailurops* contains a single species, *A. ursinus*, the bear cuscus (Flannery *et al*. 1987), found on Sulawesi and nearby islands. Two taxa whose phylogenetic placements are key for understanding evolutionary relationships are *Ailurops ursinus* and *Strigocuscus celebensis*.

Recent morphological analyses consider *Ailurops* the sister taxon to all other phalangerids while *S. celebensis* is often associated with trichosurins (Tate 1945; Menzies and Pernetta 1986; Flannery *et al*. 1987; George 1987; Flannery 1994; Norris 1994; Crosby and Norris 2003). Tate (1945), using morphological data, considered *Ailurops* to be within one of his three *Phalanger* groups. *S. celebensis* was recognised as a species within the *Phalanger orientalis* group. Unfortunately, his study did not include trichosurins. A subsequent analysis of morphological characters by Menzies and Pernetta (1986) confirmed the position of *S. celebensis* as a *Phalanger* in a group corresponding to the *orientalis* group as defined by Tate (1945). Flannery *et al*. (1987) conducted a morphological cladistic analysis with the phalangerid genera *Trichosurus* (including *Wyulda squamicaudata* as *Trichosurus squamicaudata*), *Spilocuscus*, *Phalanger*, *Ailurops* and *Strigocuscus*, and divided the Phalangeridae into two subfamilies. The subfamily Phalangerinae was separated into two tribes: Trichosurini (*Trichosurus* and *Strigocuscus*) and Phalangerini (*Phalanger* and *Spilocuscus*). *A. ursinus* was placed into its own subfamily, Ailuropinae. Flannery *et al*. (1987) concluded that the most parsimonious reconstruction placed the bear cuscus as sister to all other species in the family. This study altered the taxonomic assignation of *Strigocuscus celebensis* within *Phalanger*, considering it instead to be a plesiomorphic trichosurin. Other morphological studies have subsequently found support for *Ailurops* as the sister taxon to other phalangerids and *S. celebensis* as a species within Trichosurini (George 1987; Flannery 1994; Norris 1994; Crosby and Norris 2003).

However, Kirsch and Wolman (2001), using DNA hybridisation, concluded that *Ailurops* may not be the sister

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taxon to all other phalangerids. The tree that best fit their data placed *Trichosurus* as the sister group to all other phalangerids with *Ailurops* as sister to Phalangerini. Unfortunately, distances between *Ailurops* and *Trichosurus* were anomalously short, making interpretation of the results inconclusive. Ruedas and Morales (2005) examined phalangerid relationships using mitochondrial 12S rRNA. Their results yielded support for an association of *Ailurops*, *Strigocuscus*, *Phalanger* and *Spilocuscus* to the exclusion of the trichosurin genera *Trichosurus* and *Wyulda*. Within the former clade, *Ailurops* grouped with *Strigocuscus* and *Phalanger* with *Spilocuscus*.

Previous molecular sequence analyses that included *Ailurops* and *Strigocuscus* were based on a single mitochondrial gene, 12S rRNA. Given that mitochondrial gene trees can conflict with nuclear gene trees (Flynn and Nedbal 1998; Murphy *et al*. 2001; Arnason *et al*. 2002), it is important to test the conclusions of Ruedas and Morales (2005) using sequences from nuclear genes. Here, we examine the phylogenetic relationships of the bear cuscus (*Ailurops ursinus*) and the small Sulawesi cuscus (*Strigocuscus celebensis*) using sequences from exon 11 of the breast cancer and ovarian cancer susceptibility gene 1, *BRCA1*. Furthermore, we construct a timescale for the evolution of phalangerids using the relaxed molecular clock methodology of Thorne *et al*. (1998), Kishino *et al*. (2001), Thorne and Kishino (2002) and Drummond *et al*. (2006). The phylogeny and timescale are used to interpret the biogeographical history of phalangerids.

Materials and Methods

DNA extraction, amplification and sequencing

DNA was extracted using DNeasy Tissue system kits from QIAGEN. A 2.5-kb fragment from exon 11 of *BRCA1* was amplified in four overlapping segments and sequenced. The forward and reverse primers were as follows: MBF1 (5′ATG TGG CAC RAY AYT CAT GCC A 3′); MBR6 (5′TCA GTC TTC YCA GAA AYG CTA GAA TTT C 3′); MBF498 (5′GAA AGT TAA TGA GTG GTT TTC CAG AA 3′); MBR11 (AGT TCT GAA AGT GGA TTC TTT); MBF9 (5′CTG ACC TRC AGC CTG AGG ATT TCA T 3′); MBR20 (5′CAG AGC AAA TGA CTG GCG CTT 3′); MBF18 (5′ACT CAG GAC AGT RCC TCR TTG TTT C); and MBR28 (5′GCC TGA GCT AAT TTT CCA TTT CCT CC 3′); MBR27 (5′CTT TGT TGA CAG ACT TGG GGT TGC 3′). Accession numbers for nine new *BRCA1* sequences (EF025758–EF025766) are given in Table 1. Additional sequences were obtained from GenBank (Table 1).

Sequence alignments and regions of ambiguity were determined using the program SOAP v1.1b1 (Löytynoja and Milinkovitch 2001). Gap-opening penalties from 11–19 (steps of 2) and extension penalties from 3–11 (steps of 2) were used, yielding 25 different alignments that varied by opening and extension penalties. Sites that were not conserved across all alignments were considered ambiguous and eliminated (Gatesy *et al*. 1993). The dataset was 2424 bp in length and consisted of 15 taxa. In the case of the Bayesian codon model analysis, the sequences were 2415 bp in length. The final dataset was deposited into TreeBASE, study accession number S1638 and matrix accession number M2959 (http://www.treebase.org/, accessed October 2006).

Phylogenetic analyses

Phylogenetic relationships were estimated using maximum parsimony (MP), maximum likelihood (ML), and neighbour-joining (NJ) using PAUP (version 4.0b10) (Swofford 2002). The Akaike Information Criterion (AIC) from Modelltest 3.06 (Posada and Crandall 1998) was used to determine the best-fit model of sequence evolution and estimate model parameters for NJ and ML. The suggested model was a general time reversible (GTR) with a gamma (Γ) distribution of rates. ML and MP searches were heuristic with 100 randomised addition orders and tree-bisection and reconnection (TBR) branch swapping. Gaps were treated as missing data. ML and MP bootstrap analyses employed 500 replicates with 100 repetitions of random step-wise additions. The NJ bootstrap analysis was performed with 500 replicates. Support along branches was further assessed through Bremer support indices (Bremer 1994) using Treerot V2c (Sorenson 1999).

Bayesian analyses were performed using Mr Bayes v3.1.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) under the GTR + Γ model for nucleotides and a codon model that was based on the Goldman and Yang (1994) and Muse and Gaut (1994) models. Three hot and one cold Markov chains were run until the average standard deviation of split frequencies fell below 0.01. Analyses started from random trees and were sampled every 100 generations with the first 25% of trees discarded as burn-in.

Statistical analyses

We performed Kishino–Hasegawa (KH) tests (Kishino and Hasegawa 1989) to distinguish between competing phylogenetic hypotheses. The tests were done with RELL optimisation and 1000 bootstrap replicates. The relationships of *A. ursinus* and *S. celebensis* within Phalangeridae were tested. The competing hypotheses included the three cladograms from Flannery *et al*. (1987; fig. 19*a*–*c*) and the phylogeny from Ruedas and Morales (2005). ML trees for the KH tests were estimated using a heuristic search with NJ starting trees and TBR branch swapping. Only species in the original analyses were included in the constraints. The following constraints were used in constructing the trees: (1) Flannery *et al*. (1987) fig. 19*a*: (*Vombatus*, *Phascolarctos*, *Bettongia*, *Gymnobelideus*, *Macropus*, *Pseudocheirus*, (*A. ursinus*, ((*S. celebensis*, (*P. gymnotis*, (*T. caninus*, *T. vulpecula*))), ((*P. lullulae*, *P. orientalis*), (*S. maculatus*, *S. rufoniger*))))); (2) Flannery *et al*. (1987) fig. 19*b*: (*Vombatus*, *Phascolarctos*, *Bettongia*, *Gymnobelideus*, *Macropus*, *Pseudocheirus*, (*P. orientalis*, (*P. lullulae*, ((*S. maculatus*, *S. rufoniger*), (*A. ursinus*, (*S. celebensis*, ((*T. vulpecula*, *T. caninus*),

Table 1. Accession numbers for taxa included in the BRCA1 dataset

Species	BRCA1
Vombatus ursinus	AF284031
Phascolarctos cinereus	AY243445
Macropus rufus	AF284033
Pseudocheirus herbertensis	AY243448
Bettongia penicillata	DQ354445
Gymnobelideus leadbeateri	EF025766
Phalanger orientalis	AY243449
Trichosurus vulpecula	EF025765
Trichosurus caninus	EF025761
Spilocuscus maculatus	EF025764
Spilocuscus rufoniger	EF025763
Phalanger gymnotis	EF025762
Phalanger lullulae	EF025760
Strigocuscus celebensis	EF025759
Ailurops ursinus	EF025758

P. gymnotis))))))); (3) Flannery *et al*. (1987) fig. 19*c*: (*Vombatus*, *Phascolarctos*, *Bettongia*, *Gymnobelideus*, *Macropus*, *Pseudocheirus*, (*A. ursinus* (*S. celebensis*, ((*T. caninus*, *T. vulpecula*), (*P. gymnotis*, ((*S. rufoniger*, *S. maculatus*), (*P. orientalis*, *P. lullulae*))))))); (4) Ruedas and Morales (2005): (*Macropus*, (((*A. ursinus*, *S. celebensis*), ((*P. lullulae*, *P. gymnotis*), (*S. maculatus*, *S. rufoniger*))), *T. vulpecula*)).

Molecular-dating analyses

Multidivtime (Thorne *et al*. 1998; Kishino *et al*. 2001; Thorne and Kishino 2002) and BEAST v.1.3 (Drummond and Rambaut 2003; Drummond *et al*. 2006) were used to estimate divergence times. Both methods use a relaxed molecular clock under a Bayesian Markov Chain Monte Carlo framework and allow for the incorporation of multiple constraints from the fossil record. In contrast to *multidivtime*, BEAST v.1.3 co-estimates the phylogeny and divergence times simultaneously and does not assume autocorrelation of molecular rates among lineages. *Multidivtime* allows for minimum and/or maximum constraints on nodes whereas BEAST v.1.3 requires minimum and maximum values for nodes that are constrained.

Multidivtime

The phylogeny shown in Fig. 2 was used in the analyses with *Phascolarctos* and *Vombatus* treated as the outgroups. Node numbers are shown in Fig. 3. The programs *estbranches* and *multidivtime* (version 9-25-03) were used to estimate branch lengths and divergence times, respectively (Thorne *et al*. 1998; Kishino *et al*. 2001; Thorne and Kishino 2002). The *multidivtime* analysis implemented the F84 (Swofford *et al*. 1996) model of sequence evolution with a Γ distribution of rates and four discrete categories. PAUP (version 4.0b10) (Swofford 2002) was used to estimate the transition/transversion parameter and rate categories of the gamma distribution for the tree shown in Fig. 2. The mean of the prior distribution for the root of the ingroup was set to 55 million years based on the absence of crown group diprotodontian fossils from Murgon, the oldest Tertiary terrestrial vertebrate–bearing deposit in Australasia (Godthelp *et al*. 1999).

The median amount of evolution from the ingroup root to the ingroup tips was used to set the mean of the prior distribution for the rate of molecular evolution at the ingroup root node. All Markov Chain Monte Carlo analyses were run for 1 million generations with a burn-in of 100 000 generations, allowing the Markov Chains to approach stationarity, and sampled every 100 generations.

The fossil constraints used were as follows:

(1) Node 13. *Paljara* sp. A from Zone A (~25.5 million years) of the Etadunna Formation is the oldest described pseudocheirid fossil (Woodburne *et al*. 1993). *Djaludjangi yadjana* from the middle Miocene of Riversleigh is the oldest reputed fossil petaurid (Brammall 1998); however, it also has been considered Petauroidea *incertae sedis* (Brammall 1998; Crosby 2004). As a result, we used 25.5 million years as a minimum for Node 13.

(2) Node 14. The oldest dated deposit to contain both potoroine (*Purtia mosaicus*: Case 1984) and macropodine kangaroos (*Nambaroo* species A and B, Genus P: Woodburne *et al*. 1993) is Zone C (25.0–25.5 million years: Woodburne *et al*. 1993) of the Etadunna Formation. Riversleigh's System A and B deposits have also produced a potoroine taxon (*Balbaroo fangaroo*: Cooke 2000). Myers and Archer (1997) suggested that these systems can be biocorrelated with the Ngama Local Fauna (Zone D; 24.7–25.0 million years: Woodburne *et al*. 1993) of the Etadunna Formation. We used 25.0 million years as the minimum for Node 14. Morphologically, the Oligo-Miocene potoroines and macropodines are similar to one another; the plesiomorphic kangaroo taxon from Zone A of the Etadunna Formation cannot be positively assigned to either a potoroine or macropodine lineage (Woodburne *et al*. 1993). As a result, we used 33.7 million years as the maximum for Node 14 (base of the Oligocene: McKenna and Bell 1997).

(3) Node 24. The oldest deposit in Australasia to produce marsupial taxa is Murgon in south-eastern Queensland (Godthelp *et al*. 1999). To date there have been no petauroid marsupials described from the deposit, which has been dated to 54.6 million years. We used 54.6 million years as the maximum age for the ingroup root (i.e. Phalangeridae + Macropodidae).

BEAST v.1.3

The mean substitution rate per year was set to the mean of the prior distribution for the rate of molecular evolution at the ingroup root node (0.001) as calculated in the *multidivtime* analyses. The standard deviation was set equal to the mean substitution rate. We used the GTR model of sequence evolution and a gamma distribution with four categories as suggested by Modelltest 3.06 (Posada and Crandall 1998) with an

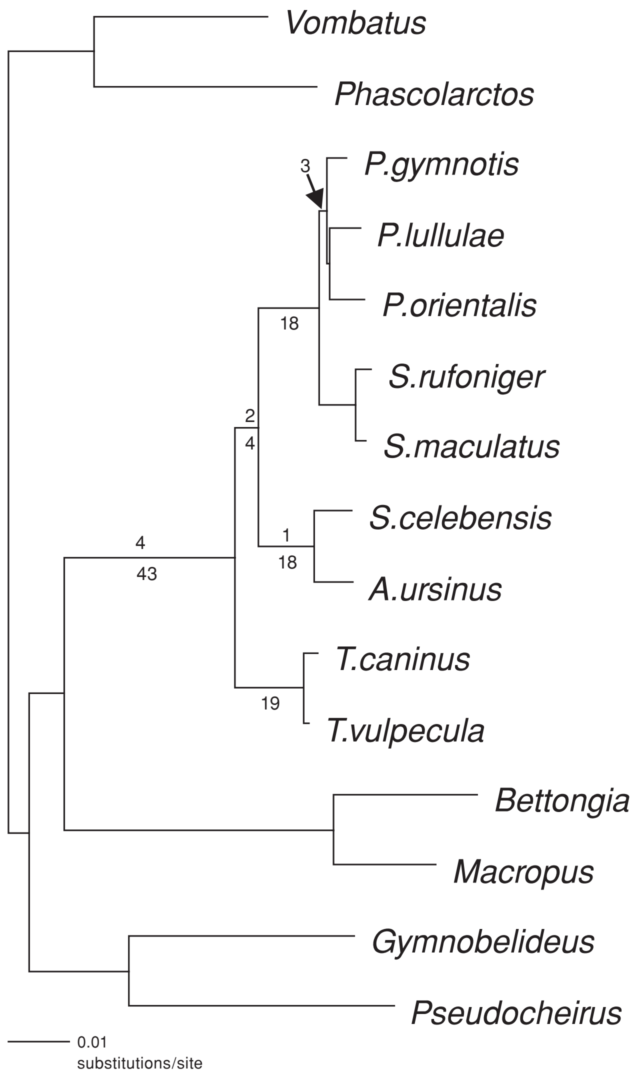

Fig. 1. The maximum-likelihood phylogram (–ln likelihood = 7934.97415) of the BRCA1 dataset using a GTR+Γ model of sequence evolution. The numbers above the branches correspond to the numbered hypotheses listed in Table 2. Bootstrap support percentages for numbered branches are given in Table 2. The values below the branches correspond to the Bremer support indices.

Table 2. Summary of bootstrap and posterior probabilities for various Phalangeridae hypotheses

Hypothesis	Parsimony (%)	Neighbour joining[A] (%)	Maximum likelihood (%)	Bayesian nucleotide 1st run	Bayesian nucleotide 2nd run	Bayesian codon 1st run	Bayesian codon 2nd run
(1) *S. celebensis* sister taxon to *Ailurops*	100.0	100.0	100.0	1.00	1.00	1.00	1.00
(2) Monophyly of *Ailurops* + *S. celebensis* + Phalangerini	90.4	95.0	95.2	1.00	1.00	1.00	1.00
(3) Monophyly of *Phalanger*	96.0	87.0	96.9	1.00	1.00	1.00	1.00
(4) Monophyly of Phalangeridae	100.0	100.0	100.0	1.00	1.00	1.00	1.00

[A] Neighbour joining analysis performed with maximum likelihood distances.

uncorrelated log-normal rate-variation model. Branching rates were assumed to follow a Yule prior distribution and the tree indicated in Fig. 2 was used as the starting tree. All analyses were run for 10 million generations, sampling every 100 generations with a burn-in of 1 million generations. Tracer 1.2 (Rambaut and Drummond 2003) was used to visually inspect chain behaviour to determine whether the chains were run long enough to reach stationarity; four independent analyses were performed to check for convergence. The following constraints were used:

(1) Maximum Root Height. We used 65 million years as the maximum age for the base of Diprotodontia (which now includes *Phascolarctos* and *Vombatus*, usually recovered as sister group to all other diprotodontians) based on absence of diprotodontian fossils in the Eocene Murgon deposit but still allowing for the possibility of a slightly earlier origin.
(2) Node 13. We used a minimum of 25.5 million years and a maximum of 54.6 million years for the base of Petauroidea.
(3) Node 14. We used the same minimum of 25 million years and a maximum of 33.7 million years for the macropodine–potoroine split as used in the *multidivtime* analyses.
(4) Node 24. We used a minimum of 25.5 million years and a maximum of 54.6 million years for the ingroup root.

Results

The maximum-likelihood phylogram and posterior probabilities for the Bayesian analysis using the nucleotide model are shown in Figs 1 and 2 respectively. The topologies place *Trichosurus* as the sister taxon to other phalangerids. Within the latter group, there is a sister group association between *A. ursinus* + *S. celebensis* and Phalangerini. *Phalanger gymnotis* is grouped in the genus *Phalanger*. The MP heuristic search yielded two most-parsimonious trees. The topology of one most-parsimonious tree was identical to the ML and Bayesian analyses while the other was nearly identical except *P. orientalis* was the sister group to a clade containing *P. gymnotis* and *P. lullulae* (results not shown). The MP, NJ, ML bootstrap values and Bayesian posterior probabilities for some of the relationships within Phalangeridae are given in Table 2. The numbers above the branches in Fig. 1 and below the branches in Fig. 2 correspond to the numbering on the hypotheses in Table 2.

All analyses placed *P. gymnotis* with all other *Phalanger* species with high support. Bootstrap values ranged from 87.0 – 96.9% and Bayesian posterior probabilities were 1.00 (Table 2). The placement of *Ailurops* as sister taxon to *S. celebensis* was extremely well supported in all analyses (bootstrap support = 100.0%; posterior probabilities = 1.00) (Table 2). An association of *Ailurops* + *S. celebensis* + Phalangerini to the exclusion of *Trichosurus* was also supported (bootstrap support = 90.4 – 95.2%; posterior probabilities = 1.00) (Table 2). Bremer support indices yielded strong support for an *A. ursinus* + *S. celebensis* association, requiring 18 steps to collapse this branch (Fig. 1). There was less support for an *A. ursinus* + *S. celebensis* + Phalangerini (Bremer support = 4) association (Fig. 1).

Table 3. Kishino–Hasegawa test results

Comparison of prior hypotheses	–ln likelihood	Δ	*P*[A]
Flannery *et al.* (1987), fig. 19*a*	8137.33120	202.35705	0.000
Flannery *et al.* (1987), fig. 19*b*	8204.68038	269.70624	0.000
Flannery *et al.* (1987), fig. 19*c*	8017.65286	82.67872	0.000
Ruedas and Morales (2005)	7934.97415	(best)	

[A] Two-sided test.

Kishino–Hasegawa tests

KH tests were used to compare the Flannery *et al.* (1987) and Ruedas and Morales (2005) hypotheses pertaining to the phylogenetic relationship of *Ailurops* and *S. celebensis*. The KH test was statistically significant and favoured the Ruedas and Morales (2005) hypothesis that *Ailurops* and *S. celebensis* are sister taxa and that this group is more closely related to Phalangerini than to *Trichosurus* (Table 3).

Molecular-dating analyses

Table 4 and Fig. 3 report the molecular divergence date estimates for the Phalangeridae. Dates from other studies are also reported in Table 4. The BRCA1 dates from this study include 95% credibility intervals for the *multidivtime* analysis (Thorne *et al.* 1998; Kishino *et al.* 2001; Thorne and Kishino 2002) and 95% highest posterior densities for the BEAST v.1.3 analysis (Drummond *et al.* 2006). The BRCA1 data estimate the basal split between Trichosurini and Phalangerini + *Ailurops* + *S. celebensis* at ~29 and 23 million years for *multidivtime* and BEAST v.1.3, respectively. The *Ailurops* + *S. celebensis* and Phalangerini split was estimated at ~24 (*multidivtime*) and 19 (BEAST v.1.3) million years. Between *Ailurops* and *S. celebensis*, *multidivtime* estimated a divergence date of ~11 million years and BEAST v.1.3 estimated a divergence date of ~8 million years.

Table 4. Estimates of divergence times (in millions of years) from molecular data

Divergence times are from Kirsch *et al.* (1997) for DNA hybridisation (1), from Hamilton and Springer (1999) for 12S rRNA transversions, from Osborne and Christidis (2002) for ND2, from Kirsch and Wolman (2001) for DNA hybridisation (2), from Ruedas and Morales (2005) for 12S rRNA, and from this study for both BRCA1 analyses. n.a., not applicable

Taxa compared	Divergence times						
	DNA hybridisation (1)	12S rRNA transversions	ND2	DNA hybridisation (2)	12S rRNA	BRCA1[A] *Multidivtime*	BRCA1[B] BEAST v.1.3
Trichosurini versus Phalangerini + *Ailurops* + *Strigocuscus celebensis* (Node 22)	19.9[C]	17.3[C]	25[C]	21[D]	24.7–27.3	28.85 (20.792–36.203)	23.30 (17.55–29.84)
Ailurops + *Strigocuscus* versus Phalangerini (Node 21)	n.a.	n.a.	n.a.	16[E]	21.1–23.3	24.20 (16.266–32.473)	18.63 (13.95–24.00)
Spilocuscus versus *Phalanger* (Node 19)	11.9	4.7	14–23	10	14.6–16.1	11.48 (5.850–19.427)	8.64 (6.00–11.68)
Strigocuscus celebensis versus *Ailurops* (Node 20)	n.a.	n.a.	n.a.	n.a.	14.9–16.4	11.18 (5.439–19.205)	7.62 (4.18–11.37)
Phalanger gymnotis versus *Phalanger orientalis* (Node 17)	7.4	4.3	11	7.5	12.5–13.8[F]	9.08 (4.323–16.182)	6.37 (4.07–8.89)
Spilocuscus maculatus versus *Spilocuscus rufoniger* (Node 18)	3.2	2.1	3	4	5.9–6.5	3.54 (0.924–8.296)	2.74 (1.28–4.87)

[A] Values in parentheses are 95% credibility intervals. [B] Values in parentheses are 95% highest posterior densities; estimates are the average of four independent runs. [C] Estimated divergence times are between Trichosurini and Phalangerini; *Ailurops ursinus* and *Strigocuscus celebensis* were not included in the study. [D] Estimated divergence times are between Trichosurini and Phalangerini + *Ailurops ursinus*; *Strigocuscus celebensis* was not included in the study. [E] Estimated divergence date is between *Ailurops ursinus* and Phalangerini; analysis did not include *Strigocuscus celebensis*. [F] Estimated dates correspond to split between *Phalanger gymnotis* and *Phalanger lullullae*.

Discussion

Evolutionary relationships within Phalangeridae

Many authors, using morphological studies, have considered *Ailurops* the plesiomorphic sister group to the remaining phalangerids (Flannery *et al*. 1987; George 1987; Norris 1994) and classified *S. celebensis* as a species within Trichosurini (Flannery *et al*. 1987; George 1987; Flannery 1994; Norris 1994; Crosby and Norris 2003). However, DNA hybridisation results (Kirsch and Wolman 2001) found support for an association of *Ailurops* and Phalangerini to the exclusion of Trichosurini. Ruedas and Morales (2005), the first authors to include *S. celebensis* in a molecular study, used mitochondrial 12S rRNA data to determine a sister group relationship between *Ailurops* and *S. celebensis*, with a sister group relationship between these taxa and Phalangerini. Our BRCA1 analyses are compatible with both DNA hybridisation and the 12S rRNA studies and provide the first set of nuclear sequence data to examine these relationships within Phalangeridae. Strong phylogenetic support was found for both the *S. celebensis* sister taxon to *Ailurops* and the *S. celebensis* + *Ailurops* sister to Phalangerini relationships. This hypothesis was found to be

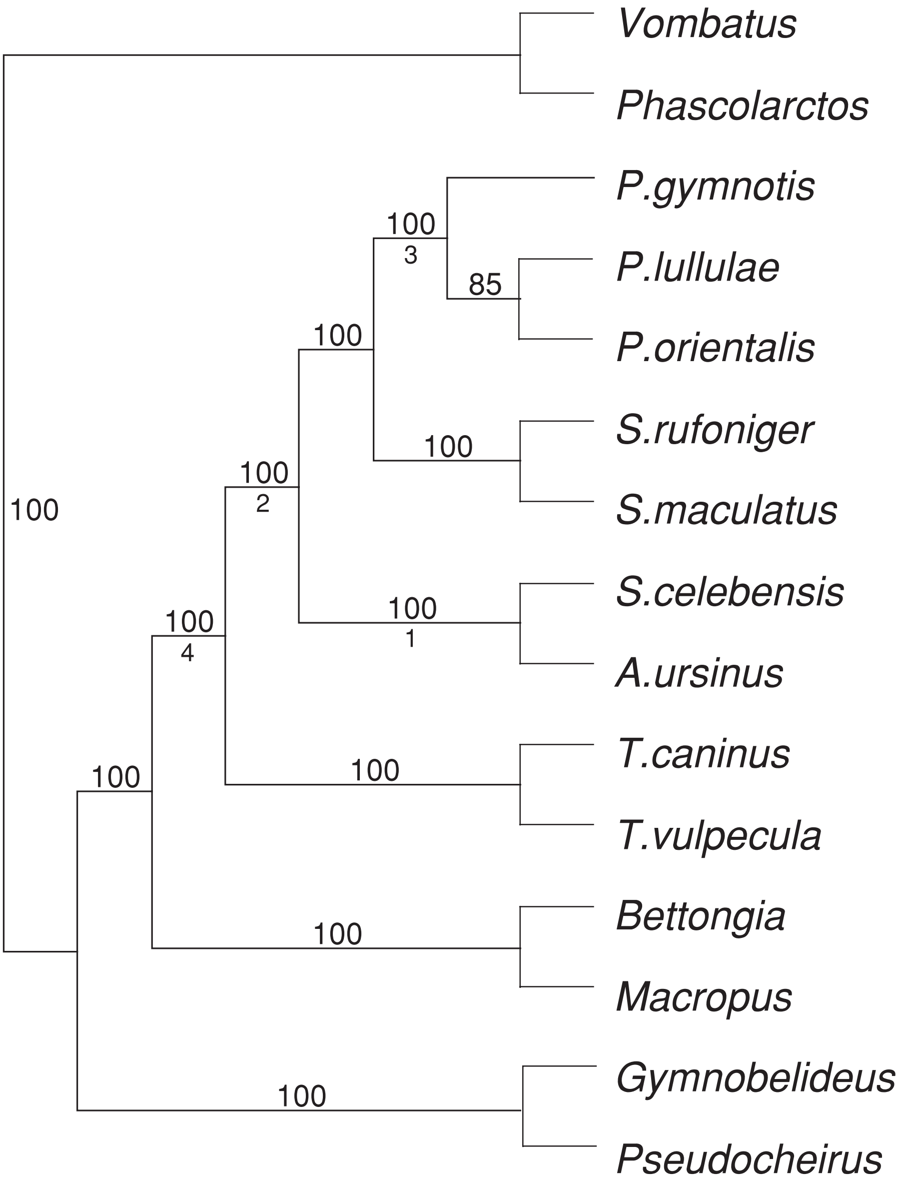

Fig. 2. Bayesian tree (GTR+Γ model of sequence evolution) from the nucleotide model analyses using the BRCA1 dataset. Posterior probabilities are expressed as percentages above the branches. Values are the same for both runs. The numbers below the branches correspond to the numbered hypotheses listed in Table 2. Posterior probabilities for numbered branches are also given in Table 2.

significantly better than the three proposed by Flannery *et al*. (1987). The *S. celebensis* and *Ailurops* association was further supported by high Bremer support indices, which were comparable to the number of steps required to collapse the *Trichosurus* branch (19 steps) and the *Phalanger* + *Spilocuscus* branch (18 steps) (Fig. 1). Furthermore, this study presents the first analyses from a nuclear gene to support the findings that *P. gymnotis* is more closely related to other *Phalanger* species than to trichosurins, a relationship previously shown through mitochondrial (Hamilton and Springer 1999; Osborne and Christidis 2002; Ruedas and Morales 2005) and DNA hybridisation (Springer *et al*. 1990; Kirsch and Wolman 2001) data.

Molecular divergence dates and biogeography

If the phylogenetic relationships proposed by Ruedas and Morales (2005) and the BRCA1 data from this study are accurate representations, then biogeography plays a

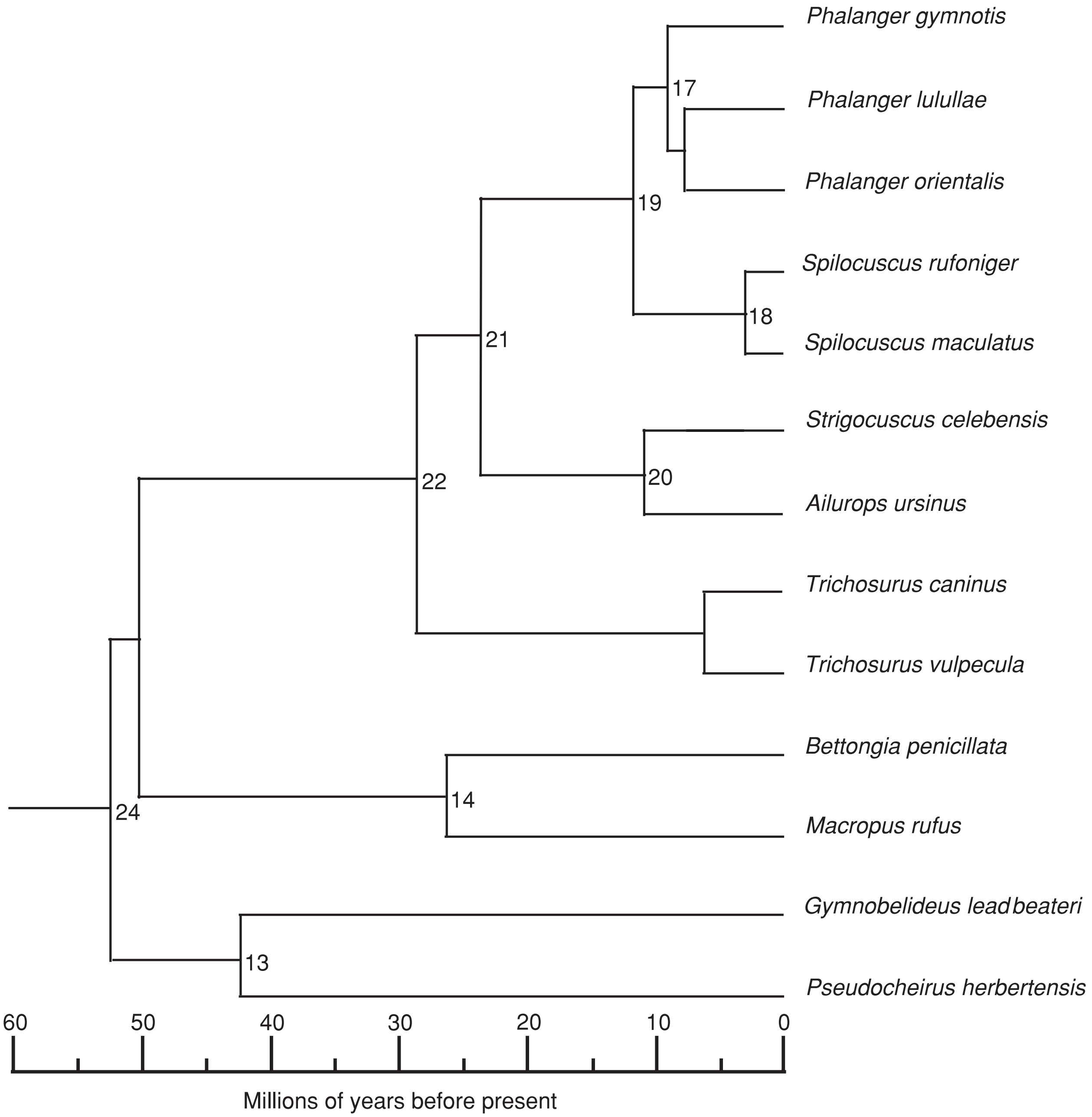

Fig. 3. Molecular time scale for the species within Phalangeridae using *multidivtime* based on the 2424-bp BRCA1 dataset and Bayesian tree of Fig. 2, three fossil constraints and a mean prior of 55 million years ago for the root of Phalangeridae. Values in the tree refer to internal node numbers that correspond with node numbers referenced in the text.

substantial role for understanding how species of Phalangeridae are related. Furthermore, the timing of the splits appears to coincide with geographical events in the region. In this study, divergence dates were estimated using programs that employ a relaxed molecular clock. These dates are comparable to previously calculated dates (Table 4). However, the relaxed molecular clock method has an advantage over previously used dating methods since it allows each branch to have its own rate of evolution. In addition, these programs allow for the incorporation of multiple fossil constraints. However, it should be noted that reconstructing the palaeogeographic history of emergent land in south-east Asia is fraught with difficulties (Hall 1998).

In a recent reconstruction by Hall (1998), New Guinea and Sulawesi were under water until ~20–25 million years ago, when small portions of New Guinea and Sulawesi became emergent. The timing of this event coincides approximately with our estimates of a Trichosurini and Phalangerini + *Ailurops* + *S. celebensis* split (Table 4). Therefore, a plausible scenario would suggest a dispersal event of the ancestor to Phalangerini + *Ailurops* + *S. celebensis* to the New Guinea region around the time these land masses became emergent.

The ancestor to *Ailurops* + *S. celebensis* would have separated from Phalangerini soon after the ancestor of Phalangerini + *Ailurops* + *S. celebensis* split from Trichosurini. Reconstruction of south-east Asia over the past 50 million years shows that crustal fragments of the Bird's Head microcontinent have been carried west on the Philippine or Molucca Sea plate (Hall 1996, 1998). Between 17 and 15 million years ago, the Buton-Tukang Besi block detached from the Bird's Head, connecting with the south-eastern peninsula of Sulawesi at ~11 million years ago (Hall 2002). An event such as this could have transported the ancestor of *Ailurops* and *S. celebensis* to its present distribution. The timing of the split between *Ailurops* and *S. celebensis* was estimated at ~8–11 million years ago. These dates would suggest a subsequent speciation event upon reaching the region or isolation on distinct emergent land masses that nevertheless would have constituted a singular microplate, in this instance Buton-Tukang Besi. The coincidence of molecular data with independent geological data from the region for a synthetic approach to biogeography reinforces our results. Examination within this synthetic framework of additional, non-related, taxa with overlapping distributions may yield a yet finer picture of a region that – through the pioneering work of Alfred Russel Wallace – gave rise both to biogeography and evolution.

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