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EVOLUTIONARY RELATIONSHIPS AMONG GENERA OF PHALANGERIDAE (METATHERIA: DIPROTODONTIA) INFERRED FROM MITOCHONDRIAL DNA

LUIS A. RUEDAS* AND JUAN CARLOS MORALES

Museum of Vertebrate Biology and Department of Biology, Portland State University, Science Building II, Room 232B, 1719 SW 10th Avenue, Portland, OR 97207-0751, USA (LAR)
Center for Environmental Research and Conservation, Columbia University, Schermerhorn Extension, 10th Floor, 1200 Amsterdam Avenue, New York, NY 10027, USA (JCM)

We sequenced the 12S rRNA gene of 2 elusive and morphologically plesiomorphic species of phalanger: the small Sulawesi cuscus (*Strigocuscus celebensis*—Gray, 1858) and the Sulawesi bear cuscus (*Ailurops ursinus*—Temminck, 1824). The sequences were integrated with previously existing data on the same gene in other species of phalangerids, as well as newly derived data from *Wyulda* Alexander, 1918. In contrast to current wisdom, we resolve *S. celebensis* not as a member of the tribe Trichosurini, but rather as a taxon sister to *Ailurops* in a reconstituted Ailuropinae in turn successively sister to Phalangerinae. Examination of our data supports an evolutionary origin for the family approximately 34 million years ago (mya), in the northwestern region of the Sahul Shelf, the continental mass underlying Australia and New Guinea. The radiation of the most plesiomorphic genera in the family, *Trichosurus* and *Wyulda*, is restricted to that region. *S. celebensis*, resolved as sister to *A. ursinus* in a clade ingroup to trichosurines, diverged from remaining ingroup lineages between 21.1 and 23.3 mya, a time when Sulawesi was available for colonization and sea currents would have enhanced the colonization potential from the east of Sulawesi and neighboring islands. We recommend Trichosurinae as a subfamilial level entity on par with Ailuropinae and Phalangerinae, circumscription of Trichosurinae to *Trichosurus* and *Wyulda*, and removal of *Strigocuscus* into Ailuropinae, leaving only *Phalanger* and *Spilocuscus* in Phalangerinae.

Key words: *Ailurops*, biogeography, *Phalanger*, Phalangeridae, 12S rRNA, Southeast Asia, *Strigocuscus*, systematics, taxonomy, *Wyulda*

Increasingly, biogeographic studies of Southeast Asia are resolving Sulawesi as a crucial nexus in the biological evolution of the region. Alfred Russell Wallace (1869) partially glimpsed the biological complexity of the island when he successively considered it as being part of his Oriental Region, then in the Australian Region, and finally, as a complex conundrum constituted by fauna of both biotic regions. Insofar as the mammal fauna of Sulawesi is concerned, Wallace's confusion may have been compounded by the fact that only 14 species of nonvolant mammals were known from Sulawesi at the time he was developing his biogeographic theories (Wallace 1869).

One of these species was the small Sulawesi cuscus (*Strigocuscus celebensis*; Fig. 1); another was the Sulawesi bear cuscus (*Ailurops ursinus*). These, together with at least 20 additional species, constitute the family Phalangeridae (as variously understood and summarized by Colgan et al. [1993], Flannery [1994, 1995a, 1995b], Flannery and Calaby [1987], Flannery et al. [1987], George [1987], Groves [1987a, 1987b, 1993], and Norris and Musser [2001]). *A. ursinus* is distributed throughout Sulawesi and nearby islands of Togian, Peleng, Muna, Buton, and Lirung (Talaud Archipelago); *Spilocuscus maculatus* Desmarest, 1817, has a range extending from Queensland (Australia), through New Guinea, to Buru and Ceram, as well as the small island of Selayar, at the tip of Sulawesi's Southeastern Peninsula; and lastly, *Phalanger pelengensis* Tate, 1945 (considered conspecific with *Strigocuscus celebensis* by Flannery et al. [1987] but specifically distinct in *Phalanger* by George [1987] following Tate [1945] and as *Strigocuscus pelengensis* by Flannery [1994, 1995a]) is restricted to the islands of Peleng and Taliabu, off the tip of Sulawesi's Eastern Peninsula. To Wallace, it thus was clear that the mammal fauna of Sulawesi was dominated by species of patent Australian affinities; the uncertainties regarding the broader biogeographic affinities lay rather with the remaining fauna, primarily insects and birds (Wallace 1869).

* *Correspondent: ruedas@pdx.edu*

Fig. 1.—*Strigocuscus celebensis* (Museum of Southwestern Biology no. 93124), from Central Sulawesi.

A phalangerid—possibly *Phalanger intercastellanus*—reputedly was the 1st Australasian marsupial discovered by Europeans, in the mid-16th century (Calaby 1994; Flannery 1994; McKay and Winter 1989), and *Phalanger orientalis* (Pallas, 1766) was the 1st australidelphian to be formally named (Flannery 1994; Flannery et al. 1987). Despite this long history of discovery, significant gaps remain in our knowledge of the biology of these organisms, especially regarding their evolutionary relationships. Within the context of Phalangeridae, the relationships of *Strigocuscus celebensis* to other species has remained an enigma. The genus *Strigocuscus* has had an inconsistent taxonomic history. Flannery et al. (1987) in their summary of the systematics of phalangerids indicated that the earliest name proposed for phalangerids was *Phalanger* Storr, 1780, a genus that variously has included species now in *Ailurops* Wagler, 1830, *Spilocuscus* Gray, 1861, and *Strigocuscus* Gray,

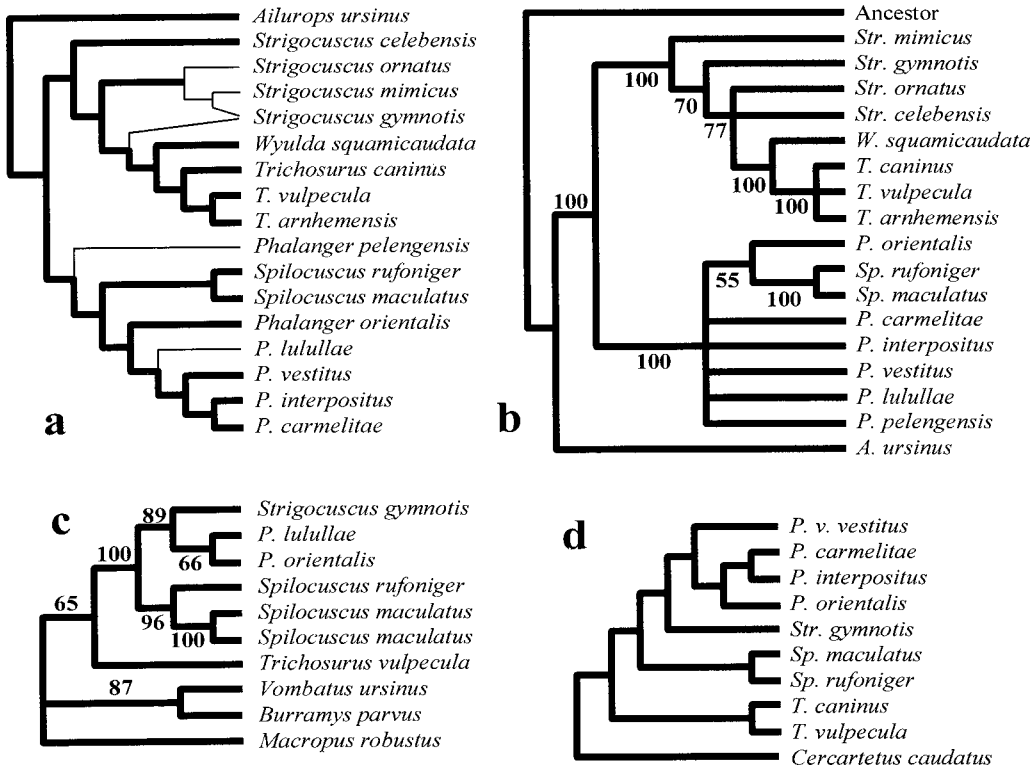

FIG. 2.—Phylogenies previously proposed for Phalangeridae. a) relationships suggested by Flannery et al. (1987). b) Consensus of 94 equally most-parsimonious trees resulting from analysis of the data set of Flannery et al. (1987). c) Bootstrap tree derived from the analysis of 12S rRNA data by Hamilton and Springer (1999). d) Topology of relationships derived from DNA:DNA hybridization by Springer et al. (1990).

1861. Revisors of all or part of Phalangeridae also have recognized the distinctiveness of *Ailurops ursinus*, but its generic context varied, being variously included in *Cuscus*, *Ailurops*, *Phalangista*, and an inclusive *Phalanger* (most recently by Feiler [1977], George [1982], and Laurie and Hill [1954]). Similarly, *Strigocuscus* has had a conflictive taxonomic history. Described by Gray (1861), the genus was erected as a ''section'' for the species *Cuscus celebensis* Gray, 1858. However, that species also is taxonomically problematic. In his description of the species, Gray (1858) indicated as habitat ''Celebes,'' the former name for Sulawesi. Furthermore, in addition to indicating that the British Museum had 1 individual from Sulawesi's main port of Macassar procured by a Mr. J. R. Wallace in 1851 (presumably Alfred Russell Wallace—although Wallace indicated that he was in Macassar in 1856 and 1857, rather than 1851 [Wallace 1869]), Gray further ascribed to the species 2 individuals originating from San Cristóbal Island, in the Solomon Archipelago. In fact, *Strigocuscus celebensis* occurs exclusively on Sulawesi and surrounding islets; only *Phalanger orientalis* is known from San Cristóbal (Flannery 1995a).

Flannery et al. (1987) suggested that *Strigocuscus celebensis* belonged in the tribe Trichosurini, along with *Trichosurus*, and defined *Strigocuscus* as being constituted by 4 species: *celebensis*, *mimicus* (Thomas, 1922), *ornatus* (Gray, 1860), and *gymnotis* (Peters and Doria, 1875). However, Flannery et al. (1987) did indicate that the species in *Strigocuscus* exclusive of *S. celebensis* should be considered incertae sedis. They further suggested that *S. celebensis* was not necessarily the sister taxon to remaining *Strigocuscus*, and that it might be the basal trichosurin, that is, that *Strigocuscus* was paraphyletic (Figs. 2a and 2b). George (1987) placed *ornatus* and *gymnotis* in *Phalanger* and considered *mimicus* a synonym of *Phalanger orientalis*; Groves (1993) followed this taxonomy. The tribe Trichosurini as conceived by Flannery et al. (1987) included *Strigocuscus* and *Trichosurus*, with *Wyulda* being considered a subgenus of *Trichosurus* by these investigators. George (1987), while recognizing that *Strigocuscus* was more closely related to *Trichosurus* and *Wyulda*, did not explicitly consider these latter genera in his review of the family. Norris (1994) and Crosby and Norris (2003) considered characters of the periotic and likewise supported inclusion of *Strigocuscus* into a Trichosurini with *Trichosurus* and *Wyulda*.

Springer et al. (1990) used DNA hybridization in an examination of 9 phalangerid species: *Trichosurus vulpecula*, *T. caninus*, *Spilocuscus rufoniger*, *S. maculatus*, ''*Strigocuscus*'' *gymnotis* (after the classification of Flannery et al. [1987]), *Phalanger interpositus*, *P. carmelitae*, *P. vestitus*, and *P. orientalis* (this latter constituting a polytypic species complex as per Norris and Musser [2001]). The specimen of *P. orientalis* used by Springer et al. (1990) subsequently was reassigned by T. F. Flannery to *P. intercastellanus* (S. Ingleby, pers. comm.). The results of Springer et al. (1990) supported a close, unresolved association between *Strigocuscus* (represented by *gymnotis*) and *Phalanger*; *Spilocuscus* was the sister taxon to the *Strigocuscus–Phalanger* clade, and *Trichosurus*

was resolved as the basal genus in family (Fig. 2d). However, *Strigocuscus gymnotis* subsequently was integrated into *Phalanger*, pursuant to the analyses of George (1987). Subsequently, Hamilton and Springer (1999) used sequence data from the mitochondrial 12S rRNA gene in an examination of 6 phalangerid species: *P. gymnotis*, *P. orientalis* (the same specimen as above, hence *P. intercastellanus*), *P. lullulae*, *Spilocuscus maculatus*, *S. rufoniger*, and *Trichosurus vulpecula*. Their sequence results were compatible with their DNA hybridization data (Fig. 2c). These conflicting data and their lacunar nature with respect to species variously assigned to *Strigocuscus* echo Flannery's (1994) perspective that the genus remains relatively unknown both ecologically and evolutionarily. Flannery (1995a:89) also noted with respect to *Ailurops* that "Because of its phylogenetic position and unusual morphology, the bear cuscus must be considered a prime candidate for field based research." Indeed, the evolutionary relationships of these 2 genera constitute critical questions, particularly given the continuing biogeographic conundrums of the region 1st outlined by Wallace.

The foregoing reveals testable hypotheses in phalangerid evolution. The major unanswered questions are the relationships of *Strigocuscus* and *Ailurops* to each other and to other phalangerid genera. The only study to address this issue (Flannery et al. 1987) yielded ambiguous results, as did the reanalysis of the same morphological characters (Springer et al. 1990). Elucidation of the phylogenetic relationships of *Strigocuscus* and *Ailurops* has important biogeographic implications. Both genera are endemic to portions of Sulawesi and adjacent islands. Hence, a basal situation for either relative to remaining phalangerids would constitute prima facie evidence that phalangerids originated in Sulawesi and subsequently colonized islands to the east. In contrast, any other evolutionary hypothesis would support the proposition of single or multiple invasions of Sulawesi by phalangerids, depending on the relationships between *Strigocuscus* and *Ailurops* and between these and remaining phalangerid genera. Herein, we use sequence data from the mitochondrial 12S rRNA gene to assess the evolutionary relationships of *Ailurops ursinus* and *Strigocuscus celebensis* within the framework of existing comparative data from other phalangerids. Specifically, we wished to test the hypotheses that either *A. ursinus* or *S. celebensis* constitute basal taxa in Phalangeridae, or whether they are in contrast members of other subfamilial or tribal level taxonomic entities within Phalangeridae.

Materials and Methods

We preserved whole in 95% ethanol a specimen of *Strigocuscus celebensis*, field number NK 103527; this specimen was deposited in the Museum of Southwestern Biology, University of New Mexico (mammal catalog number 93124). Date of collection was 16 August 2000; location of provenance was Indonesia: Sulawesi Island, Sulawesi Tengah Province, Kabupaten (≈ Regency) Tana Toraja, Kecamatan (≈ County) Rindingallo, Desa (≈ Township) Awan; Dusun (≈ neighborhood) Rantekarua; 2°54.130′S, 119°41.839′E, elevation 2,120 m. The specimen was purchased from a local hunter who indicated that it came from the environs of the nearby village (Kampung) of Baru (elevation ≈1,750 m). We preserved whole in 95% ethanol a specimen of *Ailurops ursinus*, field number NK 80066; this specimen is deposited in the Indonesian National Museum of Natural History, Cibinong, Java, Indonesia (also known as Research Center for Biology—Lembaga Ilmu Pengetahuan Indonesia; formerly known as the Bogor Museum). Date of collection was 2 June 1998; location of provenance was Indonesia: Sulawesi Island, Sulawesi Tengah Province, Poso Regency, Kecamatan (≈ County) Ulubongka, Desa (≈ Township) Marowo; ≈ 0°57′S, 121°27′E, elevation 5 m. The specimen was purchased from a local hunter who kept it in captivity with another, immature, individual (not purchased) trapped approximately 3 km inland (southeast) from Marowo, at ≈150–300 m. Tissues from NK 80066 (stored in liquid nitrogen in the field) and NK 103527 (stored in 95% ethanol in the field) are deposited in the Museum of Southwestern Biology, University of New Mexico, Division of Biological Resources. Tissues from *Wyulda squamicaudata* were obtained from The Australian Museum, specimen M 22321, collected in 1990 by H. Parnaby in Western Australia, Mitchell Plateau, Mertens Creek; 14°49′S, 125°44′E. Animals were handled in accordance with approved guidelines set out by University of New Mexico Animal Health and Welfare Committee per guidelines established by the American Society of Mammalogists (Animal Care and Use Committee 1998).

The DNA was extracted from an approximately 3-mm^3 sample of liver by using a DNeasy tissue extraction kit (Qiagen, Valencia, California). Total genomic DNA was isolated from all tissues by using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocols. The 12S rRNA mitrochondrial DNA (mtDNA) gene was sequenced as follows. Polymerase chain reaction was performed in a 50-μl total volume containing 2 μl (approximately 200 ng) of total genomic DNA as template, 1 μM of each of primers 378F (5′-AAG TTT GGT CCT AGC CTT-3′) and 382R (5′-TTT CAT CTT TCC CTT GCG GTA CT-3′), 0.2 mM of deoxynucleoside triphosphates, 1.0–3.0 mM of $MgCl_2$, and 0.4 U of AmpliTaq DNA polymerase (5 U/μl; Perkin-Elmer, Wellesley, Massachusetts). Thermal cycling was performed in Perkin-Elmer thermocyclers with the following protocol: hot-start at 94°C for 1 min, followed by 25–35 cycles of denaturation at 94°C for 30 s, annealing at 52–55°C for 30 s, and extension at 72°C for 60 s, ended by a final step at 72°C for 7 min, and stored at 4°C. After electrophoresis in a 1% agarose gel stained with ethidium bromide, the polymerase chain reaction product was visualized under ultraviolet light. The polymerase chain reaction product was purified by using a QuiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Cycle sequencing reactions were performed in a 10-μl total volume containing 1–2 μl of the purified polymerase chain reaction product and 1 μM of sequencing primer in a BigDye version 3.1 (Applied Biosystems, Foster City, California) reaction mixture. The sequencing primers included 378F and 382R as well as 12SAREV (5′-ATA GTG GGG TAT CTA ATC CCA GTT T-3′) and 317F (5′-GCG GTC ATA CGA TTA ACC C-3′). Thermal protocols were 96°C for 3 min followed by 30 cycles of 96°C for 10 s, 54–62°C for 45 s, 72°C for 2 min 30 s, and a final 7 min at 72°C. Sequencing reactions were purified by using a precipitation protocol with 1 μ of 125 mM ethylenediaminetetraacetic acid, 1 μl 3 M sodium acetate, and 25 μl of 100% ethanol. After adding the mixture, the samples were vortexed for 15 s, incubated at room temperature for 15 min, then centrifuged for 30 min at 3,500 rpm. The supernatant was discarded and 35 μl of 70% ethanol was added. Samples were vortexed and centrifuged again for 15 min at 3,500 rpm. The supernatant was discarded and samples were air dried for at least 15 min before adding 10 μl of Di-Hi Formamide (Applied Biosystems). Sequencing was performed on an ABI 3730 Automated DNA Sequencer (Applied Biosystems). Output files were assembled by using the AutoAssembler program (ABI) or the Sequencher program (Gene Codes Corporation, Ann Arbor, Michigan).

Sequences were aligned initially by using CLUSTAL W (Higgins et al. 1992, as modified by Thompson et al. 1994; http://workbench.ucsd.edu). Final alignment was undertaken by hand by following the model of Springer and Douzery (1996), which takes into account the secondary structure of the 12S rRNA, including stems, loops, and secondary interactions. Monophyly of Phalangeridae was assumed, by following the analyses of Hamilton and Springer (1999); based on those authors' results, we used *Burramys parvus* (Burramyidae) as outgroup (sequence AF108223 of Hamilton and Springer [1999], cropped to the 12S rRNA gene). GenBank accession numbers for remaining phalangerids used in the study are *Phalanger maculatus*, AF108220; *Burramys parvus*, AF108223; *Spilocuscus rufoniger*, AF108221; *P. lullulae*, AF108219; and *P. gymnotis* AF108218 (all as in Hamilton and Springer [1999]); we also included *P. intercastellanus* U33496 (listed as *P. orientalis* in Springer et al. [1995]).

Aligned sequences were analyzed by using PAUP* (version 4.0b10—Swofford [2000]). We used maximum likelihood under the assumptions of the model of Tamura and Nei (1993) with gamma-distributed rate classes. This model was chosen by using Modeltest (version 3.06—Posada and Crandall 1998) based on hierarchical likelihood ratio test (log-likelihood [−lnL], 3,187.4685); the information-theoretic approach (Akaike information criterion [AIC]) implemented by the same software selected the General Time Reversible model with gamma-distributed rate classes and proportion of invariable sites empirically determined (−lnL, 3,173.3958; AIC, 6,366.7915); however, we used the method of Tamura and Nei (1993) because it was more parsimonious. Individual parameters were −lnL, 3,187.4685; base frequencies: A, 0.3886; C, 0.2171; G, 0.1653; T, 0.2289; rate matrix: A–C, 1.0000; A–G, 2.9868; A–T, 1.0000; C–G, 1.0000; C–T, 15.8443, G–T, 1.0000; proportion of invariable sites, 0; gamma distribution shape parameter = 0.1501. Branch support was assessed by using nonparametric bootstrap (Felsenstein 1985), jackknife, and Bayesian analyses. Bayesian posterior probabilities were estimated by using MrBayes (version 3.0b4.0—Huelsenbeck and Ronquist 2001). The DNA substitution parameters estimated by Modeltest were used as well as letting MrBayes estimate during the analysis by using the default values. No topological differences were found in the ensuing trees, nor were differences found in Bayesian posterior probability support values between the 2 methods. We used 20 Markov chains and set the "temperature" parameter to 0.01. We allowed a Monte Carlo Markov chain length of 2×10^7 generations (M. Holder and R. M. Brown, pers. comm.), sampling every 100 generations; although burn-in occurred within 10^4 generations, only the last 10,000 trees were used to estimate the Bayesian posterior probabilities. Although criticisms exist to the effect that bootstrap probabilities are more suitable than posterior probabilities for assessing the reliability of phylogenetic trees (Suzuki et al. 2002), and caveats exist relative to choice of assumed model of evolution (Bollback 2002), a growing body of evidence suggests that the Monte Carlo Markov Chain analytical technique is extremely robust (Alfaro et al. 2003; Buckley et al. 2002; Douady et al. 2003; Huelsenbeck et al. 2001; Leaché and Reeder 2002; Murphy et al. 2001; Wilcox et al. 2002).

Tree topology also was assessed by using maximum parsimony and distance. We analyzed the data by using both unweighted and weighted schemes. In the unweighted analysis, all data were given equal weights. In the weighted analysis, nucleotides ($n = 23$) in the decoding sites were weighted 9:1, and those in the stem regions ($n = 479$) were weighted 3:1; additional variations included differential weighting of transversions over transitions. For the distance analysis, we used LogDet distances because that method is more robust in the face of long-branch attraction and unequal base frequencies (Lockhart et al. 1994).

Relative rate tests were carried out by using the program RRTree (Robinson-Rechavi and Huchon 2000), which implements the relative rates test approach of Wu and Li (1985). Potential saturation was assessed by plotting transitions and transversions against patristic distance (Mindell and Honeycutt 1990). In order to estimate divergence times within the framework of the molecular clock hypothesis, and remain comparable to previous sequence studies of phalangeroids (Osborne and Christidis 2002a, 2002b), we augmented the data set to include comparisons with 3 cetartiodactyls (Cetacea, Balaenopteridae, *Balaenoptera physalus* GenBank accession X61145 [Arnason et al. 1991]; *B. musculus*, X72204 [Arnason et al. 1993]; Artiodactyla, Bovidae, *Bos taurus*, V00654 [Anderson et al. 1982]), as well as 2 marsupials and a platypus (Diprotodontia, Macropodidae, *Macropus robustus*, Y10524 [Janke et al. 1997]; Didelphimorphia, Didelphidae, *Didelphis virginiana*, Z29573 [Janke et al. 1994]; Monotremata, Ornithorhynchidae, *Ornithorhynchus anatinus*, X83427 [Janke et al. 1996]). We employed the approach of Tosi et al. (2003) to estimate times of divergence among lineages. The molecular clocks were calibrated by using 60 million years ago (mya) for the cladogenesis between lineages leading to Cetacea and Artiodactyla as proposed by Janke et al. (1997) and Arnason and Gullberg (1996), and a divergence estimate of 75 mya for the cladogenesis between lineages leading to opossums and wallaroos as proposed by Janke et al. (1994).

Results

Our sequences for the 12S rRNA gene of *Ailurops ursinus*, *Strigocuscus celebensis*, and *Wyulda squamicaudata* are deposited in GenBank (accession numbers AY735447, AY735445, and AY735446, respectively). We sequenced 947 nucleotides in the mitochondrial 12S rRNA gene of *S. celebensis*, and 948 in *A. ursinus*; the total length of the gene (following the model of Springer and Douzery [1996]) is 949 nucleotides. The first 2 nucleotides are missing from our sequence for *S. celebensis* but are conserved (as "CA") across all phalangerids sampled and were included as missing data in the sequence for our analyses. Of the 949 nucleotide sequence, 896 nucleotides were used in the analysis. Three regions totaling 49 nucleotides in length corresponded to hypervariable regions of ambiguous homologies and were not included (304–315, 737–740, and 858–892). Stem regions accounted for 476 nucleotides, areas involved in tertiary interactions accounted for 16 nucleotides, regions participating in the decoding site accounted for 23 nucleotides, and hypervariable regions (model of Springer and Douzery 1996) that could be aligned accounted for 30 nucleotides. Remaining nucleotides constituted loop regions of the molecule. The sequence analyzed showed marginal AT-bias (%AT = 58.3). The 12S gene in phalangers in general contains a low proportion of guanine residues (mean ± *SD*, $\bar{X} = 0.183 \pm 0.002$, range 0.179–0.187), whereas adenine is in excess ($\bar{X} = 0.365 \pm 0.005$, 0.358–0.371); cytosine and thymine were subequally represented at $\bar{X} = 0.231 \pm 0.004$, 0.223–0.234 and $\bar{X} = 0.221 \pm 0.003$, 0.217–0.224, respectively. Results of a chi-square test of heterogeneity of base frequencies across taxa were not significant. The transition to transversion ratio (Table 1; Fig. 3) among ingroup taxa averaged 2.76 ± 0.843, 1.81–5.71; that between *Macropus* and remaining ingroup taxa averaged 1.61 ± 0.298, 1.20–2.24. Mindell and Honeycutt (1990) suggested that transitions begin to saturate with change when

TABLE 1.—Genetic distances (below the diagonal) calculated by using maximum likelihood (model of Tamura and Nei [1993], with gamma-distributed rate classes; molecular clock not enforced), and estimated times of divergence (above the diagonal; molecular clock enforced). Above the diagonal, the 1st figure is calculated by using an estimated time of divergence between Cetacea and Artiodactyla of 60 million years ago (mya), as proposed by Janke et al. (1997) and Arnason and Gullberg (1996). The 2nd value is that calculated by using the 75 mya date estimated for cladogenesis between *Macropus robustus* and *Didelphis virginiana* proposed by Janke et al. (1994). The temporal estimates in row 1 refer to distances calculated between *Macropus* and phalangerid species, because in our broader-scope analyses *Macropus* resolved ingroup to *Burramys*. However, for consistency with previously published analyses, the genetic distance indicated in column 1 is that between *Burramys* and the phalangerid species. Data in the cell for that comparison otherwise would be: distance, 0.29910; estimated divergence time, 39.7–43.8 mya.

	B. parvus	*P. intercastellanus*	*Sp. rufoniger*	*Sp. maculatus*	*St. celebensis*	*P. lullulae*	*P. gymnotis*	*T. vulpecula*	*W. squamicaudata*	*A. ursinus*
Macropus robustus		32.6–35.9	32.6–35.9	32.6–35.9	32.6–35.9	32.6–35.9	32.6–35.9	32.6–35.9	32.6–35.9	32.6–35.9
Phalanger intercastellanus	0.24482		14.6–16.1	14.6–16.1	21.1–23.3	9.1–10.0	12.5–13.8	24.7–27.3	24.7–27.2	21.1–23.3
Spilocuscus rufoniger	0.25868	0.07733		5.9–6.5	21.1–23.3	14.6–16.1	14.6–16.1	24.7–27.3	24.7–27.3	21.1–23.3
Sp. maculatus	0.24534	0.07965	0.03676		21.1–23.3	14.6–16.1	14.6–16.1	24.7–27.3	24.7–27.3	21.1–23.3
Strigocuscus celebensis	0.25167	0.12339	0.12305	0.12354		21.1–23.3	21.1–23.3	24.7–27.3	24.7–27.3	14.9–16.4
P. lullullae	0.26427	0.06028	0.09194	0.08588	0.13614		12.5–13.8	24.7–27.3	24.7–27.3	21.1–23.3
P. gymnotis	0.28092	0.07048	0.08719	0.08362	0.12932	0.08545		24.7–27.3	24.7–27.3	21.1–23.3
Trichosurus vulpecula	0.22000	0.11605	0.13244	0.12452	0.13901	0.11144	0.13720		14.6–16.1	24.7–27.3
Wyulda squamicaudata	0.24324	0.15748	0.16689	0.15162	0.14426	0.14322	0.16230	0.08847		24.7–27.3
Ailurops ursinus	0.23336	0.11506	0.12294	0.12187	0.09053	0.14590	0.14210	0.12741	0.13302	

they represent approximately 50% of the substitutions, that is, the transition to transversion ratio is ≈1; both ingroup and ingroup–outgroup values are well clear of this value, reinforcing our choice of 12S rRNA for the present analysis. In addition, no pairwise comparison was significant in the relative rate test.

Average uncorrected (patristic) distances (Table 2) among ingroup taxa were 7.772% (*SD,* 1.538%); minimum and maximum distances among ingroup taxa were between *Spilocuscus maculatus* and *Spilocuscus rufoniger* (3.268%) and between *Wyulda squamicaudata* and *Phalanger gymnotis* (9.968%), respectively. Among our taxa of interest, average distance between *Strigocuscus celebensis* and remaining ingroup taxa was 8.396% (*SD,* 0.743%, range 6.687–8.941% [to *Ailurops ursinus* and *Trichosurus vulpecula*, respectively]). Average distance between *Ailurops* and remaining ingroup taxa was 8.342% (*SD,* 0.839, range 6.687–9.440% [to *Strigocuscus celebensis* and *Phalanger lulullae*]).

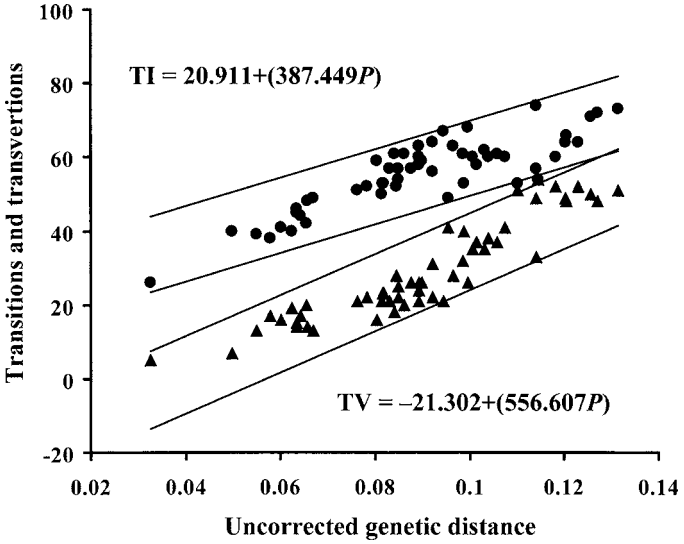

FIG. 3.—Plot of transitions and transversions (vertical axis) against patristic (uncorrected, or "p") distance for all pairwise comparisons undertaken in the present analysis. Solid lines represent the 95% confidence interval for individual values (SAS procedure REG, option CLI—SAS Institute Inc. 1990). The least-squares regression equations are highly significant ($P = 0.0001$), with R^2 values of 0.744 (transitions) and 0.848 (transversions).

As a further test of the suitability of the 12S rRNA gene in the present study, we regressed transitions and transversions against genetic distance (Fig. 3). A visual inspection would show an asymptote in the instance where either transitions or transversions had become saturated (hence lacking in phylogenetic information). This is not the case here: a linear regression was highly significant in both transition to patristic ratio (tr:p; tr = 20.91 + 387.449p, $F = 154.364$, $P < 0.0001$), and transversion to patristic ratio (tv:p; tv = −21.302 + 556.607p, $F = 296.765$, $P < 0.0001$).

Our analysis resulted in a single best tree (Fig. 4), based on maximum likelihood, with −lnL = −2,455.19062. A phalangerine subfamily remains supported by our results. *Trichosurus vulpecula* and *W. squamicaudata* are resolved as basal sister taxa within the family, in a strongly supported sister relationship to remaining phalangerids examined. In contrast to the prevailing paradigm (Flannery 1994), we did not resolve *Strigocuscus* as a constituent of a tribe Trichosurini; instead, *Strigocuscus* is, with *Ailurops*, a taxon ingroup to *Trichosurus* and *Wyulda*, and sister to the Phalangerini sensu stricto. The Monte Carlo Markov Chain implemented by MrBayes resulted in a tree with identical topology, but higher support for each node (Fig. 4). Of note under this analytical framework is that *Phalanger* obtains as equivocally paraphyletic, with *P. gymnotis* clustering with *Spilocuscus* to the exclusion of remaining species of *Phalanger*. Trees derived from distance analyses (Fig. 4) were generally congruent with those from maximum likelihood. The principal difference was that *Phalanger* this time was monophyletic with *P. gymnotis* basal to the other species. *Phalanger gymnotis* and

Table 2.—Transition to transversion ratios among taxa examined for this study.

	M. robustus	*P. intercastellanus*	*Sp. rufoniger*	*Sp. maculatus*	*St. celebensis*	*P. lullulae*	*P. gymnotis*	*T. vulpecula*	*W. squamicaudata*	*A. ursinus*
Macropus robustus										
Phalanger intercastellanus	1.32									
Spilocuscus rufoniger	1.71	2.24								
Sp. maculatus	1.20	2.56	5.20							
Strigocuscus celebensis	1.57	3.39	2.71	2.71						
P. lulullae	1.46	5.71	2.10	2.11	3.00					
P. gymnotis	1.58	3.00	2.59	3.29	3.05	3.00				
Trichosurus vulpecula	1.77	2.36	1.86	1.85	2.50	2.43	2.19			
Wyulda squamicaudata	2.24	2.25	1.91	1.81	2.23	2.27	2.62	3.43		
Ailurops ursinus	1.65	3.69	2.30	2.30	3.77	3.19	2.91	2.59	2.16	

P. intercastellanus showed marginal (50%) support in bootstrap analyses; that relationship was not supported in jackknife analyses. The relationships of *P. gymnotis* to remaining species of phalangerids remain elusive and will require additional sequence data in order to be indubitably ascertained.

Trees resulting from analyses with maximum parsimony varied in topology according to the parameters chosen. Five equally most-parsimonious trees resulted from an exhaustive search under unweighted parsimony analysis, of length = 264, consistency index (CI) = 0.7462, and retention index (RI) = 0.4463 (rescaled consistency index [RC] = 0.3330); these are summarized in Fig. 4 (top). Of the characters examined, 738 characters were constant, 90 were variable uninformative, and 76 were parsimony informative. A single tree, identical in topology to the distance tree (Fig. 4), resulted from weighted parsimony analysis, of length = 458, CI = 0.7751, and RI = 0.4876 (RC = 0.3779). Support values under weighted parsimony were even with unweighted parsimony, with the exception of the branch leading to *Strigocuscus*, which had 68% support in bootstrap analyses and 69% support in jackknife analyses.

We estimated times of divergence among lineages by assuming a molecular clock (Table 1; Fig. 5). Under this set of assumptions, we estimated the divergence between *Strigocuscus celebensis* and *Ailurops ursinus* at ≈14.9–16.4 mya and that between this lineage and remaining ingroup phalangerids at 21.1–23.3 mya. These phalangerids are estimated to have diverged from the basal clade in Phalangeridae (*Wyulda* + *Trichosurus*) at 24.7–27.3 mya. Phalangeridae were estimate to have diverged from Macropodidae at 32.6–35.9 mya and from Burramyidae at 39.7–43.8 mya.

Discussion

Flannery et al. (1987:504) noted that ''A striking feature in the evolution of phalangerids is the concentration of plesiomorphic species on islands west of New Guinea.'' Indeed, from a morphological perspective *Ailurops ursinus* is the sole acknowledged representative of the phalangerid subfamily Ailuropinae, considered the most primitive in the phalangerids. Of greater interest in light of our data, however, is the hypothesis that *S. celebensis* likewise is highly conservative from a morphological perspective, ''the most plesiomorphic member of the Trichosurini'' according to Flannery et al. (1987:504). If the 2 most plesiomorphic taxa of phalangers are in Sulawesi, it might logically be construed that in the absence of fossil data to the contrary, the origins of the family may lie in westernmost Wallacea. However, our results demonstrate that *Ailurops* and *Strigocuscus* are not basal members of the Phalangeridae, instead supporting an origin for the family in northwestern Australo-Papua, on the Sahul Shelf, with constituents incursive to the western islands.

The central question in undertaking this work was to determine these relationships in order to address broader issues with respect to biogeography of Sulawesi. Sulawesi straddles what may be the most biologically complex region on the planet, perhaps a result of the geological complexity of the region (Hall 2002): Southeast Asia from the mainland to Australo-Papua is one of the most complex geological zones known today, consisting of a very large number of microplates, continental fragments, and other major geological features (Hall 1996, 1998; Hall and Blundell 1996; Hutchinson 1989; Smith et al. 1994; Whitmore 1981, 1987; Wilson and Moss 1999). The geological complexity of the area has resulted in numerous zoogeographic puzzles. Most notably, Wallace (1859, 1860, 1863, 1869, 1876, 1880) was the 1st to point out the faunal contrasts across the Selat Lombok, thereby establishing Wallace's Line: the purported division of Oriental and Australian faunas between Bali and Lombok (although numerous studies appear to contradict the presence of clear faunal discontinuities; see summaries by George [1981], Grehan [1991], Mayr [1944], Michaux [1994], and Simpson [1977]).

In light of the biogeographical conundrums of the area, resolution of the phylogenetic relationships between phalangerids in the western reaches of the family's range and eastern phalangerids gains added relevance; if, as most reviewers acknowledge, *Ailurops* is a divergent, plesiomorphic taxon sister to remaining phalangerids, then (barring undiscovered extinct taxa) the origin of the family may well be in Sulawesi, the principal easternmost outpost of the Australian Region. Furthermore, the morphologically plesiomorphic *Strigocuscus celebensis* also is endemic to Sulawesi. Halmahera, just west of New Guinea, between Sulawesi and New Guinea, hosts the plesiomorphic putative trichosurin *Strigocuscus ornatus*. Finally, Waigeu, west of New Guinea, also hosts an enigmatic and possibly plesiomorphic *Spilocuscus*: *Sp. papuensis* (Flannery et al. 1987). The remaining larger islands between New

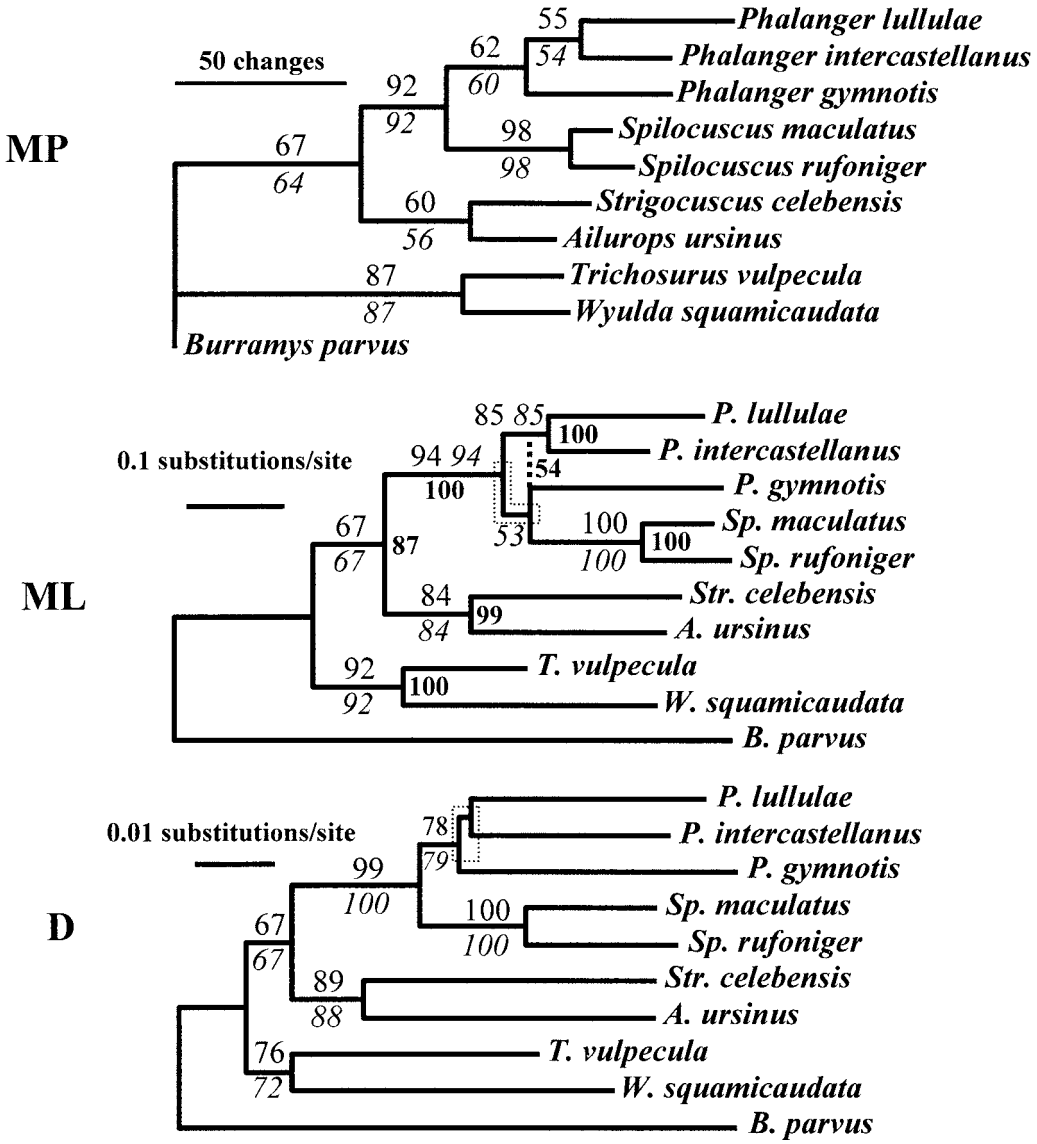

Fig. 4.—Phylogenetic relationships among phalangerids based on maximum parsimony (MP, top), maximum likelihood (ML, center), and LogDet distance (D, bottom). Details are presented in "Materials and Methods." Top) MP: consensus of 5 equally most-parsimonious trees found under unweighted parsimony. Numbers above the node indicate bootstrap support for the topology shown (1,000 iterations), numbers below the node indicate jackknife support values (1,000 iterations) for the topology shown. Center) ML: single tree resulting from the maximum-likelihood analysis. Numbers in bold beside or below the node indicate Bayesian posterior probabilities. Numbers in italic represent percentage support from bootstrap (1,000 iterations); numbers in lightface represent percentage support from the jackknife analysis (1,000 iterations). The thin dotted line surrounds the clade supported at 53% by the bootstrap analysis, uniting *Phalanger gymnotis* with *Spilocuscus*; the thick dashed line indicates the monophyletic *Phalanger* supported in 54% of Monte Carlo Markov Chain trees. Bottom) Single tree resulting from distance analysis; topologies under different distance parameters were identical; shown is that resulting from General Time Reversible distance. Numbers in tree represent the same parameters as in MP tree. Relationships among individual species in the *Phalanger* clade surrounded by the thin dotted line were not supported by jackknife or bootstrap analyses.

Guinea and Sulawesi (Moluccas) all host a number of species, often endemic to particular islands, belying the putatively poor dispersive abilities of phalangers. However, these regions at one time all were constituent elements of the Sahul Shelf (Australia, New Guinea, and off-shore island groups). Therefore, when phalangerids arose becomes a crucial question in resolution of the origins of the family and of regional biogeography.

Thus, a plausible evolutionary scenario for Phalangeridae based on currently known morphological attributes would have the family presumably originating somewhere on the northeastern edge of the Sahul Shelf (where the most plesiomorphic taxa remain), with remaining phalangerids dispersing eastward. This is not necessarily an unexpected scenario. The center of diversity for extant phalangerids is New Guinea: portions of present-day Sulawesi are constituted from fragments derived from the northern Sahul Shelf. In particular, Buton and its outlying Tukangbesi Islands on the Southeastern Peninsula, as well as a portion of the Eastern Peninsula, and the Banggai

Islands, all are fragments derived from the westernmost Sahul Shelf; that is to say, they are islands once forming part of a Greater Australo-Papuan region. Fragmentation of eastern Sahul occurred starting 20–25 mya (Hall 2002). The tectonic model of Hall (2002) further hypothesizes that the areas currently constituting Sulawesi's Southeastern Peninsula, the northern Central Core, and proximal Eastern Peninsula, the smaller peripheral islands associated with these fragments, as well as the Wallacean islands between Sulawesi and New Guinea, all would either have constituted a part of greater Sahul, or fragments nearby the Sahul Shelf, at least as far back as 30 mya. Our estimates of the time of diversification of the Phalangeridae based on a molecular clock model indicate that Phalangeridae would have undergone an initial diversification from a common ancestor with Macropodidae about 34 mya (32.6–35.9 mya). Within Phalangeridae, the trichosurine lineage diverged from a combined Sulawesi phalanger clade and Phalangerinae approximately 24.7–27.3 mya. Hall (2002) hypothesized that at that time the geological masses constituting the current distributions of the more plesiomorphic phalangerids would have been part of an archipelago in the northwestern Sahul Shelf. Therefore, it might be further hypothesized that a combination of dispersal onto geological elements constituting the precursors of the islands of the western Malay Archipelago, together with the rapid spreading apart of those islands, would have led to rapid cladogenesis of phalangerids in that region at that time.

The most primitive constituent of the phalangerid radiation is resolved to be the Trichosurinae, including *Trichosurus vulpecula* and *Wyulda squamicaudata*. The current distribution of these genera is restricted to the Australian landmass, with *Wyulda* in particular highly restricted to a small area of northwestern Australia, the Kimberley region, Western Australia Province. Thus, at the initial diversification of Phalangeridae, areas wherein phalangerids now occur all were within the bounds of the Sahul Shelf.

Our sequence data point to *Ailurops ursinus* and *Strigocuscus celebensis* being sister taxa ingroup to Trichosurinae, and diverging from a common ancestor with Phalangerinae some 21.1–23.3 mya. This hypothesis of relationships, together with that of *Trichosurus* + *Wyulda*, resolves the conundrum of Flannery (1994:152): "How this group [*Trichosurus* + *Wyulda* + *Strigocuscus*] came to have such an odd distribution . . . is not presently understood." During that time period, the geological model of Hall (2002) postulates that the portion of present-day Sulawesi constituting the northern Central Core region split from the Sahul Shelf, closely approaching present-day Sulawesi's Northern (or Minahassa) Peninsula. Hence, colonization of other portions of Sulawesi by morphologically plesiomorphic phalangerids allied to (or conspecific with) *Ailurops* or *Strigocuscus* would have been eminently feasible; indeed, all the present-day Wallacean islands (Buru, Ceram, Ambon, and others) remained solidly on the Sahul Shelf. Furthermore, in terms of land areas, Hall (1998) hypothesized that portions of Sulawesi east of the Sunda Shelf (Southwestern Peninsula and western Central Core) as well as between New Guinea and Sunda Shelf (portions of the Southeastern and

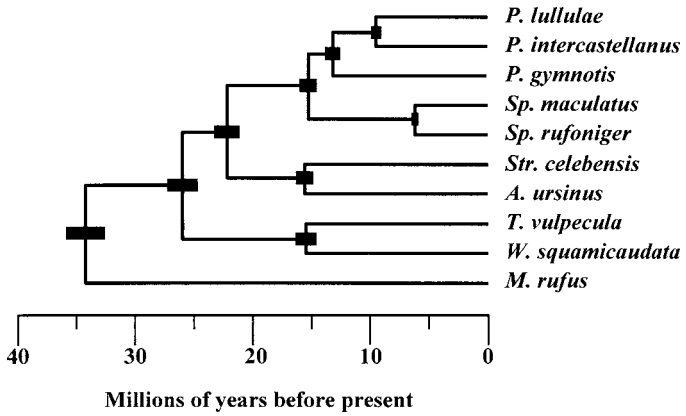

FIG. 5.—Evolutionary relationships resulting from a maximum-likelihood analysis constrained to a molecular clock under the assumptions of the model of Tamura and Nei (1993) with gamma-distributed rate classes. Estimated times of divergence for each node are listed in Table 1. Solid blocks at each node represent upper and lower estimates for cladogenesis derived from estimated divergences between Cetacea and Artiodactyla (60 million years ago [mya]—Janke et al. 1997) and opossums and wallaroos (75 mya—Janke et al. 1994).

Eastern peninsulas) were available for dispersal. In that time period, more of Sulawesi became dry land. At approximately 25 mya, southeastern Sulawesi and most of the Central Core region of the island were above sea level, and by 20 mya the Eastern Peninsula and southern reaches of the Northern Peninsula also are hypothesized to have been dry. In addition, the prevailing currents (as hypothesized by Fine et al. [1994], Hall [1998], and Kennett et al. [1985]) would at that time have facilitated colonization of a proto-Sulawesi. It is important to note that these ocean circulation models hypothesize a strong east–west South Equatorial current through the Indonesian Seaway at approximatley 30 mya, increasing the difficulties of dispersion from the Sahul Shelf to the Sunda Islands of the Malay Archipelago but cutting these areas off from each other. By approximately 25 mya, shallow areas constituting present-day Sulawesi, Philippines, and Halmahera, underwent a rapprochement with areas constituting the present-day island of New Guinea, lessening the strength of the current by diverting much of it northward and restricting the potential flow through the Indonesian Seaway. At the same time, areas constituting present-day Sulawesi from the Sahul Shelf were approaching the Oceanic Sulawesi components. After 20 mya, the strength of the South Equatorial current through the Indonesian Seaway would have increased anew as the geological fragments constituting portions of the Philippines distanced themselves from portions of present-day Halmahera and New Guinea, allowing for increased flow volume.

In our analyses, the *Spilocuscus* lineage diverged from *Phalanger* approximately 14.6–16.1 mya; we estimate cladogenesis between *P. gymnotis* and remaining *Phalanger* at approximatley 12.5–13.8 mya, with the most recent divergence in *Phalanger* being between *P. intercastellanus* and *P. lulullae* timed at approximately 9.1–10.0 mya. We estimate the 2 species of *Spilocuscus* to have diverged approximately 5.9–6.5 mya. Additional incursions at this point from the Sahul Shelf

westward to the Moluccas would have been facilitated by the closing of the Indonesian Seaway and the shift of the South Equatorial Current to near present-day circulation patterns starting at about 10–15 mya.

Taxonomic Conclusions

The current taxonomy of the Phalangeridae is not consistent with the results of our analysis of sequence data from the mitochondrial 12S rRNA gene. *Ailurops* always has been considered as the sole genus in the monotypic subfamily Ailuropinae to the exclusion of remaining phalangers, which were considered to constitute a polytypic Phalangerinae. Within Phalangerinae, a case had been made for a tribal-level group Trichosurini, including *Trichosurus*, *Wyulda*, and *Strigocuscus*. For example, numerous craniodental characters (Flannery et al. 1987), as well as characters from petrosal morphology (Crosby and Norris 2003; Norris 1994) support such an arrangement. Phalangerini was construed to contain *Spilocuscus* and *Phalanger*. In contrast, the relationships we recovered among *Ailurops*, *Strigocuscus*, *Wyulda*, and remaining phalangers point to *Ailurops* and *Strigocuscus* as morphologically plesiomorphic constituents of Phalangeridae, but not evolutionarily basal to all. Instead, Trichosurini, including only *Trichosurus* and *Wyulda*, is the basal clade in the family. In order to maintain a biologically meaningful taxonomy, it accordingly becomes expedient to consider two potential solutions. Phalangeridae being constituted by Trichosurinae and Phalangerinae, with the latter divided into 2 tribes: Phalangerini (*Phalanger*, and *Spilocuscus*, *Strigocuscus*), and Ailuropini (*Ailurops* and *Strigocuscus*). Alternatively, each of these clades could be considered a subfamilial-level entity. We advocate the latter solution because in the absence of subfamilial distinctions, tribal groups are irrelevant; and because subfamilial recognition of these groups more accurately reflects not only the temporal depth of their lineages but the biological wealth and distinctiveness of these animals, particularly the morphological identity of *Ailurops*. In this scheme, the taxonomic status of *Phalanger* vis-à-vis *Spilocuscus* could be resolved using additional data, although *Spilocuscus* is ineluctably admitted a phalangerin genus. The taxonomic arrangement reflecting the evolutionary relationships we have illuminated therefore is

Family Phalangeridae Thomas, 1888
- Subfamily Phalangerinae Thomas, 1888
  - *Phalanger* Storr, 1780
  - *Spilocuscus* Gray, 1861
- Subfamily Trichosurinae (Flynn, 1911)
  - *Trichosurus* Lesson, 1828
  - *Wyulda* Alexander, 1918
- Subfamily Ailuropinae Flannery, Archer, and Maynes, 1987
  - *Ailurops* Wagler, 1830
  - *Strigocuscus* Gray, 1862

The foregoing classification simplifies a complex biogeographic question: the unlikely presence of 2 putatively distinct lineages (*Ailurops* and *Strigocuscus*) on a single island so removed from the hypothetical locus of origin for phalangerid genera (Flannery 1994). Our solution is that these are sister taxa and likely dispersed in a singular event. Nothwithstanding, the novel classification also raises some questions.

Morphological data, most notably of Flannery et al. (1987), but especially of petrosal morphology (Crosby and Norris 2003; Norris 1994) are strongly at odds with our conclusions based on analysis of molecular data. However, the morphological data themselves may be equivocal. Our data are molecular, thus an in-depth discussion of the morphological data is not pertinent. However, we suggest that morphological characters employed heretofore are symplesiomorphies (as indeed pointed out by Flannery et al. [1987:484]) that have been retained in *Ailurops* and to a lesser extent *Strigocuscus*, rather than characters useful in defining these genera relative to remaining phalangerids, or in establishing relationships between them and remaining phalangerid genera.

Our point here is not to criticize morphological versus molecular data, nor to demonstrate the infallibility of molecular over morphological data. Rather, our purpose is to provide a sound, testable hypothesis of evolutionary relationships and biogeography that can be tested objectively by using the best appropriate data and analyses. Our heterodox hypothesis of relationships in the Phalangeridae still leaves many unanswered questions, not the least of which is whether other data sets will support our proposition. What are the relationships among the species of *Spilocuscus* and *Phalanger*? Given the equivocal morphological relationships among taxa in the family, where do other species currently assigned to these genera truly lie in terms of their evolutionary relationships? Can—or should—the craniodental characters in Phalangeridae be reassessed in light of our data? Examination of our data provides a robust, evolutionarily based springboard whence to address these and other questions relative to systematics and biogeography of this fascinating, cryptically diverse, and enigmatic group of animals. To paraphrase Gray (1862:315): we lay before the readership these hypotheses in the hope of doing something toward settling relationships among species of this very difficult group of animals.

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