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DNA Hybridization, Cladistics, and the Phylogeny of Phalangerid Marsupials

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Summary. Single-copy DNA/DNA hybridization experiments and numerical cladistic analyses of anatomical characters were used to investigate relationships among nine phalangerid (Marsupialia) species from four different genera. Both rate-dependent and rate-independent analyses of molecular data indicate that species of *Trichosurus* form one clade and that *Strigocuscus, Phalanger,* and *Spilocuscus* form a second. Within the latter group, *Spilocuscus* is excluded from a *Strigocuscus–Phalanger* clade, which, in turn, is not fully resolved on a jackknife strict consensus tree. Minimum-length Dollo, Wagner, and Camin–Sokal parsimony trees based on 35 anatomical characters, in contrast, suggest placement of *Strigocuscus* with *Trichosurus* rather than with *Spilocuscus* and *Phalanger.* However, there are two derived characters that support the alternative arrange of *Strigocuscus* with *Spilocuscus* and *Phalanger* and one character that further unites *Strigocuscus* and *Phalanger.* Thus, DNA hybridization results are not inconsistent with the distribution of derived character states among anatomical characters, only with minimum-length trees based on character data.

Key words: DNA/DNA hybridization — Cladistics — Phylogeny — Phalangeridae — Marsupials

Introduction

The family Phalangeridae includes brush-tailed possums (*Trichosurus*), scaly-tailed possums (*Wyulda*), and cuscuses (*Phalanger, Strigocuscus, Spilocuscus, Ailurops*). Flannery et al. (1987a) recognize 17 extant species, which occur throughout most of mainland Australia, Tasmania, and New Guinea, as well as the great arc of lesser islands surrounding New Guinea.

The systematic study of phalangerids has a long history, but only the study of Flannery et al. (1987a) included all extant taxa. Most other studies concentrated on *Phalanger* without discussing how *Trichosurus* and *Wyulda* fit into the phalangerid radiation.

Early systematic studies include those of Temminck (1827), Waterhouse (1846), Gray (1858, 1862), Jentink (1885), Thomas (1888), Schwartz (1934), Tate and Archbold (1937), and Tate (1945), and are reviewed in Flannery et al. (1987a).

Tate (1945) recognized three species groups in *Phalanger,* which are as follows:

1) A *Phalanger orientalis* group that includes *P. orientalis* (*P. interpositus, P. ornatus,* and *P. lullulae* as synonyms), *P. gymnotis, P. vestitus* (*P. carmelitae* as a synonym), and *p. celebensis*;
2) A *Phalanger maculatus* group including *P. maculatus* (*P. krameri* and *P. rufoniger* as synonyms) and *P. atrimaculatus*;
3) A *Phalanger ursinus* group containing the subspecies of *P. ursinus.*

More recent systematic investigations include those of Hayman and Martin (1974), Kirsch (1977), Feiler (1977, 1978a,b,c), George (1979, 1982, 1987), Ziegler (1983), Archer (1984), Baverstock (1984), Flannery and Archer (1987), Flannery and Calaby

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(1987), Flannery et al. (1987a,b), and Groves (1987a,b).

Cytogenetic studies on phalangerids reported by Hayman and Martin (1974) indicate that the chromosome number is 20 in *Trichosurus* species, but only 14 in *Phalanger gymnotis* and *Phalanger vestitus*. Serological studies include those of Kirsch (1977) and Baverstock (1984). Kirsch found that the three species of *Trichosurus* plus *Wyulda* were closely related. Baverstock's results, which are based on microcomplement fixation studies of albumin, indicate that *Phalanger* is highly divergent and possibly not monophyletic. In agreement with Kirsch, his results show that *Wyulda* is more closely related to *Trichosurus* than to species of *Phalanger*. His results also suggest that *P. carmelitae* and *P. gymnotis* are more closely related to *T. vulpecula* and *W. squamicaudata* than to either *P. vestitus* or *P. maculatus*.

George (1982) divides species of *Phalanger* into the following four groups:

1) Subgenus *Spilocuscus* including *P. maculatus, P. rufoniger,* and *Phalanger* sp. from Waigeu Island;
2) Subenus *Ceonix* including *P. ursinus*;
3) Subgenus *Phalanger* (in part) including *P. celebensis, P. ornatus,* and *P. gymnotis*;
4) Subgenus *Phalanger* (in part) including *P. orientalis, P. lullulae, P. carmelitae, P. vestitus,* and *P. interpositus*.

In a more recent revision of the living species of cuscuses, however, George (1987) elevated subgeneric groups to generic status, and adopted the following taxonomic arrangement:

1) Genus *Spilocuscus* containing *S. maculatus, S. rufoniger,* and *S. papuensis*;
2) Genus *Ailurops* containing *A. ursinus*;
3) Genus *Strigocuscus* containing *S. celebensis*;
4) Genus *Phalanger* containing *P. pelengensis, P. rothschildi, P. ornatus, P. leucippus, P. gymnotis, P. orientalis, P. lullulae, P. interpositus, P. carmelitae,* and *P. vestitus*.

Ailurops, Strigocuscus, and *Spilocuscus* represent genera not recognized by Tate (1945). In contrast to George's (1982) arrangement, group three (i.e., *Strigocuscus*), contains only one species.

Flannery et al. (1987a) employed a cladistic analysis of 35 anatomical characters to investigate phylogenetic relationships in the Phalangeridae, although numerical cladistic algorithms were not utilized. Based on this analysis, Flannery et al. proposed three different phylogenetic hypotheses. The first hypothesis is shown in Fig. 1 and lays the foundation for Flannery et al.'s classification (see Table 1), which recognizes five genera. The genus *Wyulda,*

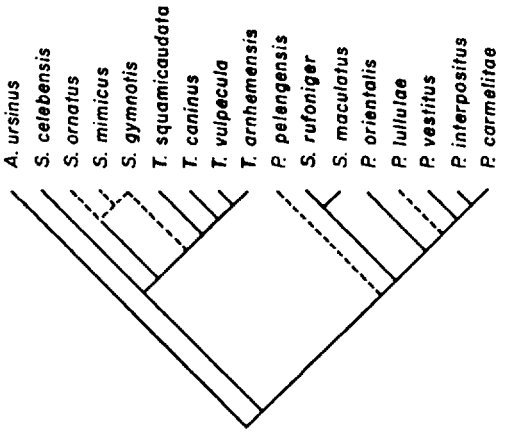


Fig. 1. Flannery et al.'s (1987a) cladogram depicting interrelationships among the Phalangeridae. According to this hypothesis, *Ailurops ursinus* is an outgroup to all other phalangerids. Dotted lines indicate uncertain relationships or alternative possible relationships. Redrawn from Flannery et al. (1987a).

which has been recognized by many other authors, is subsumed under *Trichosurus* in Flannery et al.'s taxonomic scheme.

Flannery et al. (1987a) propose that *Ailurops ursinus* is the most plesiomorphic of all extant phalangerids and is excluded from a clade containing all other species. *Ailurops ursinus* is placed in its own subfamily, the Ailuropsinae, whereas the remaining species are placed in the Phalangerinae. Within this subfamily, Flannery et al. recognize two tribes, Trichosurini and Phalangerini. Trichosurini includes *Strigocuscus* and *Trichosurus*; Phalangerini includes *Phalanger* and *Spilocuscus*. Placement of *Strigocuscus* with *Trichosurus* in the Trichosurini reflects Flannery et al.'s view that *Phalanger* (sensu Tate 1945) is not monophyletic. George (1987) also suggests that *Strigocuscus* is more closely related to *Trichosurus* (sensu Flannery et al. 1987a) than to other phalangerids, but his use of *Strigocuscus* is much more restrictive and only includes *S. celebensis*.

A further point of Flannery et al.'s classification is the recognition of two groups of subspecies within *Phalanger orientalis* (sensu Tate 1945). First, subspecies from northern New Guinea and the islands (*orientalis, vulpecula, ducatoris, kiriwinae, breviceps, intercastelanus,* and *meeki*) appear to be closely related and are placed in *Phalanger orientalis*. Second, subspecies from southern New Guinea and Australia (*mimicus, brevinasus,* and *peninsulae*) are tentatively placed in *Strigocuscus mimicus*. Hence, Flannery et al. place some of Tate's (1945) conspecifics in different tribes.

Alternative hypotheses of Flannery et al. (1987a), both of which they regard as nonparsimonious, are depicted in Fig. 2. The first alternative hypothesis places *P. vestitus, P. carmelitae,* and *P. interpositus,*

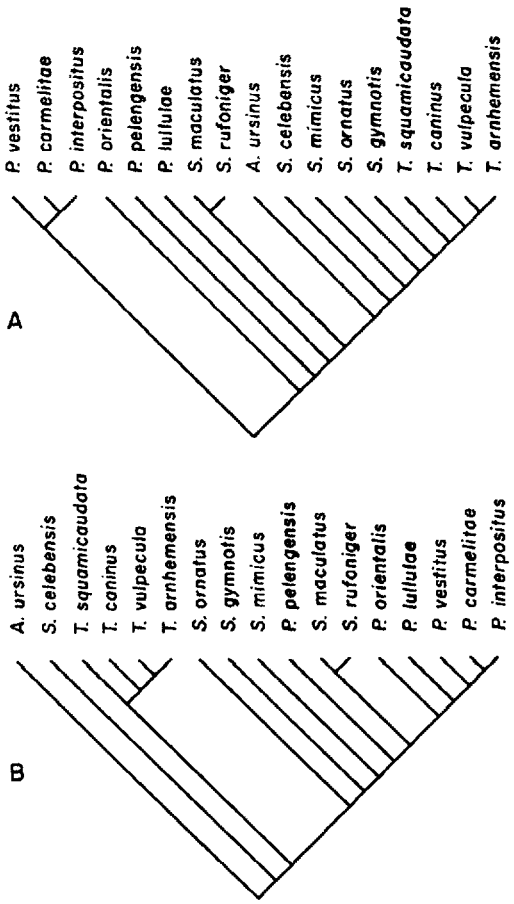


Fig. 2. Two alternative cladograms presented by Flannery et al. (1987a). In the first hypothesis (**A**), *Phalanger vestitus, Phalanger carmelitae,* and *Phalanger interpositus* form an outgroup to all other phalangerids. In the second hypothesis (**B**), trichosurins are a polyphyletic group with forms close to *Strigocuscus gymnotis* and *Strigocuscus mimicus* giving rise to a phalangerin clade.

all of which are montane New Guinean species, in a clade that is peripheral to all other extant phalangerids. The second alternative hypothesis depicts trichosurins as a polyphyletic group, with forms close to *S. gymnotis* and *S. mimicus* giving rise to a phalangerin clade.

In the present study, we follow the primary classification of Flannery et al. and present the results of single-copy nuclear DNA/DNA hybridization experiments conducted on phalangerids, using *Cercartetus caudatus* (family Burramyidae) as an outgroup. Previous DNA hybridization studies indicate that burramyids may be a sister taxon to phalangerids among extant families (Springer 1988; Springer and Kirsch 1989). For the phalangerid taxa included in our DNA hybridization experiments (*Trichosurus vulpecula, Trichosurus caninus, Strigocuscus gymnotis, Spilocuscus rufoniger, Spilocuscus maculatus, Phalanger orientalis, Phalanger interpositus, Phalanger vestitus,* and *Phalanger carmelitae*), we have also performed numerical cladistic analyses on the 35 characters given in Flannery et al. (1987a) (see Table 2).

Table 1. Flannery et al.'s (1987a) classification of phalangerid marsupials

Family Phalangeridae
Subfamily Ailuropsinae
Ailurops ursinus
Subfamily Phalangerinae
Tribe Trichosurini
Strigocuscus celebensis
Strigocuscus mimicus
Strigocuscus ornatus
Strigocuscus gymnotis
Trichosurus squamicaudata
Trichosurus caninus
Trichosurus vulpecula
Tribe Phalangerini
"*Phalanger*" *pelengensis* incertae sedis
Spilocuscus rufoniger
Spilocuscus maculatus
Phalanger orientalis
Phalanger lullulae
Phalanger vestitus
Phalanger interpositus
Phalanger carmelitae

Materials and Methods

DNA Hybridization Data

Tissue Collection. Tissue samples for all species were preserved in ~95% ethanol. Collectors' field numbers and museum catalogue numbers are available for all specimens.

DNA Hybridization Protocol. DNA/DNA hybridization experiments were performed using the hydroxylapatite column-chromatography technique (Britten and Kohne 1968; Kohne 1970; Kohne and Britten 1971; Sibley and Ahlquist 1981, 1983). Briefly, tissue samples were washed free of ethanol, frozen overnight, lyophilized for 24 h, immersed for 30 s in liquid nitrogen, and ground into a powder with a mortar and pestle. Long-stranded DNA was then extracted from powdered tissue samples using the method of Marmur (1961). Pronase was used to degrade soluble proteins, and RNA was removed by treatment with RNase (Maniatis et al. 1982).

Long-stranded DNA was fragmented with a Branson sonifier/cell disruptor. The resulting fragment-size distributions were assessed by comparison with restriction-digested lambda phage on 1% agarose gels (Nathans and Smith 1975). Fragment length distributions ranged from 100 to 2000 bp and centered on 600–800 bp.

Single-copy DNAs to be used as radiolabeled tracers were prepared by boiling sonicated DNA for 10 min in 0.48 M neutral phosphate buffer, allowing the single-stranded DNAs to reassociate to Cot 200 (Ecot 1130) at 60°C in 0.48 M phosphate buffer, diluting the samples to 0.12 M phosphate buffer, and passing the samples over hydroxylapatite columns to remove repeated sequences. Following elution from hydroxylapatite columns, single-copy DNAs were dialyzed against deionized water, frozen, and lyophilized for 18–24 h.

Single-copy DNAs were then labeled with radioiodine (Commorford 1971; Davis 1973; Tereba and McCarthy 1973; Orosz

Table 2. A list of characters and the distribution of character state polarities in selected phalangerid taxa employed in cladistic analyses

A) List of characters

1) Squamosal is dorsally restricted
2) Basicranium is well pneumatized
3) No groove is present between mastoid and ectotympanic
4) Orbital wing of maxilla present
5) I3/ reduced in size
6) Molar lophids relatively well developed
7) Paracristid of M/3–5 more buccally placed and fissure is present
8) Buccal kink of cristid obliqua is well developed
9) Metaconid and protodonid of M/2 are merged
10) P2/ single rooted
11) Lachrymal is retracted from face
12) Rostrum is narrowed
13) The ventral rim of the orbit is visible from below
14) Ectotympanic is excluded from anterior of postglenoid process
15) P3/ is at oblique angle to the molar row
16) P3/ has at least four cuspules
17) Tail tuberculated and almost completely naked
18) P3/ very large
19) Tail with brush of black hairs
20) P3/ as high posteriorly as anteriorly
21) M/2 metaconid posteriorly displaced
22) Alisphenoid extends far posteriorly
23) Ventral edge of periotic elongate
24) Squamosal overlaps ectotympanic
25) M2/ preprotocrista does not contact parastyle
26) Orbital wing of maxilla greatly enlarged
27) I3/-C1/ diastema lost
28) Molars complexly crenulated
29) Alisphenoid and basoccipital meet over long suture
30) Males with mottled pattern on back
31) Frontals domes
32) M1/ has metacone
33) Alisphenoid–basoccipital suture very long
34) I3/ is extremely small
35) Very large protoconule and neometaconule and present

B) Distribution of character state polarities

	Character number																															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Strigocuscus gymnotis	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0
Trichosurus caninus	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Trichosurus vulpecula	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
Spilocuscus maculatus	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0
Spilocuscus rufoniger	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0
Phalanger orientalis	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	1	0
Phalanger vestitus	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	1
Phalanger interpositus	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1	1
Phalanger carmelitae	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1	1

Data taken from Flannery et al. (1987a). 1 = derived; 0 = primitive

and Wetmur 1974; Scherberg and Refetoff 1975; Anderson and Folk 1976; Chan et al. 1976; Prensky 1976; Springer 1988). Briefly, lyophilized DNA samples were resuspended in 50 µl of 0.2 M NaAc adjusted to pH 5.7 with glacial acetic acid. Aliquots (100 µg) of DNA in 0.2 M NaAc were then diluted into a total volume of 130 µl 0.2 M NaAc (pH 5.7) and combined with 6 µl of 0.002 M KI and 11 µl of bromcresol green dye. Samples were then adjusted to pH 4.7 with 0.2 M NaAc (pH 4.0). Five millicuries of ^{125}I in a 10–20-µl volume of NaI was obtained from Amersham and diluted with 350 µl of 0.2 m NaAc (pH 5.7), 0.06 mM KI. After allowing the isotope to equilibrate for 1 h, 40 µl (0.625 mCi) was added to each reaction mixture, followed by the addition of 60 µl of 0.0018 M thallium (III) chloride (TlCl). Reaction mixtures were then heated at 60°C for 15 min and cooled on ice for 5 min. Thirty microliters of 1.0 M Tris was added to each sample, the samples were again heated to 60°C (5

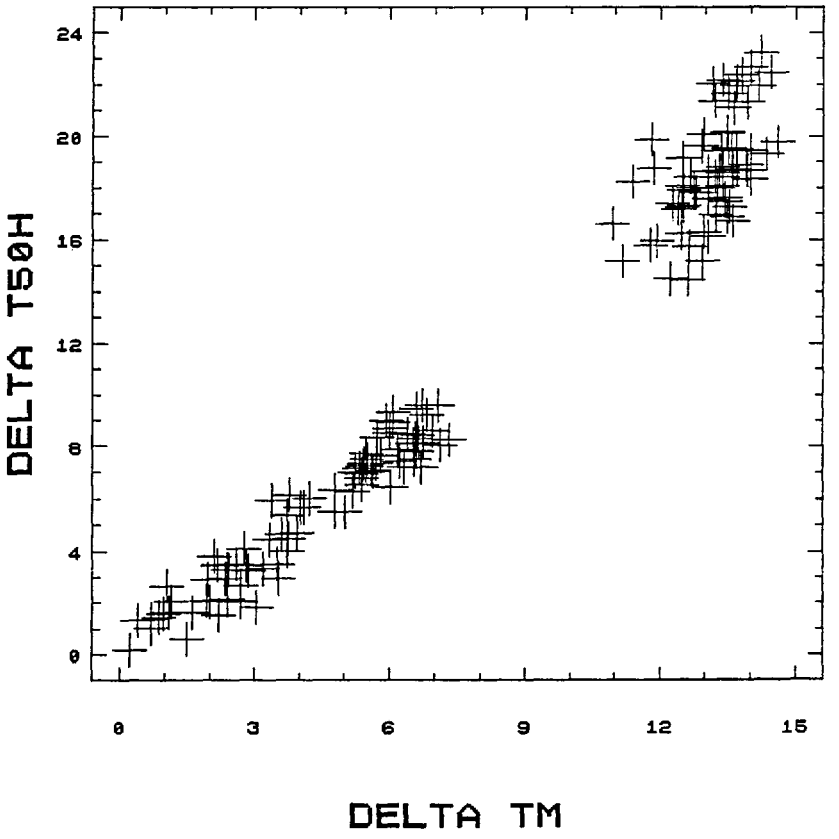


Fig. 3. Delta Tm values versus delta T50H values for 156 interspecies DNA hybridization experiments. Labeled taxa include *Phalanger vestitus, Phalanger orientalis, Phalanger interpositus,* and *Trichosurus vulpecula,* all of which are associated with relatively uncompressed distances. Delta Tm values <7 represent intrafamilial distances. Delta Tm values >10 correspond to interfamilial distances in the order Diprotodontia. Based on the data in this figure, we have calculated a power regression to describe the relationship between delta Tm and delta T50H (see text).

min), and the reaction mixtures were transferred to dialysis tubes and dialyzed overnight against 0.4 M NaCl, 0.01 M phosphate buffer, 0.0002 M EDTA. Labeled DNAs were then transferred to screw-top vials and stored at −20°C.

DNA/DNA hybrids were formed by combining 0.5 μg of tracer DNA with 250 μg of unlabeled driver DNA. Hybrid mixtures were then boiled and incubated at 60°C to approximately Cot 6000 (Ecot 34,000) in 0.48 M phosphate buffer to permit the formation of hybrid duplexes. After incubation, hybrid mixtures were diluted to 0.12 M phosphate and the hybrids were loaded onto hydroxyapatite columns immersed in a custom-built, fully automated Thermal Elution Device (TED) modeled after Sibley and Ahlquist's (1981) DNanalyzer. Column temperatures were then raised to 60°C and three 8-ml washes of 0.12 M phosphate buffer were passed through each column to collect unhybridized fragments and free iodine. The temperature was then raised in 2° increments from 60°C to 98°C, except that the first and last steps were 4° increments. Eluates containing single-stranded fragments produced by the melting of duplexes were collected at each temperature by passing 8 ml of 0.12 M phosphate buffer heated to that temperature over the columns. The radioactivity of each eluate was counted and the median melting temperature (Tm or T50 of some authors) calculated using linear interpolation.

Matrix Construction. Delta Tm values were obtained by averaging Tm values for all homologous hybrids from a particular tracer preparation that were incubated together and subtracting heterologous Tm values. Average delta Tm values were then calculated and summarized in a pairwise matrix of distances (Table 3). Because delta Tm values associated with particular tracer preparations are sometimes compressed, and lead to nonrandom measurement error, we have employed an algorithm to reduce the effects of nonrandom linear compression on a matrix of distances (Springer 1988; Springer and Kirsch 1989). The resulting matrix of corrected delta Tm values is given in Table 4.

Measurement error aside, reduced normalized percentages of hybridization (NPH) and homoplasy both render delta Tm values nonadditive in expectation and shorten patristic branch lengths on topological reconstructions (Springer and Krajewski 1989). Corrections for reduced NPH and homoplasy are available and have been employed by several authors (Koop et al. 1986; Catzeflis et al. 1987; Springer and Kirsch 1989), although these corrections are less important for small distances than for large distances, especially if we are only interested in branching pattern.

Problems with correcting delta Tm values for reduced NPH to generate delta T50H values are discussed in Sheldon (1987), Marks et al. (1988), Sarich et al. (1989), and Springer and Krajewski (1989), and focus on the large standard error of NPH measurements for replicate hybridization experiments and on the possible influence of kinetics (i.e., NPH differences that result from different rates of reassociation in homologous versus heterologous reactions may inflate delta T50H values). Most importantly, delta T50H values may be so fraught with measurement error that they obscure the branching pattern revealed by delta Tm values. An alternative strategy is to use a regression equation to convert delta Tm values into delta T50H values. This approach is much less sensitive to the effects of measurement error, yet it allows us to obtain better estimates of branch lengths on patristic topologies. For our data, we used a power regression based on data presented in Fig. 3 (156 data points) to convert a half-matrix of corrected delta Tm values (not shown) to a half-matrix of delta T50H values. This equation is

$$\text{delta T50H} = 1.25 \times \text{delta Tm}^{1.04}$$

For some of the data points in Fig. 3, NPH values for heterologous hybrids are higher than for homologous hybrids. In these cases, we have normalized a homologous hybrid against a heterologous hybrid, calculated the new T50H value for the homologue (the heterologue T50H remains the same as the Tm), and subtracted the heterologue T50H from the homologue T50H to obtain a delta T50H value. This results in delta T50H values

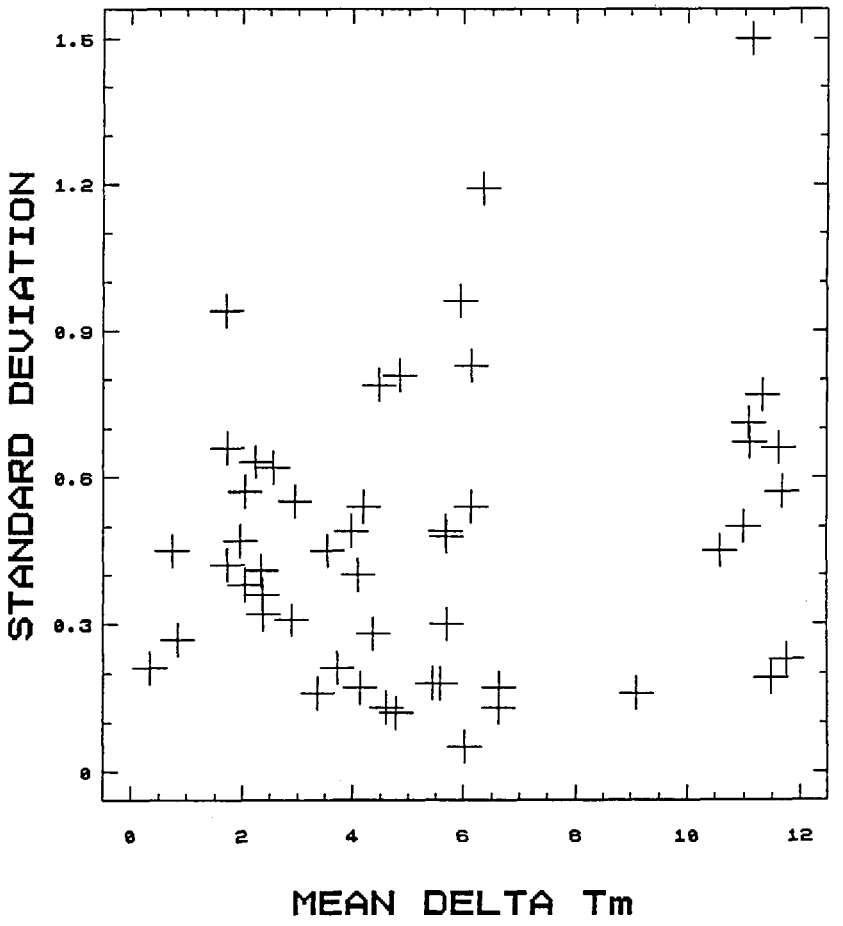


Fig. 4. A plot of the mean versus the standard deviation for delta Tm measurements (this paper) when $n > 2$. The mean and standard deviation are not significantly correlated at $P = 0.05$. In these instances, Felsenstein (documentation for PHYLIP, version 2.8) suggests employing the Cavalli-Sforza and Edwards option of FITCH.

that are less than delta Tm values. In the context of experimental error, however, this is not unexpected, as NPH values are so variable. Indeed, to treat the data otherwise would bias the distribution of measurement error against one of its tails.

Finally, we corrected the half-matrix of delta T50H values for homoplasy using the equation given in Jukes and Cantor (1969). The resulting half-matrix, which has been corrected for nonrandom measurement error, reduced NPH, and homoplasy, is given in Table 5.

Of the included species, only the DNA of *Phalanger carmelitae* was not labeled. We were also unable to hybridize tracer *Spilocuscus rufoniger* DNA with driver *Strigocuscus gymnotis* DNA. In all of these instances, reciprocal values were used to fill empty cells before the matrices were subjected to phylogenetic analysis.

Tree Construction. Both uncorrected and corrected matrices were analyzed using the Cavalli-Sforza and Edwards (1967) least-squares option of FITCH, a pairwise tree-construction algorithm included in J. Felsenstein's PHYLIP (version 2.8) package that makes no assumptions about rate equality. Felsenstein (documentation for PHYLIP package, version 2.8) suggests employing this option when the measurement error for small distances is the same as it is for large ones. For our data, standard deviation and mean delta Tm values are not significantly correlated for cells with greater than two replicates (see Fig. 4). We have also used the Cavalli-Sforza and Edwards option of KITSCH (PHYLIP, version 2.8) and UPGMA clustering (F.J. Rohlf's NTSYS package for the IBM-PC, version 1.20) to generate trees that assume equal rates.

Stability of branching patterns on phylogenetic trees has been assessed using the jackknife approach of Lanyon (1985). Briefly, nine modifications of the original data set (pseudoreplicates) were generated by omitting a different ingroup taxon in each iteration. Best-fit trees were then generated for each pseudoreplicate data set. Finally, these trees were combined to produce a strict-consensus tree (Sokal and Rohlf 1981) to summarize the points upon which all trees agree.

Anatomical Data

Cladistic Analysis. Character data provided in Table 2 were analyzed using Wagner, Dollo, and Camin–Sokal parsimony algorithms available on PHYLIP (version 2.8) and/or PAUP (version 2.4). Both Camin–Sokal and Wagner parsimony were employed in conjunction with PENNY, a branch and bound program available on PHYLIP that is guaranteed to find the complete set of all most parsimonious trees (Hendy and Penny 1982). Character state polarities follow Flannery et al. (1987a).

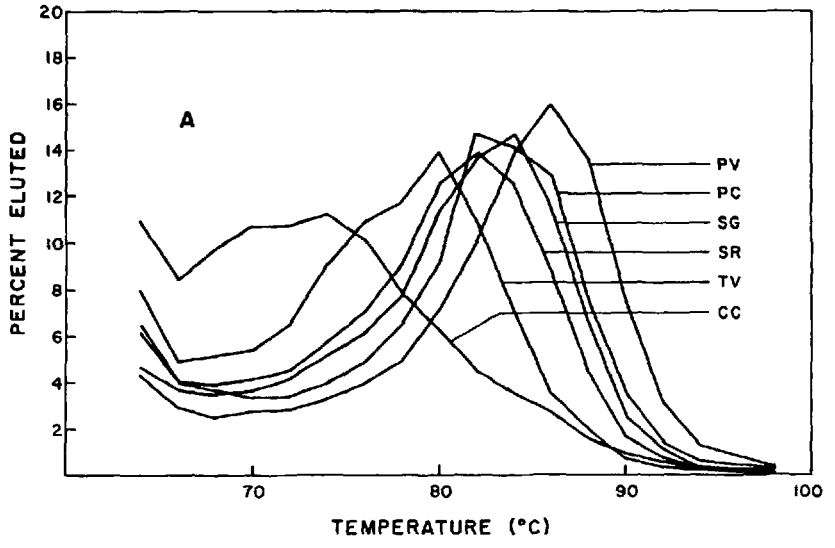
Results

Thermal Melting Curves

A sample of our raw data (i.e., radioactive counts) is given in Appendix 1. Representative melting curves are shown in Fig. 5.

Delta Values and Matrix Reciprocity

A matrix of uncorrected delta Tm values is presented in Table 3. This matrix includes 32 reciprocal



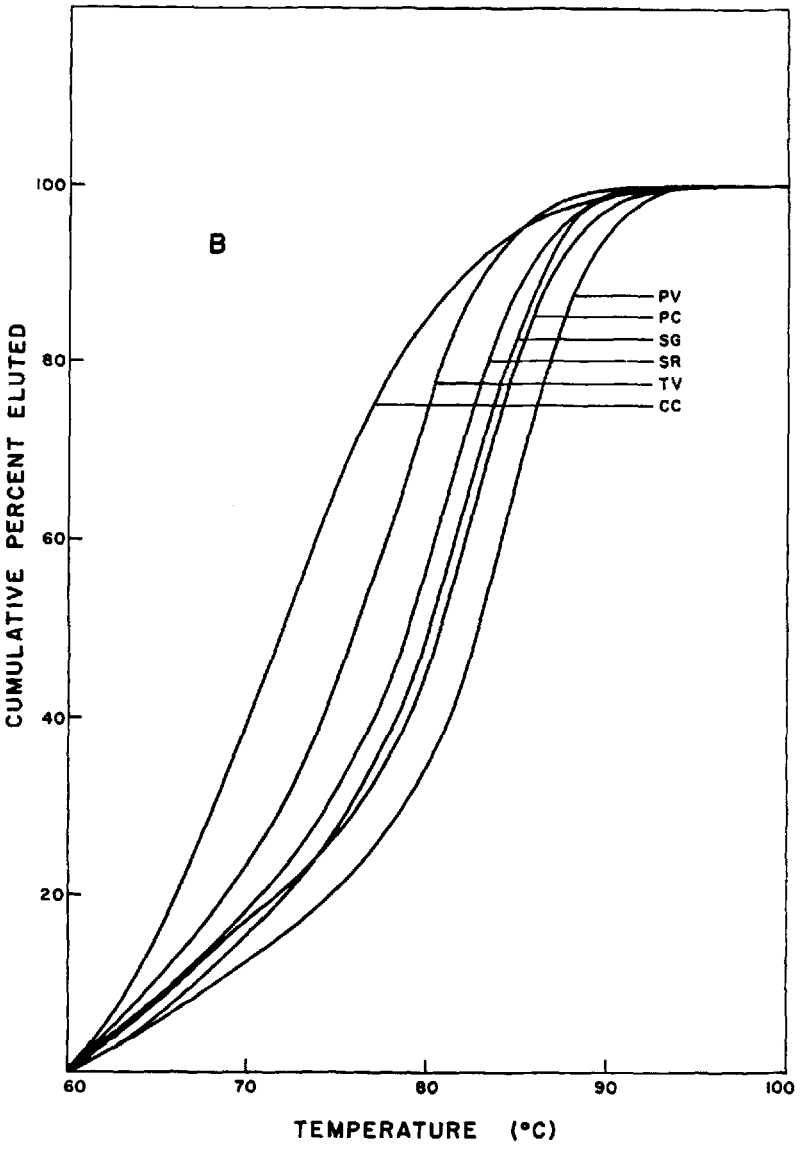


Fig. 5. Representative melting curves for phalangerid DNA hybrids. The labeled taxon is *Phalanger vestitus* (= PV). Driver DNAs are as follows: *Phalanger carmelitae* = PC; *Strigocuscus gymnotis* = SG; *Spilocuscus rufoniger* = SR; *Trichosurus vulpecula* = TV; *Cercartetus caudatus* = CC. **A** The percentage of radioactive counts plotted for each temperature. **B** The cumulative percentage of radioactive counts is plotted as a function of temperature.

Table 3. Mean uncorrected delta Tm values among phalangerids

	C.cau	T.can	T.vul	S.mac	S.ruf	S.gym	P.ves	P.int	P.ori
C.cau	0	9.19	10.59	9.10	9.58	10.32	11.08	11.86	11.33
		−, 1	0.45, 3	0.16, 4	0.45, 2	−, 1	0.71, 4	−, 1	0.23, 2
T.can	11.49	0	0.83	4.46	4.23	5.61	6.12	6.35	6.39
	0.19, 3		0.27, 6	−, 1	0.10, 2	0.25, 2	0.54, 5	1.19, 3	0.64, 4
T.vul	11.11	0.33	0	4.14	4.47	4.76	5.94	6.12	5.67
	0.67, 5	0.21, 3		0.17, 3	0.79, 3	0.42, 2	0.96, 5	0.83, 3	0.49, 6
S.mac	11.31	4.78	5.69	0	0.76	2.89	3.98	3.13	3.72
	0.11, 2	0.12, 4	0.30, 4		0.38, 2	0.31, 4	0.49, 8	0.66, 2	0.21, 5
S.ruf	11.77	5.23	6.63	0.57	0	3.29	4.37	3.36	3.54
	0.23, 7	0.69, 2	0.13, 3	0.21, 2		0.98, 2	0.28, 4	0.16, 3	0.45, 4
S.gym	11.14	4.85	5.69	2.74	—	0	2.35	2.38	2.94
	1.50, 3	0.81, 3	0.48, 5	−, 1			0.36, 9	0.32, 4	0.55, 5
P.ves	11.69	4.42	5.57	2.44	2.62	1.60	0	1.72	2.23
	0.57, 3	0.08, 2	0.18, 5	1.0, 2	0.16, 2	0.80, 2		0.66, 4	0.63, 5
P.int	11.00	4.19	6.01	2.15	3.19	1.80	1.97	0	1.71
	0.50, 3	0.54, 3	0.05, 4	−, 1	0.13, 2	0.06, 2	0.47, 5		0.94, 5
P.ori	11.33	4.61	6.63	3.07	2.93	2.03	2.56	2.04	0
	0.77, 4	0.13, 3	0.17, 7	−, 1	0.06, 2	0.57, 3	0.62, 4	0.38, 7	
P.car	11.63	4.10	5.44	2.58	3.03	1.45	1.72	0.74	2.34
	0.66, 6	0.52, 3	0.18, 5	0.98, 2	−, 1	0.06, 2	0.42, 6	0.93, 3	0.41, 4

Standard deviations and replicate numbers are given below means. Tracer DNAs are listed above columns. Driver DNAs are listed to the left of rows. C.cau = *Cercartetus caudatus*, T.can = *Trichosurus caninus*, T.vul = *Trichosurus vulpecula*, S.mac = *Spilocuscus maculatus*, S.ruf = *Spilocuscus rufoniger*, S.gym = *Strigocuscus gymnotis*, P.ves = *Phalanger vestitus*, P.int = *Phalanger interpositus*, P.ori = *Phalanger orientalis*, P.car = *Phalanger carmelitae*

Table 4. Mean corrected delta Tm values among phalangerids and outgroup taxa

	C.cau	T.can	T.vul	S.mac	S.ruf	S.gym	P.ves	P.int	P.ori
C.cau	0	12.93	11.05	13.06	13.72	13.19	11.08	12.22	12.59
T.can	12.64	0	0.87	6.40	6.06	7.17	6.12	6.54	7.10
T.vul	12.22	0.46	0	5.94	6.40	6.08	5.94	6.30	6.30
S.mac	12.44	6.73	5.93	0	1.09	3.69	3.98	3.22	4.13
S.ruf	12.95	7.36	6.92	0.82	0	4.20	4.37	3.46	3.93
S.gym	12.25	6.82	5.93	3.93	—	0	2.35	2.45	3.27
P.ves	12.86	6.22	5.81	3.50	3.75	2.04	0	1.77	2.48
P.int	12.10	5.90	6.27	3.09	4.57	2.30	1.97	0	1.90
P.ori	12.46	6.49	6.92	4.41	4.20	2.59	2.56	2.10	0
P.car	12.79	5.77	5.67	3.70	4.34	1.85	1.72	0.76	2.60

Tracer DNAs are listed above columns. Driver DNAs are listed to the left of rows. See Table 3 for species abbreviations

comparisons. Based on the formula given in Sarich and Cronin (1976), mean percent nonreciprocity for this matrix is 11.37%. Individual percent nonreciprocity values range from 0.00 to 43.10%. The latter value represents the pairwise combination of *T. vulpecula* and *T. caninus*. The average delta Tm value between these taxa is 0.58°. Because measurement error and distance are not correlated for our DNA hybridization data, smaller distances are expected to have higher percent nonreciprocities.

A matrix of corrected delta Tm values is given in Table 4. Average percent nonreciprocity for this matrix is 5.10%, less than half the value for the uncorrected matrix. The range of nonreciprocity values for the corrected matrix is 0.08–30.83%. A fully corrected half matrix is given in Table 5.

Phenograms and Best-Fit Trees

A UPGMA tree based on a half-matrix of uncorrected delta Tm values is shown in Fig. 6. This tree divides phalangerids into two groups, one containing species of *Trichosurus*, and another containing *Spilocuscus*, *Strigocuscus*, and *Phalanger*. Within the latter group, *Spilocuscus* is excluded from a group containing *Strigocuscus* and *Phalanger*. The branching pattern on the best-fit KITSCH tree is identical to the UPGMA tree.

Figure 7 depicts best-fit Cavalli-Sforza and Edwards (FITCH) trees for both the uncorrected and fully corrected matrices (Tables 2 and 4). A best-fit tree based on corrected delta Tm values (Table 3) is not shown but has a branching pattern identical

Table 5. Half-matrix of delta values corrected for nonreciprocity NPH, and homoplasy

	C.cau	T.can	T.vul	S.mac	S.ruf	S.gym	P.ves	P.int	P.ori	P.car
C.cau	0									
T.can	20.19	0								
T.vul	18.06	0.83	0							
S.mac	20.12	9.42	8.43	0						
S.ruf	21.24	9.65	9.57	1.21						
S.gym	20.06	10.11	8.54	5.20	5.78	0				
P.ves	18.67	8.79	8.34	5.10	5.57	2.89	0			
P.int	19.02	8.87	8.98	4.25	5.51	3.15	2.44	0		
P.ori	19.71	9.79	9.49	5.88	5.58	3.92	3.34	2.62	0	
P.car	20.19	8.17	8.01	5.04	5.99	2.41	2.23	0.95	3.45	0

See Table 3 for abbreviations

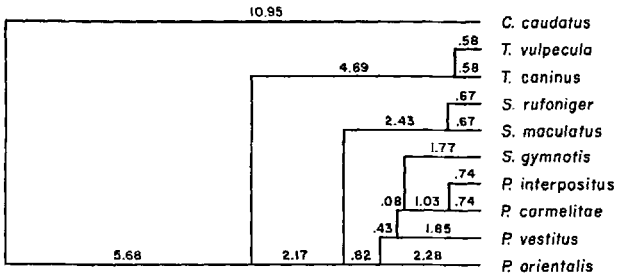


Fig. 6. A UPGMA tree based on a folded half-matrix of uncorrected delta Tm values in which reciprocal values were averaged.

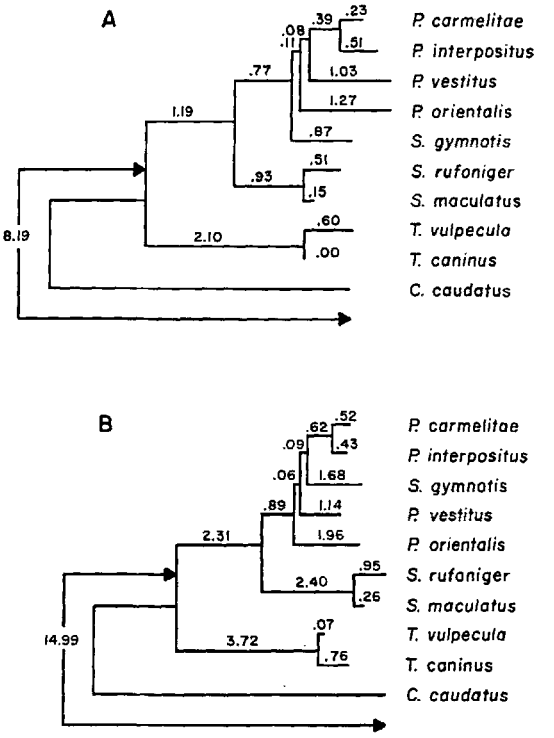


Fig. 7. **A** A best-fit Cavalli-Sforza and Edwards tree based on the uncorrected delta Tm values given in Table 3. The sum of squares for this tree is 28.96. **B** A best-fit Cavalli-Sforza and Edwards tree based on a half-matrix of distances that have been corrected for nonrandom compression, NPH, and homoplasy. The sum of squares for this tree is 14.61.

to that for fully corrected data. Finally, a jackknife strict consensus tree for the uncorrected matrix is shown in Fig. 8.

All of the trees in these figures indicate that *Trichosurus* forms one clade and that *Strigocuscus, Spilocuscus,* and *Phalanger* form a second clade. Within this latter group, *Spilocuscus* is excluded from a clade containing *Strigocuscus* and *Phalanger.* Finally, *Phalanger* is monophyletic only on the best-fit tree for uncorrected data, although *P. interpositus* and *P. carmelitae* group together in all cases.

Cladistic Analysis

When Wagner parsimony was assumed, the branch and bound algorithms of both PAUP and PHYLIP discovered two trees requiring 42 steps. One of these trees is shown in Fig. 9. It unites *Strigocuscus gymnotis* with *Trichosurus* and *Spilocuscus* with *Phalanger.* In the latter group, *Spilocuscus* and *Phalanger* are separate monophyletic groups. This agrees with the phylogenetic hypothesis preferred by Flannery et al. (1987a). The second tree (not shown) excludes *P. vestitus* and *P. orientalis* from a clade containing *Spilocuscus, P. carmelitae,* and *P. interpositus.*

Minimum length trees under the assumption of Camin–Sokal parsimony, of which there are two, require 44 steps. One of these is identical to the Wagner tree shown in Fig. 9. The only difference on the second is a switch of positions between *P. orientalis* and *P. vestitus.* The shortest Dollo parsimony tree that we were able to find also contains 44 steps and is shown in Fig. 10. This tree unites *Strigocuscus gymnotis* and *Trichosurus* but does not separate *Spilocuscus* and *Phalanger.*

Discussion

Molecules versus Morphology

Phylogenetic hypotheses suggested by our DNA hybridization studies are in moderate agreement with

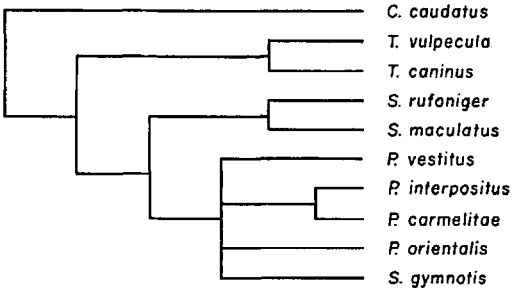


Fig. 8. A strict-consensus jackknife tree produced from best-fit Cavalli-Sforza and Edwards trees derived from nine pseudoreplicate matrices for uncorrected delta Tm values.

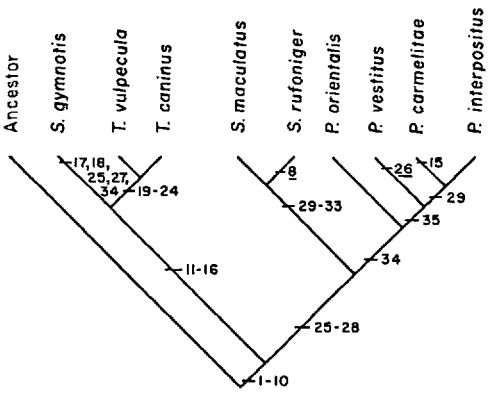


Fig. 9. One of two minimum-length Wagner parsimony trees (42 steps) for the character state data presented in Table 2. Characters supporting this tree are listed next to branches. Character reversals (1 → 0) are underlined.

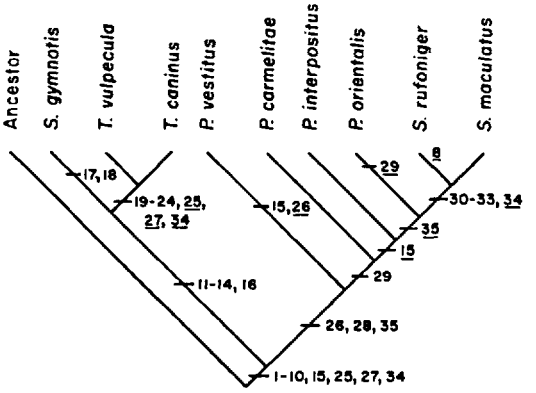


Fig. 10. A minimum-length Dollo parsimony tree containing 44 steps. Numbers on the tree represent the numbers of the characters (see Table 2) that change along each branch. Reversals (1 → 0) are underlined.

Flannery et al.'s classification and with several of the cladistic analyses presented here, except for the placement of *Strigocuscus gymnotis*. Flannery et al. (1987a) unite *Strigocuscus* and *Trichosurus* in the tribe Trichosurini. Wagner, Camin–Sokal, and Dollo parsimony also support this arrangement. DNA hybridization results, on the other hand, suggest placement of *Strigocuscus* in the tribe Phalangerini along with *Spilocuscus* and *Phalanger*. Indeed, an alternative hypothesis of Flannery et al. (1987a) unites *Spilocuscus, Phalanger,* and *Strigocuscus* (except *S. celebensis*) to the exclusion of *Trichosurus*. In this alternative arrangement, which is consistent with George's (1987) usage of *Strigocuscus,* Flannery et al. (1987a) unite *Spilocuscus* and *Phalanger* to the exclusion of *Strigocuscus*. DNA results, however, support Tate (1945) and George (1987) and suggest that *Strigocuscus* and *Phalanger* form a clade to the exclusion of *Spilocuscus*.

In the *Phalanger–Strigocuscus* group, our DNA hybridization results only provide mixed support for the monophyly of *Phalanger,* although all trees support a sister-group relationship between *P. interpositus* and *P. carmelitae*. All of our cladistic analyses also support this sister-group relationship, as do the three phylogenetic hypotheses proposed by Flannery et al. (1987a).

One of the minimum-length Wagner trees, as well as the minimum-length Dollo tree, fails to separate *Spilocuscus* and *Phalanger*. This is strongly contradicted by our molecular results.

All of the most parsimonious trees suggest that *Strigocuscus gymnotis* is a trichosurin. Putative shared derived characters that support this arrangement are as follows: retraction of lachrymal from face, rostrum narrowed, ventral rim of orbit visible from below, ectotympanic excluded from anterior postglenoid process, P3/ at oblique angle to molar row, and P3/ possesses at least four cuspules (Flannery et al. 1987a). On the other hand, there are only two derived characters (M2/ preprotocrista not contacting parastyle, I3/-C1/ diastema lost) that support the union of *Strigocuscus, Spilocuscus,* and *Phalanger,* and only one character (I3/ extremely small) that unites *Strigocuscus* and *Phalanger* to the exclusion of *Spilocuscus*. Still, the fundamental tenet of cladistic methodology is that only shared derived characters provide evidence of monophyly, not that characters must evolve in a minimum-length fashion. The inconsistency between DNA hybridization results and numerical cladistic results, then, does not focus on the fundamental tenet of cladistic analysis, but on parsimony, which we regard as an ancillary criterion rather than a canon of phylogenetic truth. If we are willing to accept a slightly less parsimonious alternative, it is easy to reconcile the DNA results and the cladistic analyses of anatomical characters.

Many authors equate cladistic analysis with parsimony analysis and do not distinguish between phylogenetic hypotheses that are consistent with the distribution of derived character states versus phylogenetic hypotheses that require the additional constraint of parsimony. Indeed, uncritical acceptance of parsimony as an infallible guide to phylogeny is

all too often standard practice. Rather, the general applicability of parsimony algorithms for phylogenetic inference based on anatomical characters should be critically evaluated, as the process of character state evolution is often poorly understood. If characters do not evolve in a minimum-length fashion, as may be the case for many anatomical characters, then parsimony may be positively misleading.

One method of assessing the utility of parsimony for phylogenetic analysis with morphological characters is to map these characters onto a molecular phylogeny. For our data, it requires a minimum of 47 steps (Wagner parsimony) to map the anatomical characters in Table 2 onto the DNA tree in Fig. 7. In fact, all trees that unite (1) *Strigocuscus* and *Phalanger* to the exclusion of *Trichosurus* and *Spilocuscus,* and (2) *Strigocuscus, Phalanger,* and *Spilocuscus* to the exclusion of *Trichosurus* require at least 47 steps. This is only five additional steps, but there are 934 trees containing ≤ 47 steps, 535 of which contain ≤ 46 steps. PAUP will only save the first 100 trees, but examination of these was sufficient to show that there are a variety of trees to choose from, e.g., *Spilocuscus* united with *Strigocuscus* and *Trichosurus* rather than *Phalanger.*

If we are willing to accept the major findings of the DNA hybridization tree that hold up under jackknifing, the unfortunate message is that to have some assurance of getting the correct tree with parsimony and anatomical characters, we need to relax our minimum-length requirements by a sufficient number of steps to include a host of incorrect trees, many of which are strikingly different from each other. Whether or not this finding will be obtained for other taxonomic groups and other character systems remains to be determined, although Springer (1988) arrived at a similar conclusion for interfamilial relationships in the order Diprotodontia when he compared DNA hybridization based phylogenies with numerical cladistic analyses of dental characters. Flannery and Rich (1986) provide a further example of extraordinary and unsuspected convergence that was only detected because of an excellent fossil record. Still, anatomical characters can be used to formulate sound phylogenetic hypotheses without invoking parsimony. Tate (1945), for example, recognized *Spilocuscus* and *Strigocuscus–Phalanger* as distinct groups long before parsimony was explicitly employed. Even Flannery et al. (1987a) expressed reservation concerning their placement of *Strigocuscus gymnotis.* There was obviously convergence—the question was how much and where. In the light of DNA hybridization studies, how much and where are not always arrived at through parsimony.

If we accept the DNA tree, we may also ask which characters exhibit the most homoplasy and are consequently less reliable indicators of phylogeny. Characters 11–16 are obvious candidates, as they have arisen independently in both the phalangerin (*S. gymnotis*) and trichosurin (*Trichosurus*) clades. Three of these characters (11–13) pertain to the arrangement of bones in the orbital–rostral region of the face. One character (14) pertains to the postglenoid region. Finally, two characters (15, 16) describe the orientation and morphology of the third upper premolar (P3/). Thus, homoplasy occurs in both dental and cranial characters and is not restricted to one or the other. P3/ itself, however, appears to be particularly malleable.

The Phalangerid Radiation

Flannery and Archer (1987) described two new trichosurin phalangerids (*Strigocuscus reidi* and *Trichosurus dicksoni*) from putative Middle Miocene sediments on Riversleigh Station, northwestern Queensland. Flannery and Archer (1987, p. 535) state that "*S. reidi* is closely related to and possibly ancestral to *S. gymnotis.*" Furthermore, Flannery et al. (1987b) described a new species of *Strigocuscus* (*S. notialis*) from the Early Pliocene Hamilton local fauna that is also closely related to *S. gymnotis.* Flannery and Archer (1987) and Flannery et al. (1987b) note that phalangerin phalangerids, which today almost completely dominate the phalangerid assemblages of New Guinea, are apparently absent from Miocene and Early Pliocene deposits of Australia. Our results, however, suggest that *S. gymnotis,* and by extension, *S. reidi* and *S. notialis,* are phalangerins. Thus, phalangerins and trichosurins are both present in Miocene and Pliocene deposits in Australia. Our preliminary results also suggest that extant phalangerins comprise a larger clade (i.e., contain more species) than extant trichosurins.

Based on the distances between many phalangerin taxa, many of which are less than three degrees, much of the phalangerin radiation occurred during the Pliocene/Pleistocene (Springer, unpublished). This is consistent with George's (1987) suggestion that tectonic activity in northern Australia and New Guinea supported rainforest differentiation, which, in turn, accelerated speciation rates and allowed ecological diversity to develop at this time.

Unfortunately, our study does not encompass *Ailurops ursinus, Strigocuscus celebensis, Strigocuscus mimicus, Phalanger lullulae,* and several other species. We hope to include these taxa in future studies and present a more complete picture of the phalangerid radiation based on DNA hybridization data. In particular, the testing of recent phylogenetic hypotheses in phalangerid systematics hinges on the

placement of *Ailurops ursinus* and *Strigocuscus celebensis.*

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Appendix 1

A sample of raw data, in the form of radioactive counts, for 37 DNA hybridization experiments, is presented below. DNA hybrids were loaded onto hydroxylapatite columns developed with 0.12 M neutral phosphate buffer at 60°C and washed three times at this temperature (8 ml per wash) to remove unhybridized DNA and unincorporated ^{125}I. The temperature was then raised in 2°C increments (except that the first and last steps were 4°C increments) and the columns were washed with 8 ml of 0.12 M phosphate buffer at each of 17 different temperatures (64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, and 98). Radioactive counts provided below correspond to these 17 temperatures. As noted in the text, delta Tm measurements were based on average homologous Tms for a particular tracer preparation. Data presented below, however, include only one homologous hybrid for a particular tracer preparation.

Tracer
Phalanger orientalis: Mean homologous Tm = 84.28.

Driver
Phalanger orientalis: 504, 314, 302, 261, 342, 332, 441, 541, 865, 1311, 1904, 2414, 2417, 1614, 896, 294, 149.
Phalanger carmelitae: 484, 447, 297, 313, 352, 394, 501, 616, 1138, 1515, 1899, 1677, 1244, 616, 260, 54, 78.
Phalanger vestitus: 541, 384, 319, 322, 347, 400, 529, 720, 1082, 1461, 1927, 1679, 1154, 572, 209, 56, 31.
Phalanger interpositus: 536, 400, 365, 386, 400, 435, 563, 695, 1185, 1595, 1941, 1764, 1185, 504, 232, 74, 80.
Trichosurus vulpecula: 726, 590, 562, 562, 719, 917, 1253, 1482, 1925, 1900, 1449, 987, 479, 213, 120, 118, 81.
Strigocuscus gymnotis: 401, 295, 308, 348, 523, 503, 643, 782, 1403, 1703, 2003, 1786, 1108, 473, 205, 179, 153.
Spilocuscus maculatus: 420, 350, 367, 394, 415, 559, 706, 1003, 1555, 1843, 1851, 1353, 772, 340, 125, 45, 57.
Spilocuscus rufoniger: 314, 353, 425, 402, 500, 599, 925, 1219, 1849, 2271, 2314, 1785, 1093, 495, 193, 105, 63.
Cercartetus caudatus: 734, 800, 870, 906, 949, 1024, 1039, 934, 669, 490, 395, 331, 249, 117, 65, 45, 43.

Tracer
Spilocuscus maculatus: Mean homologous Tm = 80.85.

Driver
Spilocuscus maculatus: 1391, 980, 862, 796, 920, 1079, 1256, 1511, 2096, 2773, 3330, 3250, 2118, 1217, 457, 193, 216.
Spilocuscus rufoniger: 1036, 850, 644, 637, 705, 846, 951, 1116, 1596, 1957, 1986, 2124, 1406, 645, 259, 133, 135.
Trichosurus vulpecula: 1426, 785, 965, 850, 1150, 1545, 1894, 2159, 2451, 1945, 1328, 817, 444, 199, 143, 102, 142.
Trichosurus caninus: 1223, 974, 950, 1007, 1202, 1464, 1803, 2070, 2342, 2108, 1356, 719, 354, 185, 102, 103, 97.
Phalanger orientalis: 1305, 895, 824, 973, 1010, 1214, 1570, 1838, 2394, 2388, 2022, 1171, 581, 268, 138, 82, 177.
Phalanger interpositus: 1217, 892, 885, 883, 943, 1196, 1380, 1779, 2513, 2780, 2507, 1611, 823, 331, 152, 109, 107.
Strigocuscus gymnotis: 1465, 1017, 885, 983, 1056, 1235, 1481, 1857, 2493, 2585, 2246, 1458, 758, 310, 154, 140, 120.
Cercartetus caudatus: 582, 368, 429, 388, 525, 341, 375, 318, 268, 197, 158, 145, 63, 55, 63, 38, 40.

Tracer
Strigocuscus gymnotis: Mean homologous Tm = 81.11.

Driver
Strigocuscus gymnotis: 1622, 992, 884, 819, 796, 861, 980, 1082, 1711, 2228, 2942, 2914, 2250, 1098, 451, 215, 120.
Trichosurus caninus: 2286, 1332, 1155, 1089, 1095, 1286, 1596, 1758, 2113, 1846, 1363, 785, 395, 201, 103, 124, 65.
Phalanger orientalis: 666, 419, 305, 350, 366, 451, 500, 575, 871, 1075, 1138, 919, 602, 261, 95, 32, 48.

Phalanger carmelitae: 2079, 1221, 1001, 901, 869, 948, 1167, 1471, 2207, 2827, 3047, 2641, 1575, 625, 280, 120, 93.
Phalanger interpositus: 3237, 1938, 1728, 1658, 1728, 1909, 2223, 2985, 4157, 5108, 5275, 4116, 2302, 1034, 405, 182, 101.
Spilocuscus maculatus: 2617, 2351, 1661, 1516, 1946, 2026, 2542, 3108, 4322, 4573, 4060, 2767, 1406, 639, 229, 164, 182.

Tracer
Trichosurus vulpecula: Mean homologous Tm = 81.92.

Driver
Trichosurus vulpecula: 1353, 851, 605, 590, 504, 575, 677, 761, 1265, 1794, 2419, 2525, 2064, 1024, 357, 78, 45.
Trichosurus caninus: 1503, 898, 672, 675, 675, 741, 971, 1203, 1908, 2610, 3172, 2823, 1872, 755, 240, 39, 27.
Strigocuscus gymnotis: 2588, 1741, 1485, 1693, 1749, 2375, 2904, 3119, 3596, 2983, 2047, 1047, 482, 308, 72, 64, 87.
Phalanger carmelitae: 1118, 684, 592, 618, 787, 914, 1230, 1499, 1822, 1630, 1175, 569, 261, 40, 18, 3, 0.
Phalanger interpositus: 1061, 668, 589, 553, 630, 736, 933, 1154, 1332, 1177, 744, 394, 170, 59, 3, 0, 0.
Phalanger vestitus: 1972, 1238, 1034, 1128, 1272, 1556, 2015, 2288, 2811, 2384, 1579, 841, 372, 107, 33, 20, 0.
Spilocuscus maculatus: 1611, 904, 841, 815, 920, 1095, 1418, 1690, 2060, 1825, 1220, 624, 299, 97, 48, 26, 25.

Tracer
Phalanger vestitus: Mean homologous Tm = 82.87.

Driver
Phalanger vestitus: 1342, 1358, 1282, 1103, 1254, 1387, 1616, 1992, 3113, 4416, 6070, 6810, 5692, 3220, 1366, 495, 210.
Phalanger carmelitae: 2057, 1429, 1325, 1239, 1317, 1515, 1899, 2485, 3781, 5004, 5645, 4995, 3000, 1346, 499, 260, 174.
Phalanger interpositus: 2004, 1340, 1240, 1288, 1436, 1701, 2187, 2778, 4154, 5564, 6136, 4794, 2707, 1089, 402, 172, 92.
Strigocuscus gymnotis: 1350, 1041, 1009, 1053, 1198, 1469, 1778, 2238, 3279, 3997, 4253, 3321, 1890, 739, 282, 99, 85.
Spilocuscus rufoniger: 2766, 1753, 1688, 1774, 1962, 2461, 3052, 3921, 5468, 6027, 5405, 3814, 1940, 733, 341, 131, 71.
Trichosurus caninus: 2701, 1818, 1959, 2078, 2497, 2962, 3766, 4245, 4703, 3588, 2328, 1220, 534, 207, 112, 67, 27.
Cercartetus caudatus: 2038, 1567, 1812, 1996, 1990, 2076, 1895, 1458, 1149, 848, 651, 499, 283, 159, 111, 57, 81.