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Evolution

DNA hybridization evidence for the Australasian affinity of the American marsupial *Dromiciops australis*

(marsupial phylogeny/molecular systematics/evolutionary rates/historical biogeography)

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ABSTRACT **DNA hybridization was used to compare representatives of the major groups of marsupials and a eutherian outgroup. Because of the large genetic distances separating marsupial families, trees were calculated from normalized percentages of hybridization; thermal-melting statistics, however, gave identical topologies for the well-supported clades. The most notable results were the association of the only extant microbiotheriid, *Dromiciops australis*, an American marsupial, with the Australasian Diprotodontia, and of both together with the Dasyuridae. Estimates of the rate of divergence among marsupial genomes suggest that the *Dromiciops*–Diprotodontia split occurred ≈50 million years ago, well after the establishment of the major clades of marsupials but before deep oceanic barriers prohibited dispersal among Australia, Antarctica, and South America. Because *Dromiciops* is nested within an Australasian group, it seems likely that dispersal from Australia accounts for its present distribution.**

Dromiciops australis, the monito del monte of southern Chile and adjacent Argentina, had been considered a small-bodied opossum until Reig (1) noted dental and other similarities to members of the Tertiary family Microbiotheriidae and suggested that *Dromiciops* is a living representative of this otherwise extinct taxon. Subsequent study of skull anatomy (2) confirmed Reig's proposal, and serology (3) indicated the distinctness of *Dromiciops* vis-à-vis didelphids. Later, Szalay (4) demonstrated similarities between the tarsi of Australasian marsupials and *Dromiciops* and proposed that *Dromiciops* represents the sister group of all Australasian species; together they comprise a monophyletic taxon (Australidelphia) apart from other marsupials (Ameridelphia) in his system. Much recent work has supported Szalay's view: Sharman (5) noted details of *Dromiciops*' chromosomes comparable to those of several Australasian families; Gallardo and Patterson (6) found male sex-chromosome mosaicism like that in the Australian diprotodontian *Petauroides volans*; and Temple-Smith (7) documented similarities among the spermatozoa of *Dromiciops*, phalangers, and kangaroos. Reig *et al.* (8), however, criticized Szalay's interpretation of tarsal anatomy, and argued that Australasian affinity was incompatible with craniodental evolution, suggesting instead that *Dromiciops* is the terminus of a lineage that evolved independently of Australasian marsupials.

As part of a DNA hybridization study of the relationships of opossums (Marsupialia: Didelphidae), we constructed a matrix including representatives of all major marsupial groups and a eutherian, *Procyon lotor*. Since one of our aims was to root the didelphid tree, more exemplars of Didelphidae (*Didelphis, Metachirus, Monodelphis, Caluromys*) were included than of any other marsupial group. Because of the limited range of thermal-melting statistics (9), and the fact that a recent study of *Dromiciops* using $\Delta t_{50}H$ (median melting temperature of hybridized plus potentially hybridizable sequences) (10) did not resolve its affinities, normalized percentage hybridization (NPH) was our preferred basis for computing topologies relating the distant lineages. NPH has been criticized because it is imprecise (11), but modifications of our elution protocol reduce the standard deviations of NPH values up to 5-fold, and trees generated from the measure have the same branching order as those calculated from thermal-melting statistics used within their respective ranges. The most interesting relationship indicated by our study is the placement of *D. australis* as the sister taxon of the Australasian Diprotodontia. Demonstration of this affinity fulfills the century-long quest for a "special relationship" between particular Australasian and American marsupials (3, 12, 13) and indicates a more complex zoogeographic history for marsupials than is commonly assumed.

MATERIALS AND METHODS

DNA Samples and Labeling. One to four whole-genome DNA extracts from each species listed in Table 1 were purified and fragmented, and single-copy fractions were labeled with ^{125}I by modifications of the methods of Sibley and Ahlquist (14) described by Kirsch *et al.* (15).

Thermal Elution and Calculation of Distances. Hybrids were fabricated and eluted according to a protocol modified from Kirsch *et al.* (15): two 8-ml room-temperature washes preceded the first elevated-temperature elution (5 ml at 52°C), followed by 22 elutions of 5 ml each at 2°C increments up to and including 96°C. Room-temperature elutions presumably wash free iodine and small (unhybridizable) DNA fragments from the hydroxyapatite columns, enabling their discrimination from both hybridized and potentially hybridizable sequences (which may remain double-stranded at low temperatures due to nonspecific base pairing), and thereby improving the precision of measured percentage hybridization. NPH values were calculated by comparing counts eluted at and above 56°C to total counts from 52°C upward and standardizing against the average percentage hybridization of the homoduplex. Parallel calculations of median melting temperature of hybridized sequences (t_m) and $t_{50}H$ values followed usual procedures (9); modes cannot be estimated reliably for very distant comparisons. The NPH values were subtracted from 100 to give distances, and the t_m and $t_{50}H$ values were expressed as Δ values (i.e., deviations from the appropriate homoduplex melting temperatures).

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Abbreviations: NPH, normalized percentage hybridization; t_m, median melting temperature of hybridized sequences; $t_{50}H$, t_m adjusted for percentage hybridization.

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Table 1. Uncorrected reciprocal (100−NPH) distances among nine marsupials and a eutherian and one-way distances from three additional marsupials

	Pro	Cal	Did	Met	Mon	Dro	Cae	Das	Ech	Pha	Dro2	Ech2	Las	Pet	Mur
Procyon	0	45.20	65.20	51.80	56.70	70.70	51.38	74.10	61.73	65.00	64.13	30.70	41.60	35.60	61.60
lotor	0.28/2	--/1	--/1	--/1	--/1	--/1	--/1	--/1	5.03/3	--/1	6.69/3	--/1	--/1	--/1	3.26/3
Caluromys	71.15	0	13.20	11.73	13.83	49.30	34.13	46.65	48.10	35.45	35.60	30.85	26.05	24.95	47.90
philander	5.30/2	0.91/5	0.72/4	1.88/4	1.69/6	0.28/2	0.35/2	0.49/2	--/1	0.07/2	--/1	1.06/2	1.48/2	0.64/2	--/1
Didelphis	76.30	19.64	0	11.60	18.48	49.15	35.53	53.43	47.20	44.43	44.20	34.90	23.60	28.30	48.37
virginiana	0.42/2	5.38/5	3.60/4	3.11/4	4.40/6	5.05/4	0.07/2	9.94/3	2.38/3	2.57/3	3.87/3	0.42/2	0.00/2	2.40/2	1.25/3
Metachirus	72.10	15.18	7.20	0	12.72	47.30	46.48	53.55	42.40	42.55	44.00	26.70	24.30	35.10	48.20
nudicaudatus	--/1	3.93/6	3.52/4	1.91/4	2.79/6	2.83/2	--/1	1.77/2	--/1	1.63/2	--/1	--/1	--/1	--/1	--/1
Monodelphis	69.40	11.57	10.40	10.43	0	47.20	43.18	51.00	39.90	38.35	37.30	29.10	35.10	23.10	41.40
dimidiata	--/1	2.47/4	3.09/4	1.52/4	1.36/6	0.28/2	--/1	0.57/2	--/1	3.46/2	--/1	--/1	--/1	--/1	--/1
Dromiciops	72.10	25.80	41.00	36.30	36.30	0	41.48	39.80	36.43	27.90	0	43.35	17.35	23.10	39.80
australis	1.56/2	--/1	--/1	--/1	--/1	3.46/6	5.09/2	--/2	4.82/3	4.81/2	1.49/5	0.92/2	2.19/2	0.71/2	3.21/3
Caenolestes	76.47	28.00	47.60	35.30	40.10	49.14	0	51.95	38.85	42.05	41.40	36.17	25.80	28.70	46.15
fuliginosus	0.49/3	6.93/2	5.23/2	0.85/2	7.50/2	2.60/5	0.82/4	1.48/2	3.18/2	0.64/2	1.98/2	7.54/3	2.40/2	1.13/2	1.34/2
Dasyurus	74.20	32.50	46.90	36.45	41.50	44.58	36.68	0	43.10	35.33	40.37	42.20	23.30	23.75	9.20
hallucatus	1.56/2	4.81/2	--/1	0.64/2	0.71/2	5.80/4	1.41/2	0.93/4	1.93/3	1.46/3	4.14/3	2.55/2	3.39/2	1.48/2	4.56/3
Echymipera	76.45	29.80	51.80	38.85	39.70	49.80	40.11	48.88	0	41.30	43.00	0	22.40	30.10	43.80
clara	0.92/2	5.80/2	1.13/2	0.07/2	--/1	2.40/2	2.16/3	8.74/4	1.47/4	5.47/3	5.46/3	2.09/3	0.00/2	0.85/2	3.90/3
Phalanger	74.10	28.10	38.10	30.80	34.05	28.17	34.11	38.74	47.80	0	29.00	31.30	12.10	11.25	29.10
orientalis *	2.83/2	3.11/2	--/1	--/1	0.35/2	4.24/6	2.69/3	4.71/5	18.81/2	0.70/4	2.97/2	2.30/3	3.11/2	4.17/2	--/1
Correction	0.814	1.255	1.028	1.189	1.012	0.811	1.074	0.842	0.992	0.928					

Average SD = 2.85 (0.45, 1.00)

Correlation of SD with distance = 0.10 (-0.27, 0.25)

Average percent. asymmetry (before correction) = 11.34% (4.90%, 4.39%)

Average percent. asymmetry (after correction) = 5.91% (1.73%, 2.86%)

Columns are tracers, abbreviated as the first three letters of each species name (given in the rows), except that Dro2 and Ech2 are second labels of *Dromiciops* and *Echymipera*, Las is *L. latifrons*, Pet is *P. xanthopus*, and Mur is *M. longicaudata*. SD and number of replicates are given in the second line of each cell, and correction factors for asymmetry are listed under each of the first 10 columns of data. Average SD (for cells with more than one replicate), correlations of SD with distance, and average percentage asymmetries before and after correction are recorded for the 10 × 10 matrix (10 leftmost columns of data); corresponding values of these statistics for the Δt_m and $\Delta t_{50}H$ matrices, respectively, are given in parentheses. Matrices of Δt_m and $\Delta t_{50}H$ values are available from J.A.W.K. on request.
*Because of limited material, some drivers were *Phalanger rufoniger*.

Tree-Building and Testing. Best-fit trees were calculated by using the FITCH program in Felsenstein's PHYLIP version 3.3 (16), both before and after correction for asymmetric reciprocal cell values (17). The consistency of the 100−NPH matrix was tested by jackknifing (18) and measurement imprecision was explored through bootstrapping (19). Strict and Adams consensuses were calculated among the 100−NPH, Δt_m, and $\Delta t_{50}H$ trees, using the CONTREE program

of Swofford's PAUP version 2.4.2 (20). The 100−NPH matrix was augmented by data obtained with second labels of *Dromiciops* and *Echymipera*, using linear regression to adjust for interrun variation (21), and again subjected to asymmetry correction, FITCH, and bootstrapping.

RESULTS

Fig. 1*a* shows the tree resulting from FITCH analysis of the symmetrized 100−NPH data. The basal internodes joining the peramelid *Echymipera* and the caenolestid *Caenolestes*, on the one hand, and the didelphids plus the three remaining marsupials, on the other, are very short (<1%), occur only in a small majority of bootstrap pseudoreplicates, and are not supported by jackknifing (Fig. 1*b*); the pairing of *Caenolestes* and *Echymipera* may be due to the attraction of long, undivided branches calculated from data that have not been corrected for nonadditivity (22). FITCH trees based on Δt_m or $\Delta t_{50}H$ values (Fig. 1*c* and *d*) show different associations of the four basal clades, and the strict and Adams consensus trees generated from the three measures are identical to Fig. 1*b*. We therefore conclude that our data do not resolve relationships among the four oldest marsupial branches.

The more terminal lineages, however, are separated by substantial internodes and have the same branching order for all three measures of distance. The most unexpected linkage was the association of *Dromiciops* with the phalangerid *Phalanger*, rather than with Australasian marsupials as a whole. Tests with other labeled diprotodontians (the wombat *Lasiorhinus latifrons* and the rock wallaby *Petrogale xanthopus*), and with another extract of *Dromiciops*, were consistent with the diprotodontian affinity of *Dromiciops*, in that *Dromiciops* was as close or closer to diprotodontians than were nondiprotodontians. Another feature of the consensus topology is that *Dasyurus* shows greatest affinity with *Phalanger* and *Dromiciops*, a relationship further supported by hybrids made with labeled DNA from a second dasyurid, *Murexia longicaudata*: distances to *Dromiciops*, *Phalanger*, and *Dasyurus* are, with one exception, shorter than those to other marsupials. Moreover, the tree calculated from the symmetrized 100−NPH matrix after addition of distances from (and to) *Murexia* has the same branching order as that of Fig. 1*c*, with *Murexia* annexed to the *Dasyurus* clade. Results with the second labeled extract of *Dromiciops* and an additional label of *Echymipera*, combined with those obtained with the original tracers, produced trees that again supported the association of *Dromiciops* with *Phalanger* and of both with *Dasyurus*. Relationships among didelphids remained as shown in Fig. 1 throughout all analyses.

As another evaluation of the symmetrized 100−NPH data, we employed the user-tree option of FITCH to generate trees with *Dromiciops* forced to be the sister group of (*i*) *Phalanger*, (*ii*) *Dasyurus* plus *Phalanger*, (*iii*) the didelphid clade, and (*iv*) all other marsupials. These associations correspond, respectively, to the conclusion of this paper, the hypothesis of Szalay (4), the view of Reig *et al.* (8), and the possibility that *Dromiciops* represents an entirely distinct lineage. The sums-of-squares increased from 740 to 776 when *Dromiciops* was removed from the *Phalanger* branch and was placed as the sister group to *Phalanger* plus *Dasyurus*; to 935 when

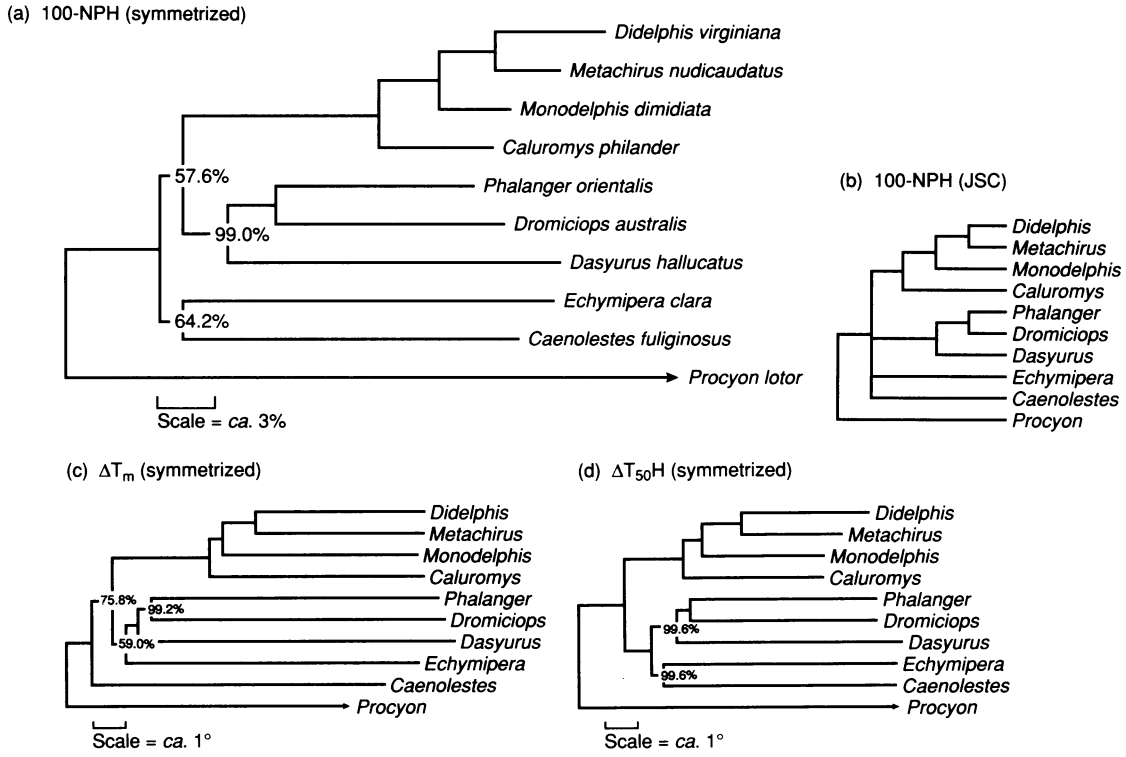

FIG. 1. (*a*) FITCH tree generated from symmetrized data of Table 1 ($P = 0$ and global branch swapping enabled). Approximately to scale. Data were bootstrapped 500 times to explore the stability of branching order (19). Numbers at nodes are percentages of pseudoreplicate trees supporting each branchpoint, when these differed from 100%; these percentages should not be regarded as measures of statistical significance. Similar results [with high percentages exhibiting the didelphid clade and that of *Dasyurus*, *Phalanger*, and *Dromiciops*; and low percentages giving the two basal marsupial dichotomies (*Echymipera* with *Caenolestes* and didelphids with *Phalanger*, *Dromiciops*, and *Dasyurus*)] were obtained with 100−NPHs based on counts at and above 70°C and, when data from second labels of *Echymipera* and *Dromiciops* were included, by using NPH values calculated either from 56°C or 70°C. (*b*) Jackknife strict consensus (JSC) of FITCH trees generated from either unsymmetrized or symmetrized data of Table 1; this is also the strict and Adams consensus of *a*, *c*, and *d*. Not to scale. (*c* and *d*) Trees generated from symmetrized Δt_m and $\Delta t_{50}H$ distances, respectively; FITCH options and bootstrap conventions are the same as for *a*. Approximately to scale.

Dromiciops and didelphids were united; and to 950 when *Dromiciops* was designated sister group of the remaining eight marsupials. Repetition of these tests with allowance of negative branches improved the sums-of-squares for the second, third, and fourth arrangements but gave negative internodes involving *Dromiciops* in these three cases.

DISCUSSION

Possible Artifacts of NPH Measurements. Theoretical objections to the use of NPH have been raised (9), which, if valid, might compromise these apparent relationships. One possibility is that the complexities of single-copy genomes vary among the species, which would be manifest in asymmetric reciprocal NPH cell values, although asymmetry due to tracer quality may mask such effects (23). Spurious pairing of *Dromiciops* and *Phalanger* could occur if both had primitively or homoplastically more complex single-copy sets than other marsupials and would be reflected in uniformly greater distances from *Phalanger* or *Dromiciops* tracers to other taxa than in reciprocal comparisons. One of the two *Dromiciops* labels meets this condition, while the *Phalanger* and other *Dromiciops* tracers do not. Alternatively, if *Phalanger* and *Dromiciops* had little repetitive DNA the higher effective concentrations of single-copy sequences in hybrids would increase NPH; however, diprotodontians at least do not have unusually low amounts of repetitive DNA (23). Other confounding factors might be kinetic differences between the rates of reassociation of low- vs. high-stability sequences (24) or the elution of poorly matched double-stranded sequences (25), but these effects should influence absolute rather than relative distances. Finally, it may be that our measurements index mostly paralogous sequences, since the majority of heteroduplex counts are located over the low-temperature peak (Fig. 2) and so confound phylogenetic interpretation (26). We reanalyzed the data using 100−NPH and Δt_{50}H values based on counts eluted only above 70°C (thus excluding most potential paralogues) and obtained identical topologies for the more terminal branches.

Another implication of the paralogy argument (26) is that the low-temperature peak should limit the range of DNA hybridization comparisons, yet the average of the greatest marsupial–marsupial (100−NPH) distances is only 43.80%, while the average marsupial–*Procyon* distance is 62.51%.

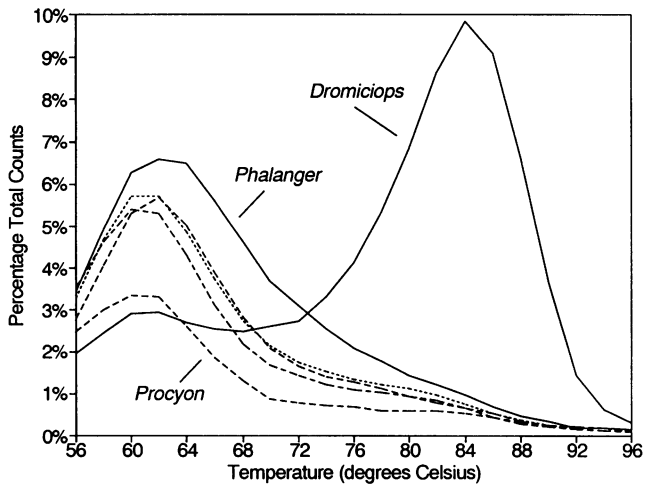

FIG. 2. Hybrids of labeled *D. australis*. Plotted values are counts divided by total (52°C–96°C inclusive) to express percentage hybridization as well as distribution of counts. Homoduplex curve is at right; highest and lowest heteroduplex curves are *P. rufoniger* and *P. lotor*, respectively; *Dasyurus hallucatus*, *Didelphis virginiana*, and *Echymipera clara* are intermediate.

Clearly, the range of the 100−NPH measure is not exceeded by intramarsupial comparisons. Accordingly, the irresolution among the four major marsupial lineages probably indicates that short time intervals were involved in their cladogenesis.

Phylogenetic Implications. If the relationship between *Dromiciops* and diprotodontians is authentic, then *Dromiciops* must be the anatomically plesiomorphic sister group of the monophyletic Diprotodontia, since the latter possess several derived characters absent in *Dromiciops*, such as procumbent incisors, syndactyl pes, and the fasciculus aberrans (27, 28). All hybridizations with labeled diprotodontians do, as predicted, show them to be more similar to each other than any is to *Dromiciops*. For example, the average reciprocal (100−NPH) distance between the rock wallaby and the wombat is 9.75%, and the average from these taxa to *Phalanger* is 11.68%, compared to an average of 20.23% to *Dromiciops*.

It appears, therefore, that Reig *et al.* (8) were mistaken in their interpretation of craniodental anatomy, and that Szalay (4) was correct in associating *Dromiciops* with Australasian marsupials. Notwithstanding his insight, special resemblances in tarsal anatomy between some small diprotodontians and *Dromiciops* noted by Szalay (4), and corresponding similarities in chromosomes and spermatozoa observed by others (5–7), have been discounted as symplesiomorphic retentions from a common australidelphian ancestor (29). These resemblances may well be synapomorphic for *Dromiciops* and Diprotodontia and would repay further study in the context of our DNA hybridization tree. By implication, our data also argue for the convergent evolution of syndactyly in diprotodontian and perameloid marsupials, since they fail to associate representatives of these two groups.

Pattern and Timing of Marsupial Distribution. There now seems little doubt that metatherians originated in Laurasia and spread to Gondwanaland. The presence of primitive didelphimorphs in the Cretaceous of North America, and the dearth of marsupials in the rich faunas of Los Alamitos (Campanian) in Patagonia (30), support the derivation of Tertiary Gondwanan marsupials from northern taxa and their prior absence from southern continents. Yet few would doubt the causal relationship between continental drift and the distribution of lineages of living metatherians. In this connection, special affinities such as that of *Dromiciops* with diprotodontians—anatomically the most derived Australasian marsupials—constitute especially strong support for a southern dispersal route (31), as opposed to multiple invasions from the north (32).

Our best estimate of the overall rate of marsupial DNA evolution, using the calibration procedure of Catzeflis *et al.* (33) applied to kangaroos and bandicoots, is ≈0.5% per million yr (23, 34), placing the *Dromiciops*–Diprotodontia divergence at ≈50 million yr before the present. This date is consistent with the fossils of undoubted microbiotheres (15–19 million yr old), although not with the earlier occurrence of other taxa that have (with less justification, we believe) been assigned to Microbiotheriidae (35). Because recent reconstructions of the history of Gondwanaland suggest that occasional travel among Australia, Antarctica, and South America was possible until at least 38 million yr ago (36, 37), we submit that the *Dromiciops*–Diprotodontia separation represents a late dispersal or vicariant event. Given the sister group relationship of *Dromiciops* plus diprotodontians with dasyurids, dispersal would more likely have been from rather than to Australia (Fig. 3). The sudden appearance in the Oligocene of South America of groeberiids and argyrolagids, if they are indeed marsupials and not peculiar endemic eutherians (38), might also be explained by migration from Australia or Antarctica.

Alternatively, both diprotodontians and dasyurids may have separately emigrated to Australasia, while micro-

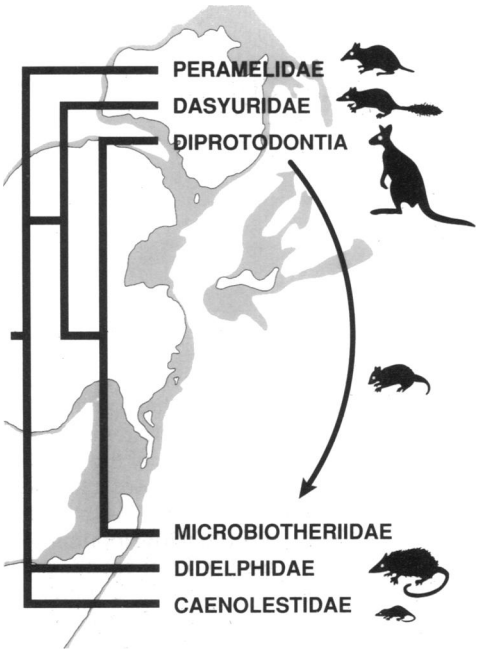

FIG. 3. Phylogeny of marsupials in the context of Gondwanan geography, ≈56 million yr before the present (geographic reconstruction after ref. 36, p. 353). Shading indicates shallow seas surrounding and uniting Australia (at top) with Antarctica and South America. The terminal linkage of Microbiotheriidae with Diprotodontia suggests dispersal of microbiotheres from Australia.

biotheriids remained *in situ*. Since a prediction of this hypothesis is that dasyurid-like marsupials should be found in South America, it may be profitable to reconsider the long-debated relationship between the Australian thylacines and carnivorous South American borhyaenoids (12, 39–41).

Whatever scenario proves correct, the phylogeny presented here clearly requires that the history of Gondwanan marsupials involved more than a single dichotomous sundering of a once-widespread ancestral group. If the dispersal of microbiotheres from Australia could occur so late in geologic time, it seems unlikely that the earlier, still-unresolved, divergences among marsupials were effected by vicariant events alone.

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