

First combined cladistic analysis of marsupial mammal interrelationships

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Abstract

We combine osteological, dental, and soft tissue data with sequences from three nuclear and five mitochondrial genes, sampling all major living clades of marsupials plus several extinct taxa, to perform a simultaneous analysis of marsupial interrelationships. These data were analyzed using direct optimization and sensitivity analysis on a parallel supercomputing cluster, and compared with trees produced with conventional parsimony and likelihood algorithms using a static alignment. A major issue in marsupial phylogeny is the relationships among australidelphians. Optimal analyses using direct optimization and those based on the static alignment support the basal positions of peramelians (bandicoots) and *Dromiciops* ('monito del monte') within Australidelphia, and in all but one case these analyses support a monophyletic Eometatheria, a group consisting of all australidelphians excluding peramelians. *Dromiciops* is basal within Eometatheria in analyses that maximize congruence across partitions, including the equally weighted parameter set. The topologies resulting from direct optimization under all parameter sets show some differences, but all show a high degree of resolution. Direct optimization supports high-level clades supported by analyses of partitioned molecular (e.g., *Notoryctes* as sister group of Dasyuromorphia) and morphological (e.g., Diprotodontia) data.

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1. Introduction

The evolutionary history of metatherian mammals has been independent from that of eutherians for at least 125 million years (Luo et al., 2003). Although marsupials are taxonomically less numerous than placentals, they comprise a diverse radiation and elucidating their phylogeny has been a major challenge. In order to resolve basic questions about biogeography and evolution, systematists have analyzed biochemical data, gene sequences, and morphological characters. Here,

we present a combined analysis of molecular and morphological data, producing one of the most comprehensive tests of marsupial phylogeny to date.

The history of marsupial systematics is complex and many contradictory hypotheses have been formulated. One of the more robust hypotheses concerns the South American 'monito del monte' (*Dromiciops gliroides*) and its position adjacent to Australasian rather than South American marsupials, as recognized in the clade Australidelphia. Since Szalay first proposed this clade in 1982, it has been confirmed by many biochemical, molecular, and morphological studies (Amrine-Madsen et al., 2003; Horovitz and Sánchez-Villagra, 2003; Kirsch et al., 1991, 1997; Luckett, 1994; Luo et al., 2003; Nilsson et al., 2003; Phillips et al., 2001; Springer et al., 1998; Szalay and Sargis, 2001), although some

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separate analyses of sequence data have not recovered this group (Palma and Spotorno, 1999). More recently, controversy has centered around several other issues, including: (1) finding the most basal branch of the marsupial tree, (2) the phylogenetic position of the bandicoots (peramelians), and (3) the position of the ‘monito del monte’ (*Dromiciops*) within the Australasian radiation. Particularly noteworthy is the Eometatheria hypothesis (Kirsch et al., 1997), a clade consisting of *Dromiciops* and Australasian marsupials excluding peramelians.

Support for Eometatheria comes from DNA–DNA hybridization studies (Kirsch et al., 1997) and analyses of mitochondrial rRNA gene sequences (Burk et al., 1999). Based on analyses of five nuclear genes, Amrine-Madsen et al. (2003) expressed uncertainty about the Eometatheria hypothesis, figuring instead trees joining peramelians with other Australasian taxa, with *Dromiciops* at the base of Australidelphia (following Szalay, 1982; but see Szalay, 1994).

In addition to its possible basal position within Australidelphia, *Dromiciops* has been alternatively allied with a group of Australian carnivorous marsupials (dasyuromorphs; Szalay and Sargis, 2001) or diprotodontians (Horovitz and Sánchez-Villagra, 2003; Kirsch et al., 1991). The latter group comprises the most diverse order of marsupials, including koalas, wombats, possums, wallabies, and kangaroos. Molecular (e.g., Springer et al., 1998) and morphological (Horovitz and Sánchez-Villagra, 2003) analyses have produced incongruent results concerning the placement of the marsupial mole, *Notoryctes*, which on morphological grounds has been allied with each of the other Australasian orders (see Horovitz and Sánchez-Villagra, 2003) or with a clade consisting of *Dromiciops* and Diprotodontia (Sánchez-Villagra, 2001). Molecular studies, however, have favored an association of *Notoryctes* with Dasyuromorphia (Amrine-Madsen et al., 2003; Burk et al., 1999; Retief et al., 1995; Springer et al., 1998) and have in addition supported the grouping of Dasyuromorphia, *Notoryctes*, and Peramelia (Amrine-Madsen et al., 2003).

The recent publication of morphological data coded at a low taxonomic level (Horovitz and Sánchez-Villagra, 2003) and the accumulation of gene sequence data (e.g., Burk et al., 1999) permits us in this study to undertake a cladistic analysis of marsupials that incorporates these multiple classes of data simultaneously. In this paper, our main vehicle for analyzing marsupial systematics is based on 230 morphological characters and DNA sequences from three nuclear and five mitochondrial genes, using direct optimization (Wheeler, 1996) and sensitivity analysis (Wheeler, 1995) on a supercomputing cluster (Janies and Wheeler, 2001), following the procedure described in Asher et al. (2003).

2. Methods

2.1. Character and taxon sample

The morphological data consist of 230 characters and 545 character states for 31 terminal taxa, consisting of two monotremes, a prototribosphenidan, four eutherians, and 24 metatherians, among which are six fossil taxa (Horovitz and Sánchez-Villagra, 2003). All orders of modern marsupials are represented in the data set: Didelphimorphia (*Didelphis* and *Monodelphis*), Paucituberculata (*Caenolestes*), Microbiotheria (*Dromiciops*), Peramelia (*Perameles* and *Echymipera*), Dasyuromorphia (*Dasyurus*, *Phascogale*, and *Dasyuroides*), Notoryctemorphia (*Notoryctes*), and Diprotodontia (*Burramys*, *Petaurus*, *Phalanger*, *Pseudocheirops*, *Trichosurus*, *Vombatus*, *Phascolarctos*, *Thylogale*, *Macropus*, *Dendrolagus*, and *Dorcopsis*). In order to maximize representation in our molecular sample (Table 1), six of these taxa are chimaeras, and are referred to in the following discussion based on their compound generic sample. Specifically, we combined sequences to represent the following composite taxa: *Burramys*–*Cercartetus*, *Dasyurus*–*Bradypus*, *Perameles*–*Isoodon*, *Petaurus*–*Dactylopsila*, *Phascogale*–*Antechinus*, and *Pseudocheirops*–*Pseudocheirus*. Morphological data, static alignments, and other supplementary data are available online as [supplementary data](#).

We used three nuclear genes (exon 1 of interphotoreceptor retinoid binding protein or IRBP, partial sequences for phosphoglycerate kinase or PGK-1, and complete sequences for protamine P1) and five mitochondrial genes (12S rRNA, 16S rRNA, tRNA-Val, cytochrome *b*, and NADH dehydrogenase subunit 2), for a total of about 6.9kb (Table 1). Most of these sequence data were generated by Springer et al. (1997, 1998), Jansa and Voss (2000), Colgan (1999), Retief et al. (1995), Burk et al. (1999), Osborne and Christidis (2001), and Osborne et al. (2002). Based on the distribution of conserved regions and leading/trailing gaps across our molecular loci as identified in a preliminary alignment obtained with ClustalX (Thompson et al., 1997), we divided the 8 genes into 18 fragments, the preliminary Clustal alignments for which are available on our [supplementary data website](#).

2.2. Direct optimization

As our primary mode of analysis we use direct optimization (Wheeler, 1996, 1998) and sensitivity analysis (Wheeler, 1995) with the program POY (De Laet and Wheeler, 2003; see ftp.amnh.org/pub/molecular/poy) on a parallel supercomputing cluster (Asher et al., 2003; Janies and Wheeler, 2001). POY optimizes character change directly onto competing topologies, integrating the process of treebuilding with molecular

Table 1

List of GenBank (<http://www.ncbi.nlm.nih.gov>) accession numbers for DNA sequences included in this study

	IRBP	PGK-1	P1	Cyt <i>b</i>	12S rRNA	tRNA-Val	16S rRNA	NADH ₂
<i>Ornithorhynchus</i>			Z26849	X83427	X83427	X83427	X83427	X83427
<i>Tachyglossus</i>			Z26848	AJ303116	AJ303116		AJ303116	AJ303116
<i>Caenolestes</i>	AF025381	AF011240	L35332	AF102816	U61072	U61072	AF102808	
<i>Burramys–Cercartetus</i>		<i>Cercartetus</i> AF011242		<i>Burramys</i> AF206307	<i>Burramys</i> AF108223	<i>Burramys</i> AF108223	<i>Burramys</i> AF108223	<i>Cercartetus</i> AF425978
<i>Dasybus–Bradypus</i>	<i>Bradypus</i> U48708			<i>Dasybus</i> Y11832	<i>Dasybus</i> Y11832	<i>Dasybus</i> Y11832	<i>Dasybus</i> Y11832	<i>Dasybus</i> Y11832
<i>Dasyurus</i>		AF011239	L35341	U07582	AF009890	AF166349	AF166349	
<i>Dasyuroides</i>			AF010271	U07579	AF009888			
<i>Dendrolagus</i>		AF011237	AF187537		AF027990	AF027990	AF027990	
<i>Didelphis</i>	Z11814	AF011232	L17007	Z29573	Z29573	Z29573	Z29573	Z29573
<i>Dorcopsis</i>			AF187540		AF027995	AF027995	AF027995	
<i>Dromiciops</i>	AF025384	AF011238	L35449	AF102815	U61073	U61073	U97341	
<i>Echymipera</i>	AF025383	AF011230		U34682	U97342	U97342	U97342	
		AF044498						
<i>Erinaceus</i>	AF025390			X88898	X88898	X88898	X88898	X88898
<i>Macropus</i>	AJ429135	AF011261	L35447	Y10524	Y10524	Y10524	Y10524	Y10524
<i>Monodelphis</i>	AF257694	AF011260	L35448	U34677	AF166346	AF166346	AF166346	
<i>Notoryctes</i>	AF025385	AF011254	L35446	U87135	U61075	U61075	AF102810	
<i>Perameles–Isoodon</i>		<i>Isoodon</i> AF011227	<i>Perameles</i> L35305	<i>Perameles</i> M99466	<i>Perameles</i> AF166347	<i>Perameles</i> AF166347	<i>Perameles</i> AF166347	<i>Isoodon</i> AF425983
<i>Petaurus–Dactylopsila</i>		<i>Dactylopsila</i> AF011235			<i>Petaurus</i> U21181			<i>Petaurus</i> AF300996
<i>Phalanger</i>		AF011250			AF108222	AF108222	AF108222	AF343887
<i>Phascogale</i>	<i>Phascogale</i> AF025382	<i>Antechinus</i> AF011245	<i>Phascogale</i> L35327	<i>Phascogale</i> M99459	<i>Phascogale</i> U33497	<i>Phascogale</i> AF102809	<i>Phascogale</i> AF102809	<i>Antechinus</i> AF425984
<i>Phascolarctos</i>			U87789	AF166348	U61076	U61076	AF166344	AF425985
<i>Pseudochirops–Pseudocheirus</i>	<i>Pchirops.</i> AF025387	<i>Pchirops.</i> AF011252	<i>Pchirops.</i> L35334	<i>Pchirops.</i> AF102813	<i>Pchirops.</i> AF102812	<i>Pchirops.</i> AF102812	<i>Pchirops.</i> AF102812	<i>Pcheirus.</i> AF300998
<i>Thylogale</i>		AF011266	AF187534	AY099277	AF027991	AF027991	AF027991	
<i>Trichosurus</i>			L32744	AF357238	AF357238	AF357238	AF357238	AF357238
<i>Vombatus</i>	AF025386			NC_003322	NC_003322	NC_003322	NC_003322	AF343893

Hyphenated taxa (e.g., *Burramys–Cercartetus*) were chimaeras treated as single terminals in our analyses.

homology determination, using parsimony as the optimality criterion. We followed Giribet et al. (2001) in choosing a parameter space of 12 distinct phylogenetic analyses, consisting of 4 different weights (1:1, 2:1, 4:1, and 1:0) of transversions (TV) to transitions (TS), and 3 weights (1:1, 2:1, and 4:1) of morphological characters to TV. Gaps were weighted equally with morphological characters. Following Asher et al. (2003), we used the incongruence length difference (ILD) and a topological index to measure congruence and choose individual weighting sets within our sensitivity analysis as optimal (see supplementary data). Our search scripts in POY consisted of two passes, as follows:

Firstpass: poy -parallel -replicates 100 -seed 1 -nospr -tbr -repintermediate -terminalfile [taxaonlist] -fuselimit 10000 -fusemingroup 1 -fusemaxtrees 1000 -norandomizeoutgroup -weight [from costmatrix] marsup.ss [morphology and 18 sequence files] -molecularmatrix [from costmatrix] -time -printtree -repintermediate > [outfile] 2 > [logfile]

Secondpass: poy -random 0 -topofile [treefile from firstpass] -terminalfile [taxonlist] -ratchettbr 10 -ratchetpercent 15 -ratchettrees 2 -slop 5 -checkslop 10 -exact -maxtrees 2 -impliedalignment -norandomizeoutgroup -time -weight [from costmatrix] [morphology and 18 sequence files] -molecularmatrix [from costmatrix] -impliedalignment -phastwincladfile [phastfile] -printtree -repintermediate -plotfile [ascii-treefile] -poystriictconsensuscharfile [consensusfile] > [outfile] 2 > [logfile].

We calculate branch support indices for optimal analyses by exporting the static, implied alignments consistent with the direct optimization output by POY into PAUP v. 4.0b10 (Swofford, 2002), as described in Asher et al. (2003).

2.3. Static alignment

As a heuristic tool, and to facilitate comparisons with previous molecular publications, we also examined a statically aligned version of this dataset using parsimony and likelihood tree building algorithms. Each of

the eight genes was aligned using ClustalX (Thompson et al., 1997) with refinements based on recent literature. Specifically, the 12S rRNA alignment contained 861 positions, excluding the nine regions identified as alignment ambiguous by Springer and Douzery (1996) and an approximately eight-nucleotide region in the downstream half of stem 36 (following the terminology of Springer and Douzery, 1996). The 16S rRNA alignment contained a total of 1312 positions, excluding 19 regions identified as alignment ambiguous in the supplementary data of Murphy et al. (2001). The 72-position tRNA valine alignment output by ClustalX was not changed, nor was the cytochrome *b* alignment of 1146 bases. The NADH₂ alignment contained 991 positions and a few internal gaps, manually adjusted to preserve reading frames. In addition, approximately 50 bases near the 5' end that could not be unambiguously aligned were deleted. For the 1254 bp IRBP alignment, we followed Springer et al. (1997, Fig. 1) in placing indels in *Notoryctes*, and made other minor adjustments in the placement of indels to preserve reading frames. The 313 bp alignment of PGK1 was based on Table 2 of Colgan (1999). The 887 bp alignment of protamine P1 was based on an amino-acid alignment of Retief et al. (1995, Fig. 1) and included a ca. 250 bp non-coding region identified by Queralt et al. (1995, Fig. 1) within the amino-acid alignment figured by Retief et al.

In our parsimony and likelihood analyses of the statically aligned data for living taxa, polymorphisms were treated as such and gap characters were treated as missing data. Using PAUP 4.0b10 (Swofford, 2002), our parsimony searches were undertaken with all character changes weighted equally, and again ignoring third position transitions, on a taxon sample first including all living taxa and then on a sample excluding the seven taxa with 49% or more missing data (*Burramys–Cercartetus*, *Dasyuroides*, *Dendrolagus*, *Dorcopsis*, *Petaurus–Dactylopsila*, *Phalanger*, and *Thylogale*). Nodes were collapsed if any optimization yielded a zero-length branch (PAUP option 'amb-'). Shortest trees were identified using a 100-replicate heuristic search with a random addition sequence and TBR branch swapping.

Most of our likelihood searches excluded the seven taxa with 49% or more missing data, and used six different models of nucleotide substitution, descriptions of which can be found in Swofford et al. (1996). In order of increasing complexity (with the acronym and settings used in PAUP 4.0b10 in parentheses), these were Jukes–Cantor (JC69), Kimura-Two-Parameter (K80), Felsenstein-1981 (F81), Felsenstein-1984 (F84), Hasegawa–Kishino–Yano-1985 (HKY85), and General-Time-Reversible (GTR). In addition to running each of these models assuming no rate variation across sites, we also analyzed the reduced-taxon static alignment using the

HKY85 model allowing for variable rates across codon positions (HKY85+SS), allowing for variable rates based on a gamma distribution (HKY85+G), and using the GTR model with a gamma distribution for variable rates and an estimated proportion of invariant sites (GTR+G+I). We also analyzed the static alignment including all 25 living taxa using the HKY85 model assuming no rate variation across sites. Details on the options used in PAUP to run each of these models are described in our supplementary data. One-tailed Kishino–Hasegawa (KH) and Shimodaira–Hasegawa (SH) tests of optimal trees vs. alternatives used HKY85, GTR, HKY85+G, and GTR+G+I models based on 1000 RELL bootstrap replicates. Bootstrap values reported here for optimal topologies were obtained for the reduced-taxon dataset with 100 replicates of a 10-replicate random addition sequence using parsimony, and 78 replicates using the HKY85 likelihood model.

To examine the extent to which partitions supported conflicting signals, each gene and taxon subset represented by that gene was analyzed separately. With all character changes weighted equally under parsimony, bootstrap values were generated with 100 replicates of a simple addition sequence to determine if any of the partitions supported mutually exclusive clades with high support (DeQueiroz, 1993).

3. Results

Using direct optimization, considerable resolution is apparent across the different parameter sets concerning relationships among the major marsupial clades (Fig. 1). At the base of the marsupial tree are the didelphimorphians, followed by a group composed of *Caenolestes* and australidelphians. None of the parameter sets supported the monophyly of a South American clade including didelphimorphians and *Caenolestes* in 'Ameridelphia,' nor did any of the separate analyses of genetic (see supplementary data) or morphological (Horovitz and Sánchez-Villagra, 2003) data. The monophyly of Australidelphia, on the other hand, is supported by all parameter sets. Furthermore, peramelians are basal within Australidelphia throughout all direct optimization analyses, giving Eometatheria unanimous support. In optimal analyses (Fig. 1), *Dromiciops* occupies the basal-most branch within Eometatheria.

The ILD and topological indices favor different parameter sets as optimal (Fig. 1). The ILD index favors the equally weighted analysis, while the topological congruence index favors the topology resulting from weighting morphology x4, gaps x4, TV x4, and transitions x1 (abbreviated here and throughout as 4-4-1, with gaps and morphology weighted equally). These two

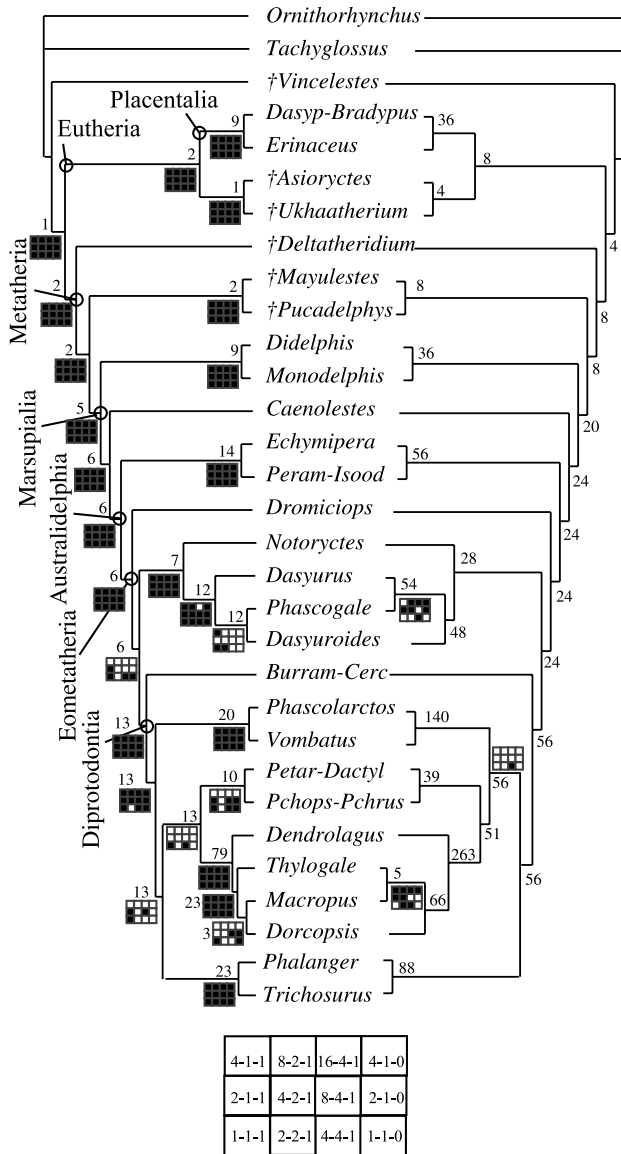


Fig. 1. Direct optimization analyses. At left is the single best topology (14,963 steps) produced by the equally weighted, combined-data analysis, favored by the ILD index. At right is the single best topology (39,287 steps) favored by the topological congruence index (morphology x4, TV x4, gaps x4, TS x1, or 4-4-1). Numbers represent branch supports calculated by exporting the static, implied alignments consistent with the direct optimization output by POY into PAUP, keeping the weights used in each analysis, and searching for the shortest trees constrained to disagree with clades present in the optimal tree. Boxes adjacent to nodes represent the occurrence of the respective clade across the 12 direct optimization analyses, as indicated in the summary below the topologies. Crosses indicate extinct taxa.

topologies are very similar, showing conflicting groups only within dasyuromorphs and diprotodontians (Fig. 1). *Dromiciops* is basal within Eometatheria in both trees. Another consistent result is that *Notoryctes* appears with Dasyuromorphia and this clade is in turn the sister group of Diprotodontia. The monophyly of Diprotodontia receives unanimous support.

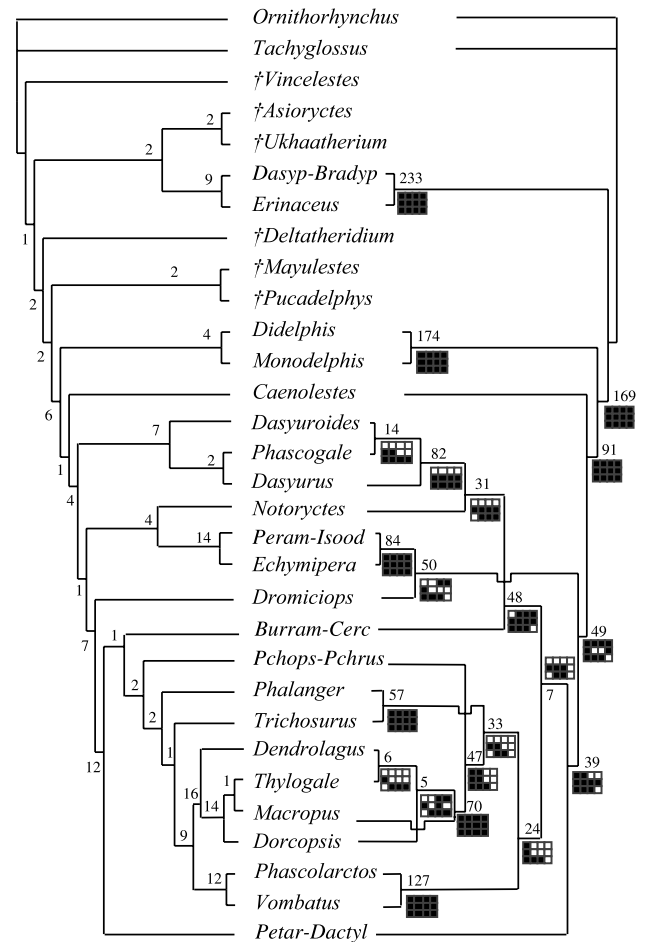


Fig. 2. Partitioned analyses. At left is the topology of the single best morphological tree (895 steps as reconstructed in NONA and POY, all changes equally weighted). At right is the single best tree favored by both ILD and topological indices of the molecular data (3 nuclear and 5 mitochondrial genes). Although these indices favored different parameter sets, both produced the same topology (21,269 steps, set 2-2-2-1; 35,478 steps, set 4-4-1). Branch supports are based on set 2-2-1, as described in Fig. 1. Notation is as in Fig. 1.

Fig. 2 shows the results of separate parsimony analyses of the morphological data and direct optimization applied to the molecular data. The molecular tree in Fig. 2 was produced by parameter sets that maximized agreement across the molecular partitions. Morphological and molecular analyses placed didelphimorphians at the base of the marsupial clade, followed by a clade composed of *Caenolestes* as sister group to Australidelphia. In these topologies, Ameridelphia is paraphyletic and Australidelphia monophyletic. Both analyses confirm the monophyly of all marsupial orders, except for that of Diprotodontia in the case of the molecular data, since *Burramys–Cercartetus* is placed closer to dasyuromorphs in the topologies favored both by ILD and topological indices (Fig. 2). While the morphological analysis places *Dromiciops* as sister group to diprotodontians, direct optimization applied to the molecular

data alone places it as sister group to peramelians. Other discrepancies between these analyses concern the placement of *Notoryctes*, which appears grouped with peramelians in the morphological tree but with dasyuromorphs according to molecular data.

Trees obtained via parsimony from the statically aligned molecular dataset (supplementary data) are similar to the trees obtained from direct optimization applied to the combined data, with one exception: when third position transitions of coding genes are not down-weighted, the chimaeric taxon *Petaurus–Dactylopsila* falls outside of Diprotodontia to become the most basal member of Eometatheria. This chimaeric taxon is known for only three of the eight genes (PGK1, 12S rRNA, and NADH₂), and with over 75% missing data is the least complete of all taxa in this study. This result is similar to the placement by direct optimization applied to the molecular data alone of another composite taxon, *Burramys–Cercartetus*, which has nearly 67% missing data (Fig. 2). Importantly, both taxa were reconstructed within Diprotodontia by direct optimization applied to the combined dataset. Other differences between the statically aligned dataset analyzed with parsimony and direct optimization include different relationships within dasyuromorphs and diprotodontians.

Among the statically aligned genetic and morphological partitions analyzed separately with parsimony, none supported mutually exclusive clades with bootstrap values exceeding 90%. Following DeQueiroz (1993), this result is consistent with the combination of these partitions into a single, simultaneous analysis. However, we note that valid topological results are often supported by combination of partitions deemed ‘incompatible’ by this and other criteria (Gatesy et al., 1999).

The trees generated by the application of both parsimony and likelihood models to the statically aligned, reduced-taxon dataset, including those that account for rate heterogeneity and the HKY85 model applied to all 25 living taxa, were nearly identical (Fig. 3). All supported a paraphyletic Ameridelphia, and reconstructed Australidelphia with *Dromiciops* and peramelians basal. The only differences among these topologies concerned relationships within diprotodontians and a potential *Dromiciops*–peramelian clade basal within Australidelphia, the latter supported uniquely by GTR + G + I. The analysis including all living taxa under the HKY85 model also supported Eometatheria with *Dromiciops* basal; but differed from the optimal, combined direct optimization trees regarding relationships within Diprotodontia (Fig. 3).

Statistical comparisons between optimal topologies discussed here and alternatives such as a monophyletic South American marsupial clade (Marshall et al., 1990), a position for *Dromiciops* more basal than that of peramelians (Szalay, 1982), or a dasyuromorph–

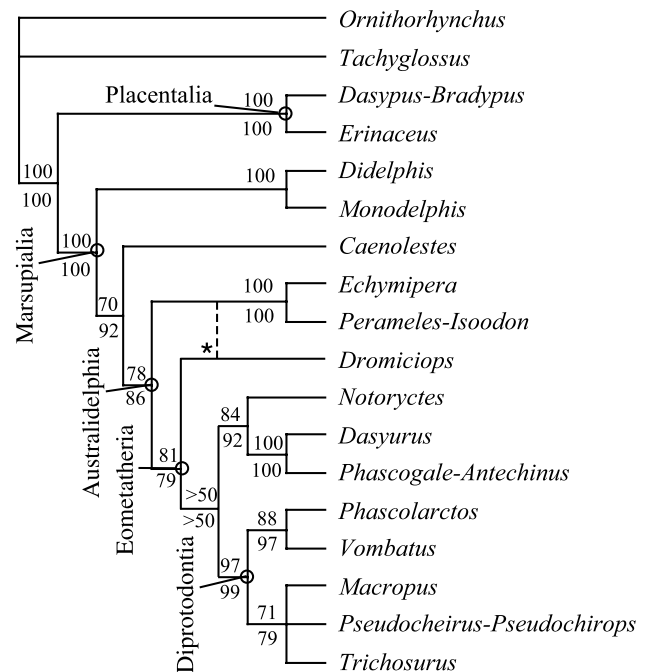


Fig. 3. Consensus tree from nine likelihood and two parsimony analyses of the static alignment based on molecular data alone, using taxa at least 49% complete. Numbers above nodes indicate bootstrap support derived from 100 replicates of a 10-rep random addition sequence based on equally weighted parsimony; numbers below nodes represent bootstrap support based on 78 replicates from the HKY85 model, starting trees obtained from neighbor joining. Hyphenated taxa represent chimaeric terminals, as shown in supplementary data. The asterisk indicates the different placement by GTR + G + I of *Dromiciops* in a clade with peramelians. All other static analyses placed peramelians as the first australidelphian clade, followed by *Dromiciops*. Parsimony and HKY85 + ss differed from the remaining analyses by placing *Trichosurus* and *Pseudocheirus–Pseudocheirus* together; the others placed the latter with *Macropus*.

Notoryctes–peramelian clade (Amrine-Madsen et al., 2003) are suspect for several reasons, such as the exclusion of nucleotide, indel, and morphological data relative to our direct optimization analyses, and due to the fact that our statically aligned sequence dataset is by itself not an adequate exploration of alternative hypotheses of molecular homology (Asher, 2003). Nevertheless, in Table 2 we present results of such tests in the hope that they will provide some indication as to the regions of the marsupial tree that might change with future improvements to taxon and character samples.

Specifically, using the KH test on the reduced extant taxon sample with HKY85, HKY85 + G, GTR, and GTR + G + I models, the hypothesis of Ameridelphian monophyly was significantly rejected ($P < 0.05$) in favor of the topology shown in Figs. 1 and 3 (Table 2). The hypothesis of a monophyletic ‘syndactyl’ clade (i.e., peramelians plus diprotodontians) was rejected under three of the four models (excluding GTR + G + I); and a peramelian–dasyuromorph–*Notoryctes* (PDN) clade, as well as a clade containing *Dromiciops* basal within

Table 2

Results of Kishino–Hasegawa (KH) and Shimodaira–Hasegawa (SH) one-tailed ‘topology tests’ using 1000 RELL bootstrap replicates, based on static alignment comparing optimal topologies (Fig. 3) with alternatives, as follows: ‘Drom–Peram’ represents *Dromiciops*–peramelian clade at base of Australidelphia (cf. Nilsson et al., 2003); ‘*Dromiciops* basal’ postulates *Dromiciops* basal within Australidelphia (cf. Szalay, 1982); ‘PDN’ postulates a perameline–dasyuromorph–*Notoryctes* clade and *Dromiciops* basal within Australidelphia (cf. Amrine-Madsen et al., 2003, Figs. 1 and 2); ‘Ameridelphia’ represents monophyly of didelphids plus *Caenolestes*; ‘syndactyl’ represents a peramelian–diprotodontian clade; and ‘Eometatheria’ represents monophyletic Australidelphia with peramelians basal Fig. 1

	–lnL	Δ–lnL	KH test P	SH test P	Model
Drom–Peram	54195.61841	15.58152	0.192	0.505	HKY85
<i>Dromiciops</i> basal	54221.09831	41.06142	0.002*	0.038*	HKY85
PDN	54241.94570	61.90881	0.003*	0.030*	HKY85
Ameridelphia	54286.04889	106.01200	0.000*	0.000*	HKY85
Syndactyl	54251.34265	71.30576	0.000*	0.009*	HKY85
Eometatheria	54180.03689	0	—	—	HKY85
Drom–Peram	50139.72014	0.37505	0.465	0.787	HKY85+G
<i>Dromiciops</i> basal	50145.87993	6.53484	0.131	0.515	HKY85+G
PDN	50152.56935	13.22426	0.061	0.198	HKY85+G
Ameridelphia	50166.30411	26.95902	0.014*	0.027*	HKY85+G
Syndactyl	50153.52842	14.18333	0.038*	0.160	HKY85+G
Eometatheria	50139.34509	0	—	—	HKY85+G
Drom–Peram	53415.34956	8.68253	0.299	0.650	GTR
<i>Dromiciops</i> basal	53440.64536	33.97832	0.004*	0.069	GTR
PDN	53457.72865	51.06162	0.005*	0.029*	GTR
Ameridelphia	53509.99883	103.33180	0.000*	0.000*	GTR
Syndactyl	53466.42263	59.75560	0.000*	0.009*	GTR
Eometatheria	53406.66703	0	—	—	GTR
Drom–Peram	49573.19042	0	—	—	GTR+G+I
<i>Dromiciops</i> basal	49580.54235	7.35193	0.116	0.433	GTR+G+I
PDN	49585.43419	12.24377	0.074	0.221	GTR+G+I
Ameridelphia	49603.83105	30.64063	0.007*	0.013*	GTR+G+I
Syndactyl	49586.62510	13.43468	0.052	0.171	GTR+G+I
Eometatheria	49575.35819	2.16777	0.381	0.706	GTR+G+I

‘–lnL’ indicates log likelihood score; ‘Δ–lnL’ indicates difference in lnL between alternative and optimal tree (a value of “0” indicates optimal topology for given model). Asterisks indicate tree with a significant difference in log likelihood at alpha 0.05. Models are identified in the text and in our supplementary data file (<http://141.20.245.230:55080/Robert.Asher/marsupMS>).

Australidelphia, were also significantly rejected using the HKY85 and GTR models, but not when rate variation was introduced using the HKY85+G and GTR+G+I models. The hypothesis of a *Dromiciops*–peramelian clade basal within Australidelphia could not be rejected by any of the models; and in fact using GTR+G+I it received a slightly higher likelihood score (without significance) than the otherwise optimal Eometatheria hypothesis, favored by direct optimization. The Shimodaira–Hasegawa test performed similarly, but was more conservative; it did not reject *Dromiciops*–basal under GTR or the syndactyl clade under HKY85+G (Table 2).

We use composite taxa because they allow us to minimize missing data in our dataset and thereby potentially help speed up analyses. However, Malia et al. (2003) have pointed out that in some cases such composite taxa can lead to misleading results. To examine this possibility we ran our statically aligned dataset twice under parsimony excluding all five of our “chimaeras,” one run with all non-composite taxa and one run excluding taxa with 49% or greater missing data. These analyses recovered the same topology as that shown in Fig. 1, support-

ing Australidelphia, Eometatheria with *Dromiciops* basal, and *Notoryctes* plus dasyuromorphs. “Ameridelphia” is still paraphyletic, with didelphids and *Caenolestes* comprising the first two branches within Marsupialia.

4. Discussion

Our analysis of marsupial higher-level relationships produces highly resolved and congruent results across a wide variety of methods. This resolution among the major australidelphian clades has implications for our understanding of character evolution and biogeography in the group, as discussed below.

Although the trees produced by the static alignment provide for several interesting heuristic comparisons, we regard those produced by direct optimization as preferable for several reasons. First, direct optimization is based on more data, as ‘alignment ambiguous’ DNA segments need not be excluded from the analysis, and morphological data, extinct taxa, and indels contribute directly to tree building. Second, indel and morphologi-

cal characters contribute to tree building not only by virtue of their own information content, but also indirectly by influencing the construction of hypotheses of molecular homology (Wheeler, 1998). Direct optimization more thoroughly explores ambiguities of molecular homology, considering millions of alternative alignments simultaneously with the topologies they imply. For these reasons, the discussion below focuses on the topologies favored by direct optimization, shown in Fig. 1.

4.1. Topology of the marsupial tree and comparison with previous studies

Concerning relationships among groups within Australidelphia, topologies resulting from direct optimization of the combined data show some differences from those resulting from partitioned analyses using direct optimization, but all show a high degree of resolution. The combined result confirms some relationships obtained by the separate morphological analysis (e.g., Diprotodontia), in other cases it confirms groups recovered by the molecular data analyzed alone (*Notoryctes* plus Dasyuromorphia), yet in other cases it presents new relationships not recovered by partitioned optimization analyses, such as the position of *Dromiciops*.

The ILD index applied to the combined data favors the equally weighted analysis, the parameter set that some authors consider the hypothesis of highest explanatory power (e.g., Giannini and Simmons, 2003; Grant and Kluge, 2003). Although the ILD and topological indices favor different optima, as in previous empirical examples (Asher et al., 2003; Wheeler, 1995) they show a similar pattern of identifying congruence across the parameter set examined in this paper, with parameter sets that ignore transitions showing least inter-partition congruence (see supplementary data).

The combined analyses support the classic view that didelphids are the most basal group of living marsupials, and is against recent proposals that have placed either peramelians (Kirsch et al., 1997; DNA–DNA hybridization data), or dasyuromorphs and *Notoryctes* (Springer et al., 1997; parsimony analysis of morphological data) as basal, or that have presented this issue as unresolved (Burk et al., 1999). The placement of *Caenolestes* as the sister group of an Australasian marsupial clade is supported by several skeletal characters (Horovitz and Sánchez-Villagra, 2003) and also receives support from the combined analyses. As in previous analyses of gene sequences (e.g., Nilsson et al., 2003), the association of *Caenolestes* and didelphimorphians (Ameridelphia) is rejected. This implies that sperm pairing, a character present in both groups, is either primitive for both and was lost among australidelphians, or evolved independently in the two South American groups (Temple-Smith, 1987).

Our combined-data analysis contrasts with the classic notion of dasyuromorphs as ancestral within the Australasian radiation (Bensley, 1903; Ride, 1964) also obtained in the parsimony analysis of morphological data by Horovitz and Sánchez-Villagra (2003). Eometatheria (Kirsch et al., 1997) receives support from all parameter sets across the combined analyses using direct optimization and analyses of the statically aligned molecular dataset (except for GTR+G+I), implying that peramelians were the first offshoot of the Australidelphia, in agreement with some previous sequence analyses (Burk et al., 1999).

The combined analyses favored by the ILD and topological indices (Fig. 1) agree with each other and all trees produced by the static alignment in favoring basal positions of peramelians and *Dromiciops* within Australidelphia. The combined analyses under other parameter sets, however, favor the position of *Dromiciops* in various other parts of the australidelphian clade. In contrast with the optimal combined analyses, the parsimony analysis of morphological data by Horovitz and Sánchez-Villagra (2003) resulted in strong support for the association of *Dromiciops* and diprotodontians. A basal position for *Dromiciops* within Eometatheria does not lack morphological support, however, as discussed below.

The combined analyses support most previous molecular studies concerning the placement of *Notoryctes* as sister group of Dasyuromorphia (Amrine-Madsen et al., 2003; Burk et al., 1999; Springer et al., 1998). Several morphological characters support this association, although this is not the most parsimonious position for *Notoryctes* based on morphology alone (Horovitz and Sánchez-Villagra, 2003).

The present analysis included only a portion of the diprotodontian radiation, with several clades missing. This caveat aside, we recovered strong support for monophyletic macropodids and for a wombat–koala clade (Aplin and Archer, 1987; Burk et al., 1999; Kirsch et al., 1997). All combined topologies rejected the monophyly of the possums (i.e., *Burramys–Cercartetus*, *Phalanger*, *Trichosurus*, *Petaurus–Dactylopsila*, and *Pseudocheirops–Pseudocheirus*), as suggested by Archer (1984; see also Osborne et al., 2002). Among these genera, only two pairs of taxa frequently comprised clades: *Phalanger* with *Trichosurus* (in the Phalangeridae) and *Petaurus–Dactylopsila* with *Pseudocheirops–Pseudocheirus* (see Fig. 1), both of which have been previously recognized (e.g., Kirsch et al., 1997).

4.2. Character evolution and biogeography

The topology of the combined analysis has implications for the reconstruction of the morphological and developmental evolution of marsupials, as well as that of their biogeographic history. Morphological changes

along the branches of the tree are shown in [supplementary data](#).

Although the most parsimonious solution according to morphological data alone would place *Notoryctes* with Peramelia and this clade nested above Dasyuromorphia, the alternative topology (*Notoryctes* as the sister group of dasyuromorphs as supported by the combined data) is supported by some morphological characters as well: they share the presence of a medial plantar tuberosity in the astragalus that protrudes proximally (character 98, which also appeared independently in a few diprotodontians), the transformation of upper incisors from spatulate to non-spatulate (character 165, independently evolved in the wombat–koala clade), the loss of the minor palatine foramen (character 204, polymorphic in *Dasyurus*), and the loss of a cecum (character 229).

Eometatheria is supported by a connection between the sustentacular and ectal facets of the calcaneum (character 123, reversed among some macropodids) and presence of a wide incisura tympanica (character 193, reversed in dasyuromorphs). The presence of a continuous lower ankle joint supporting an australidelphian clade was advanced by Szalay (1982), who placed *Dromiciops* at the base of that radiation. In the peramelians both facets of the ankle joint are linked only by a narrow area, representing an intermediate condition between the separated facets that characterize *Didelphis*, *Monodelphis*, and *Caenolestes* and the continuous facets of eometatherians (Hershkovitz, 1992; Horovitz and Sánchez-Villagra, 2003).

Syndactyly is a condition shared by peramelians and diprotodontians, in which the second and third digits of the pes are often reduced and always joined together by a common integument. The phylogenetic position of these groups indicates that this feature evolved independently in each group. Syndactyly can be attained during development by lack of interdigital cell death (Tickle, 2002), which is under simple mutagenic control (Hall, 1987; Muragaki et al., 1996).

Variation in the degree of altriciality at birth in marsupials has been recognized for some time (Hughes and Hall, 1988), and the topology of the combined tree implies that there has been plasticity within the australidelphian radiation in this and other aspects of reproductive biology and ontogeny. Although data about body weight at birth are missing for many taxa, one could infer from the information available that from the condition present in didelphimorphians and peramelians (and probably in the common ancestor of australidelphians) where the young are large (at least 100mg), dasyuromorphs and *Cercartetus* have evolved a strategy in which newborns are the smallest and least developed at birth among marsupials (30mg or less). The macropodids, wombats, and koalas, among other diprotodontians, have relatively large

young (Cockburn, 1989). In addition, the grades of organization in the eyes and ears at birth vary as well, with didelphimorphians, peramelians, and phalangerids intermediate between dasyuromorphs and macropodids in degree of development of these morphological features at birth. There is no consistent gradient in neonatal size variation and degree of development along the internested nodes of the phylogeny depicted in Fig. 1, suggesting that the traditional view that large size at birth is a derived character is not necessarily true for all clades of marsupials (Cockburn, 1989).

Peramelians are unique among marsupials in having a chorioallantoic placenta, in contrast with the more typical choriovitelline placenta. Peramelians are similar to living eutherians in this reproductive trait (Tyndale-Biscoe and Renfree, 1987). The taxonomic diversity of eometatherians (183 spp., Wilson and Reeder, 1993) is larger than that of their sister group the peramelians (21 spp.). If one were to judge the evolutionary success of either kind of placentation based on the species richness of their constituent groups, then one would infer that among marsupials, the choriovitelline placenta has been more successful. This indicates that the typical reproductive features of marsupials are not simply primitive and/or maladaptive when compared to those of placentals, but rather a successful alternative strategy to that of their sister placental taxa (Kirsch, 1977; Renfree, 1993).

The basal position of didelphimorphians in the marsupial tree is congruent with the hypothesis that early marsupials, or their taxonomically more basal metatherian ancestors, first invaded South America, not Australia, from a North American source. There are two possible scenarios for the biogeographic history of marsupials in the southern continents suggested by the optimal topologies resulting from the combined analyses. Nesting of *Dromiciops* within Australidelphia implies either back-migration from Australia to South America or multiple dispersals into Australia (Kirsch et al., 1991; contra Szalay and Sargis, 2001). Putative microbiotheres from Eocene deposits in Australia and Antarctica could be interpreted as potential evidence in support of the back-migration to South America hypothesis (Springer et al., 1998), but these fossils are very fragmentary and their taxonomic assignment only preliminary. To further complete the picture of past biogeographic distributions of marsupial groups across the globe, several unstudied or poorly known fossil taxa will have to be incorporated in the analysis, including for example *Yalkaparidon*, groeberids, caroloameghiniids, polydolopiforms, Itaborian metatherians (Szalay and Sargis, 2001), and enigmatic forms from the Early Eocene of Australia such as *Djardulia murgonensis* (Godthelp et al., 1999).

5. Conclusions

Methodological critiques of parsimony (e.g., Swoford et al., 2001) and likelihood (e.g., Goloboff, 2003) have received considerable attention for some time. More recently critiques of sensitivity analysis (Grant and Kluge, 2003) and direct optimization (Simmons, 2004) have also been made. However, in this study, disparate phylogenetic methods converge on a very similar topology, with the caveat that KH and SH tests based on likelihood analyses of the static alignment do not significantly reject some alternative topologies (Table 2), and that the positions of *Dromiciops* and peramelians are slightly different in one of the likelihood models. Nevertheless, all likelihood analyses agree with direct optimization and parsimony in reconstructing both taxa as basal within Australidelphia. Optimal topologies identified here from all of these methods, including data-rich direct optimization and likelihood models incorporating estimates of rate heterogeneity, support the same basic groupings within marsupials (Fig. 1), such as the monophyly of Australidelphia, the paraphyly of Ameridelphia, the basal positions of peramelians and *Dromiciops* within Australidelphia, and a close relationship between *Notoryctes* and dasyuromorphs. This highly congruent signal provides a solid basis upon which to better understand character evolution, adaptation, and biodiversity among marsupial mammals.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ympcv.2004.05.004](https://doi.org/10.1016/j.ympcv.2004.05.004).

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