

## Phylogenetic Studies on Didelphid Marsupials I. Introduction and Preliminary Results from Nuclear IRBP Gene Sequences

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We report and analyze nucleotide sequence variation in the first exon (1158 bp) of the nuclear gene encoding the Interphotoreceptor Retinoid Binding Protein (IRBP) among 21 species representing all 15 currently recognized genera of living didelphids. Six previously published IRBP sequences representing five nondidelphimorph marsupial orders were also analyzed to test didelphid monophyly, and 12 published sequences representing ten placental orders were used as outgroups. No gaps (indels) are necessary to align didelphid sequences, but one short region (35 bp) is alignment-ambiguous among nondidelphids. Uncorrected pairwise sequence divergence ranges from 0.7 to 5.7% among nonconspecific didelphids, from 9.2 to 15.3% between didelphids and nondidelphid marsupials, and from 24.9 to 32.1% between marsupials and placentals. Neither transitions nor transversions exhibit saturation for any codon position at any level of taxonomic comparison. Parsimony analyses of these data provide strong support (bootstrap values >95%, Bremer values  $\geq 7$ ) for the monophyly of (1) Didelphidae ("caluromyines" + Didelphinae); (2) a group containing *Caluromys* and *Caluromysiops*; (3) Didelphinae; (4) a group of large opossums that includes *Metachirus*; (5) a group containing the remaining large opossums (with  $2N = 22$  chromosomes); (6) a group containing *Marmosa* and *Micoureus*; (7) a group containing *Thylamys*, *Lestodelphys*, and *Gracilinanus*; and (8) a group containing the last three genera plus a monophyletic *Marmosops*. In addition, we found moderate support (bootstrap values >80%, Bremer values  $\geq 2$ ) for the monophyly of *Thylamys* + *Lestodelphys* and for a sister-group relationship between *Monodelphis* and *Marmosa* + *Micoureus*. Sensitivity analysis suggests that all of these clades, together with their associated levels of bootstrap and Bremer support, are robust to alternative hypotheses of positional homology within the ambiguously alignable region. Although some of the relationships supported by IRBP are not consistent with the results of published morphological analyses, our reassessment of the morphological data suggests that many conflicts are more apparent than real.

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**KEY WORDS:** marsupials; IRBP; Didelphidae; phylogeny; mammalian systematics.

### INTRODUCTION

Despite impressive advances in systematic research on Recent and fossil metatherians over the last several decades (reviewed by Aplin and Archer, 1987; Marshall *et al.*, 1990; Springer *et al.*, 1997b), many aspects of marsupial phylogeny remain problematic.

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**Table I.** Composition of the Didelphid Crown Group<sup>a</sup>


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Subfamily Caluromyinae
<i>Caluromys</i> (3 spp.)
<i>Caluromysiops</i> (1 sp.)
<i>Glironia</i> (1 sp.)
Subfamily Didelphinae
<i>Chironectes</i> (1 sp.)
<i>Didelphis</i> (4 spp.)
<i>Gracilinanus</i> (9 spp.) <sup>b</sup>
<i>Lestodelphys</i> (1 sp.)
<i>Lutreolina</i> (1 sp.)
<i>Marmosa</i> (9 spp.)
<i>Marmosops</i> (11 spp.) <sup>c</sup>
<i>Metachirus</i> (1 sp.)
<i>Micoureus</i> (4 spp.)
<i>Monodelphis</i> (15 spp.)
<i>Philander</i> (4 spp.) <sup>d</sup>
<i>Thylamys</i> (5 spp.)

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<sup>a</sup>Species counts and subfamilial contents after Gardner (1993) except as noted. Alternative recent classifications (e.g., Hershkovitz, 1992; Kirsch and Palma, 1995) allocate these genera to two or more families, but we prefer to retain Didelphidae in its traditional neontological sense, regarding issues of rank as biologically insubstantial.

<sup>b</sup>Species count after Hershkovitz (1992).

<sup>c</sup>Mustrangi and Patton (1997) showed that *Marmosops paulensis* is a species distinct from *M. incanus*; Patton *et al.* (2000) recognized *M. neblina* as a separate species from *M. impavidus*.

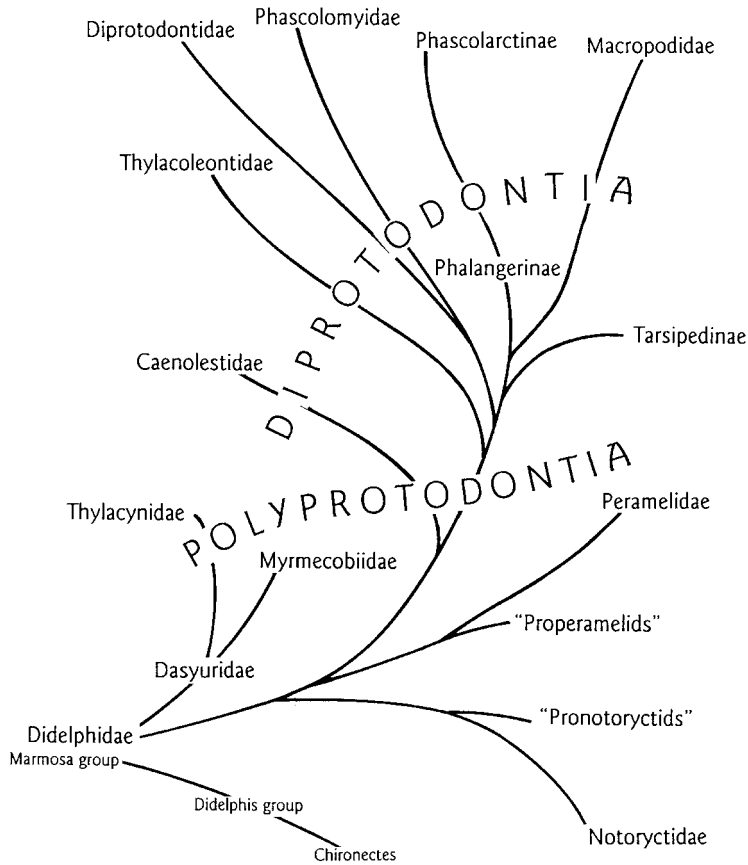
<sup>d</sup>Patton and da Silva (1997) recognized *Philander mcilhennyi* as a species distinct from *P. andersoni*, and *P. frenata* as a species distinct from *P. opossum*.

For students of the New World fauna, the most conspicuous of such unresolved issues concern the diverse assemblage of polyprotodont taxa traditionally referred to the family Didelphidae. Geographically distributed from Patagonia to Canada, the didelphid crown group includes 70 currently recognized species allocated to 15 genera in two subfamilies (Table I). Long neglected except by taxonomists and embryologists, living didelphids are increasingly attracting the attention of comparative researchers in many biological disciplines (e.g., McNab, 1978; Temple-Smith and Bedford, 1980; Charles-Dominique, 1983; Grand, 1983; Atramentowicz, 1986; Harder, 1992; Moore, 1996; Lemelin, 1999) with the result that much new information is accumulating on taxonomic variation in anatomical, behavioral, ecological, and physiological characters.

Unfortunately, no well-corroborated phylogenetic framework is currently available to confidently assess the evolutionary significance of character variation among didelphid taxa. Foremost among the problems facing comparative researchers with this objective are (1) the monophyly of the didelphid crown group and (2) the interrelationships of the taxa it contains. Brief reviews of both topics provide the essential context for our study.

### Didelphid Monophyly

Winge (1893), Bensley (1903), and Gregory (1910) regarded didelphids as ancestral to other living metatherians and some of their tree diagrams explicitly depicted Recent



**Fig. 1.** Gregory’s (1910: Fig. 14) diagram of “morphogenetic” relationships among marsupial families with didelphids depicted as a basal paraphyletic group. In an accompanying tabular summary entitled “Adaptive Radiation of the Polyprotodontia,” Gregory explicitly derived dasyurids and perameloids from primitive didelphids (p. 203), but he subsequently cautioned that the “*phylogenetic validity*” (original italics, p. 205) of such conjectures was subject to question.

didelphids as paraphyletic with respect to Old World taxa (Fig. 1). According to these authors, all of the derived morphological traits observed among Australasian marsupials could be interpreted as modifications of more plesiomorphic conditions exhibited by didelphids. Simpson’s (1945) treatment of didelphoids as basal metatherians—together with his taxonomic allocation of such Cretaceous and Paleogene taxa as *Alphadon*, *Peradectes*, and *Pedimys* to the Didelphidae—effectively summarized the prevailing mid-century view of the crown group as examples of “living fossils.”

By contrast, it is now widely recognized that Recent didelphids are morphologically derived with respect to most Cretaceous and Paleogene metatherians (Clemens, 1968, 1977; Szalay, 1982a,b, 1994; Reig *et al.*, 1987; Wible, 1990; Goin, 1993). However, all of the dental and osteological apomorphies of the didelphid crown group identified to date by comparisons with fossil taxa are either shared by other living marsupials (e.g., the ros-

tral tympanic process of petrosal; Wible, 1990) or are believed to have been subsequently transformed in microbiotherians and Australasian lineages (e.g., the double-faceted calcaneocuboid joint; Szalay, 1994). Therefore, while such traits are appropriately cited as useful criteria for diagnosing didelphids from peradectids, pediomyids, stagodontids, and other extinct lineages (or plesions), none provides compelling evidence for didelphid monophyly.

Most surveys of nonosteological character variation have likewise failed to identify plausible didelphid synapomorphies. For example, didelphids appear to be indistinguishable as a clade apart from the Australasian fauna in pelage traits (Boardman, 1951; Lyne, 1959), tongue morphology (Sonntag, 1924), female reproductive anatomy (Hill and Fraser, 1925), and karyotypes (Reig *et al.*, 1977). Although epididymal sperm pairing (an unambiguously derived trait shared by all examined didelphids but not known to occur in any Old World marsupial taxon; Temple-Smith, 1987) appears to provide an important counterexample, many didelphid genera (e.g., *Caluromysiops*, *Glironia*, *Gracilinanus*, *Lestodelphys*, *Lutreolina*, *Marmosops*, and *Thylamys*) have yet to be examined for this character and its phylogenetic significance is, therefore, ambiguous.

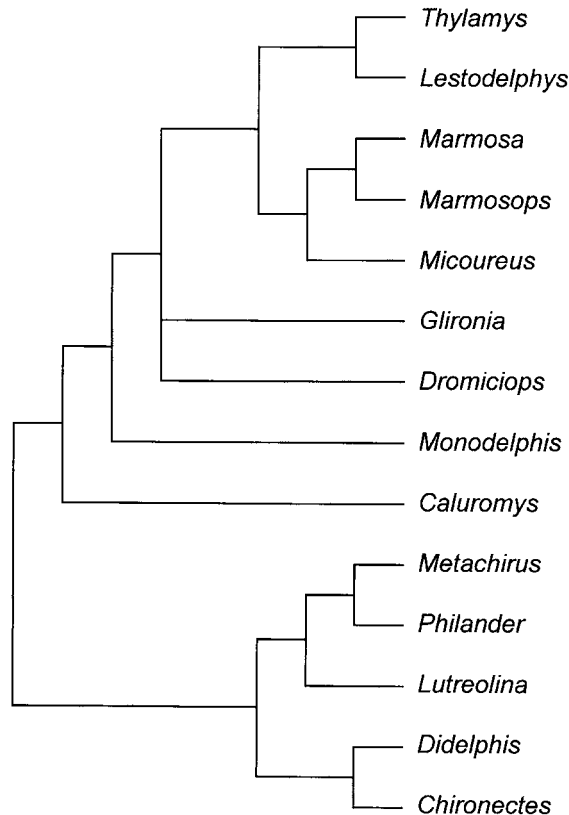
Monophyly of the didelphid crown group could still be supported as the most parsimonious interpretation of available character data even in the absence of unequivocal (unique and unreversed) synapomorphies, but the only published parsimony analyses that have included both (1) a taxonomically dense sampling of didelphids and (2) relevant Australasian exemplars suggest that didelphids are paraphyletic (Kirsch and Archer, 1982). Other parsimony analyses of marsupial character data have assumed rather than tested didelphid monophyly (Creighton, 1984; Patton *et al.*, 1996), have not included any Australasian exemplars (Reig *et al.*, 1987), or have included so few didelphids as to preclude meaningful inferences about familial monophyly (Springer *et al.*, 1994; Retief *et al.*, 1995; Rougier *et al.*, 1998; Colgan, 1999).

To date, the most suggestive evidence for didelphid monophyly comes from comparative serology (summarized by Kirsch, 1977) and DNA–DNA hybridization (summarized by Kirsch *et al.*, 1997), both of which suggest greater molecular similarity among Recent didelphids than between any didelphid and members of other extant marsupial families. Although such distance comparisons have been of considerable heuristic value in the development of current thinking about marsupial systematics, the absence of compelling character data supporting didelphid monophyly remains a significant research challenge. In effect, taxonomic assignments to the family are still based primarily on biogeography and plesiomorphic resemblance rather than well-corroborated patterns of synapomorphy.

### Didelphid Interrelationships

Many systematists using diverse kinds of data have made significant contributions to didelphid phylogenetic research and it is not possible to do justice to all of them here. Instead, we illustrate and discuss results from five studies that best exemplify the principal points of conflict and consensus concerning relationships among extant taxa. To facilitate meaningful comparisons, we have updated nomenclature to conform with current usage in all of the following synopses.

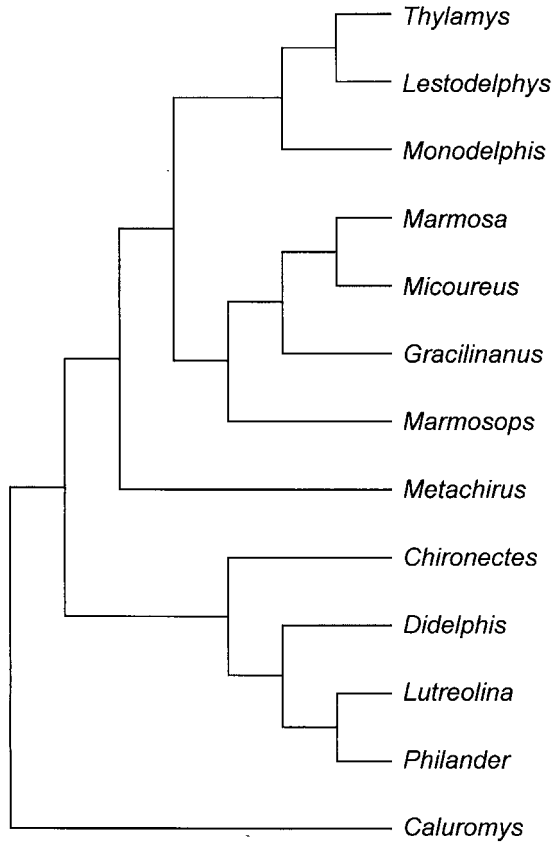
Tate (1933) provided the first hypothesis of didelphid phylogeny to include most of the forms currently treated as valid genera (Fig. 2). Although he clustered taxa by



**Fig. 2.** Tate's (1933) hypothesis of didelphid phylogeny relabelled to conform with current taxonomic usage. In the accompanying caption, Tate noted the omission of *Gracilinanus*, which he hypothesized to be related either to the *Marmosa* + *Marmosops* or the *Lestodelphys* + *Thylamys* clade.

unknown (presumably subjective) criteria, Tate's conjectures were the result of close familiarity with most of the taxa in question and effectively served as the basis for all subsequent research on didelphid relationships. Prominent in Tate's scheme was a basal clade of large opossums (*Chironectes*, *Didelphis*, *Lutreolina*, *Metachirus*, and *Philander*), on the one hand, and a monophyletic group of small "murine" or "marmosine" genera (*Gracilinanus*, *Lestodelphys*, *Marmosa*, *Marmosops*, *Micoureus*, and *Thylamys*) on the other. Among the marmosines, *Marmosa* and *Marmosops* were depicted as sister taxa, as were *Lestodelphys* and *Thylamys*. Tate placed *Dromiciops* (a microbiotherian) and *Glironia* in an unresolved trichotomy with the marmosine clade, and ranked *Monodelphis* and *Caluromys* as successively more distant sister taxa to that group. Notably absent from Tate's phylogeny is any special association between *Caluromys* and *Glironia*, the only caluromyine genera known at that time.

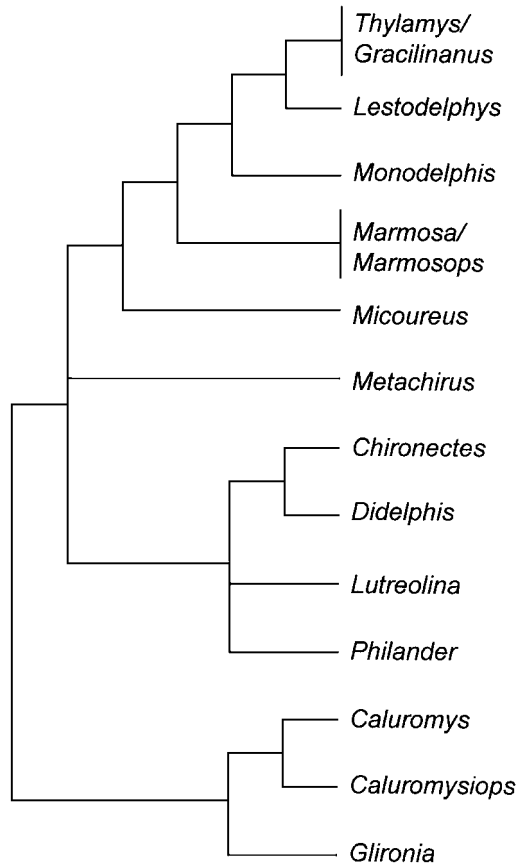
Most of the qualitative morphological traits discussed by Tate (1933) and other early students of didelphid taxonomy were included in Creighton's (1984) quantitative cladistic



**Fig. 3.** Creighton's (1984) parsimony topology rooted with *Dromiciops* as the designated outgroup. *Caluromysiops* and *Glironia* were not included in his analysis.

study. Using both compatibility and parsimony methods, Creighton analyzed 58 characters (57 morphological characters plus one karyotypic character) scored for exemplars of all Recent didelphid genera except *Caluromysiops* and *Glironia*. The tree he illustrated and discussed as the result of parsimony analysis (Fig. 3) was rooted with *Dromiciops* as the designated outgroup and differed from Tate's phylogeny in many significant respects: (1) *Caluromys* appears as the sister group to a clade containing all of the other didelphids; (2) *Metachirus* does not form a monophyletic group with the other large opossums but instead forms the basal lineage of a predominantly marmosine clade; (3) *Monodelphis* is nested within marmosines as the sister taxon to *Lestodelphys* + *Thylamys*; and (4) *Marmosa* and *Micoureus* are placed as sister taxa, successively joined by *Gracilinanus* and *Marmosops*. In fact, the only monophyletic group common to both Tate's phylogeny and this topology is *Lestodelphys* + *Thylamys*, a clade supported by three unique morphological synapomorphies in Creighton's data.

Reig *et al.* (1987) scored 45 characters (42 morphological characters, 2 characters encoding serological similarity, plus 1 karyotypic character) for 13 didelphid terminals



**Fig. 4.** Reig *et al.*'s (1987) summary cladogram, pruned of the extinct taxa included in their analysis. The terminal labeled "Marmosa" in this study consisted of species currently referred to *Marmosa sensu stricto* and *Marmosops*, whereas the terminal labeled "Thylamys" consisted of species currently referred to *Thylamys sensu stricto* and *Gracilinanus*. The parsimony analyses on which this tree was partially based were rooted with a reconstructed hypothetical ancestor (not shown) closely resembling the fossil taxa *Alphadon* and *Peradectes*.

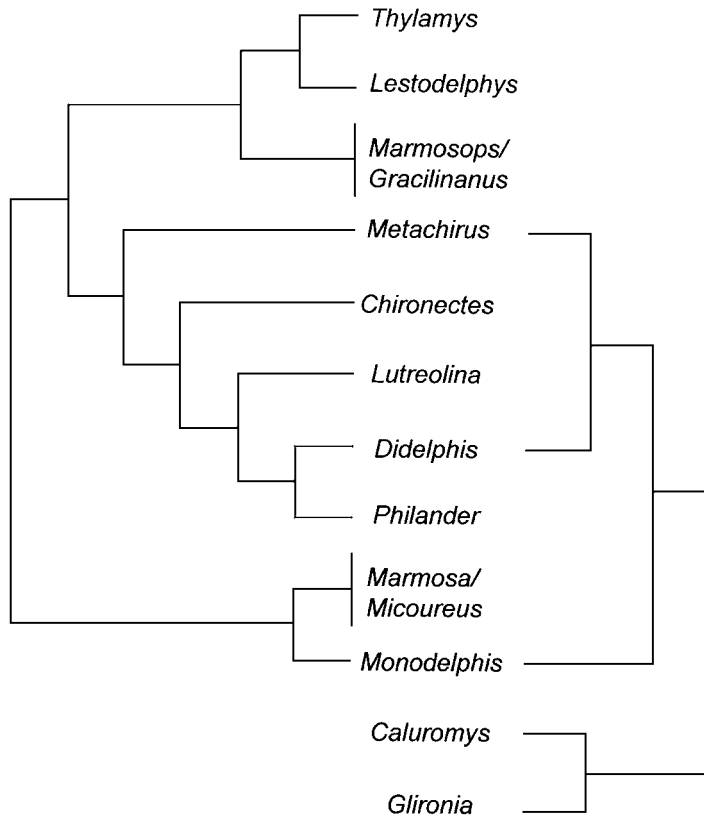
together with *Dromiciops* and 18 fossil New World metatherians. Apparently because their parsimony algorithms were incapable of handling missing data, the authors presented the results of many analytic permutations to explore the consequences of omitting various sets of taxa and characters. A summary cladogram (Fig. 4) was said to be based in part on these analyses of tabulated character data but "taking into account informal consideration of other features" (op. cit., p. 65). Noteworthy similarities between this tree and Creighton's (1984) are (1) the basal position of *Caluromys*, here associated in a clade with other caluromyines that Creighton did not study; (2) the clustering of *Mono-*

*delphis* with marmosines; and (3) a monophyletic group containing the four large opossums *Chironectes*, *Didelphis*, *Lutreolina*, and *Philander*. Notable differences include the positions of *Marmosa/Marmosops* and *Micoureus* as successively distant sister groups to  $\{[(Thylamys/Gracilinanus) Lestodelphys] Monodelphis\}$ . Although *Metachirus* clustered with the large opossums in most of Reig *et al.*'s parsimony analyses, the position of this genus within the didelphine complex was left unresolved in their summary cladogram.

Kirsch and Palma's (1995) DNA–DNA hybridization study included exemplars of 14 didelphid genera, but not all of these were treated simultaneously. Subfamilial monophyly was assumed in one analysis, resulting in a didelphine tree rooted with *Caluromys* as the designated outgroup (Fig. 5, left). Unlike the results of earlier analyses based primarily on morphological data (see above), the marmosine genera are widely separated in Kirsch and Palma's results, with one clade consisting of  $[(Thylamys + Lestodelphys) Marmosops/Gracilinanus]$  clustered with the large opossum group ( $\{[(Didelphis + Philander) Lutreolina] Chironectes\} Metachirus$ ), and another clade (*Marmosa/Micoureus*) clustered with *Monodelphis*. Relationships within the clade of large opossums likewise differed from previous results based on morphology. In a separate analysis, Kirsch and Palma tested subfamilial monophyly using three didelphine exemplars, two caluromyines, members of other marsupial families, and a eutherian outgroup (Fig. 5, right). Bootstrap resampling exercises described by the authors suggest that all of the branching patterns illustrated in Fig. 5 are strongly supported, with the exception of didelphine monophyly in the right-hand diagram.

Most recently, Patton *et al.* (1996) sequenced the entire mitochondrial cytochrome *b* gene for 15 didelphids and two outgroup taxa. The results of their parsimony analyses of both nucleotide and amino acid substitutions (Fig. 6) supported Kirsch and Palma's (1995) clades  $[(Marmosa + Micoureus) Monodelphis]$ , as well as the basal association of *Metachirus* with the other large opossums. However, the most parsimonious placement of *Caluromys* for both their DNA and amino acid sequence data is not basal to the didelphine radiation, nor do *Caluromys* and *Glironia* form a monophyletic group. Instead, the strict consensus of three maximally parsimonious nucleotide trees shows  $(Caluromys + Marmosops)$  and *Glironia* in an unresolved polytomy with  $[(Didelphis/Philander) Metachirus]$  and  $[(Marmosa + Micoureus) Monodelphis]$ . In the strict consensus of three maximally parsimonious amino acid trees, *Caluromys* clusters with  $[(Didelphis/Philander) Metachirus]$  whereas *Glironia* clusters with  $[(Marmosa + Micoureus) Monodelphis]$ . In a striking departure from previous studies, *Marmosops* occupies the basal position in Patton *et al.*'s amino acid parsimony trees. Unfortunately, bootstrap and Bremer support values calculated by the authors are not very high for most of the nodes diagrammed here.

In summary, no pattern of relationships among didelphid genera is common to all of these five studies. Setting aside Tate's (1933) tree as an object of primarily historical interest, the relationships supported by the remaining four analyses are consistent with only one monophyletic group, the now-familiar clade of four large opossums (*Didelphis*, *Chironectes*, *Lutreolina*, and *Philander*). Three out of four modern studies support a monophyletic Didelphinae (Patton *et al.*'s, 1996 does not), the sister-group relationship of *Marmosa* with *Micoureus* (Reig *et al.*'s, 1987 does not), and the basal relationship of *Metachirus* with the remaining large opossums (Creighton's, 1984 does not). All other relationships are supported by less than a majority of these studies, although it is noteworthy that the group *Thylamys + Lestodelphys* is contradicted only by Reig *et al.*'s eccen-

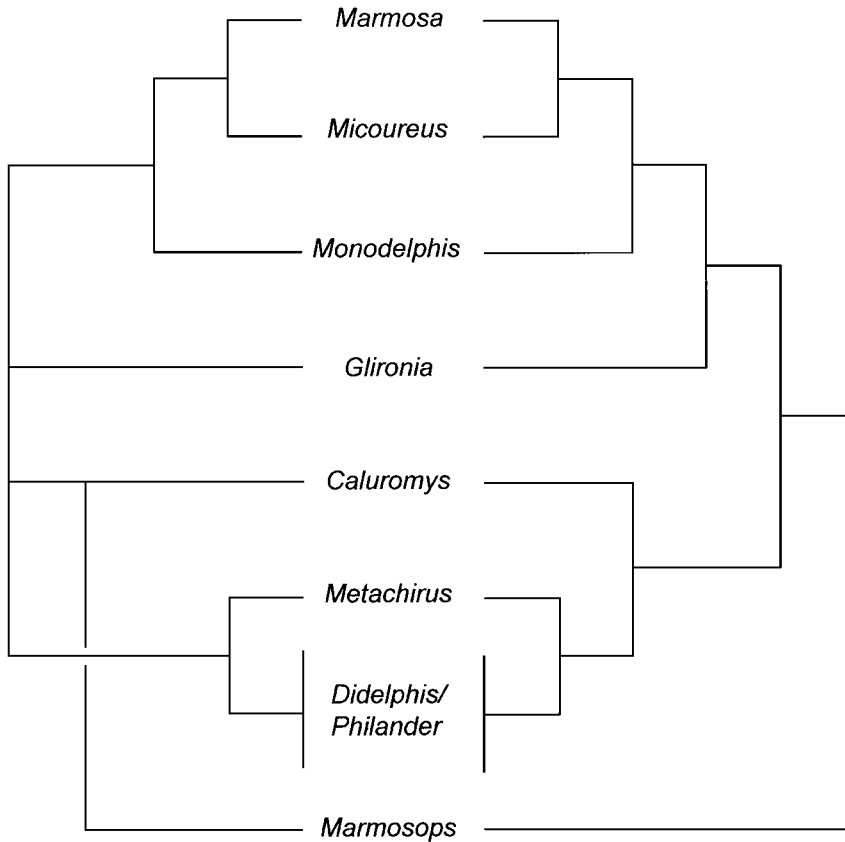


**Fig. 5.** Tree diagrams summarizing the DNA–DNA hybridization results of Kirsch and Palma (1995). The left-hand diagram is a Fitch–Margoliash tree resulting from an analysis of “symmetrized”  $\Delta T_m$  values among 17 didelphid species, rooted with *Caluromys* as the designated outgroup. The right-hand diagram is part of a similar analysis that included five didelphids, a microbiotherian (*Dromiciops*), a paucituberculate (*Caenolestes*), three Australian marsupials (*Dasyurus*, *Echymipera*, and *Phalanger*) and a placental (*Procyon*), with the latter as designated outgroup; only relationships within the didelphid clade are illustrated here. For simplicity, congeneric terminals are not distinguished in these condensed diagrams. Based on the species samples included in their analysis, Kirsch and Palma could not resolve *Marmosa* and *Micoureus* as reciprocally monophyletic groups; the reciprocal monophyly of *Gracilinanus* and *Marmosops* was likewise unresolved.

tric taxonomic application of the former name (see legend to Fig. 4). Taxa of notably uncertain placement in these results are *Glironia*, *Gracilinanus*, *Marmosops*, and *Monodelphis*. To date, *Caluromysiops* has been included in but a single morphological study, and its relationships remain uninvestigated with molecular data.

### A Preliminary IRBP Perspective

As the initial phase of a combined molecular and morphological study designed to address these and other problems in didelphid phylogeny, we sequenced part of the first



**Fig. 6.** Results of Patton *et al.*'s (1996) sequencing study of the mitochondrial cytochrome *b* gene. The left-hand diagram shows the strict consensus of three equally parsimonious trees based on analyses of all first and second position nucleotide substitutions plus third position transversions. The right-hand diagram shows the strict consensus of three maximally parsimonious trees based on analyses of translated amino acid sequences. Both trees are rooted with a paucituberculata (*Lestoros*) and a paramelomorph (*Echymipera*) as designated outgroups (not shown).

exon (ca. 1200 bp) of the gene encoding the Interphotoreceptor Retinoid Binding Protein (IRBP) for selected species of Recent didelphids. The IRBP protein is found in the eyes of all vertebrate classes (Bridges *et al.*, 1986), where it apparently functions in the transfer of retinoids during light- and dark-phase adaptation (Fong *et al.*, 1990; Pepperberg *et al.*, 1993). The protein is encoded by nuclear DNA sequence that is presumed to exist as a single copy because no duplicate genes have been reported in any taxon examined to date (Bridges *et al.*, 1986; Stanhope *et al.*, 1992, 1996; Springer *et al.*, 1997a). The region of IRBP exon 1 that we sequenced has previously been used to address relationships among mammalian orders (Stanhope *et al.*, 1992, 1996; Springer *et al.*, 1997a, 1999; Gatesy *et al.*, 1999), but only one study (Yoder and Irwin, 1999) has hitherto used this gene for phylogenetic inference at lower taxonomic levels. The primary goals of this paper are thus threefold: (1) to test the monophyly of Didelphidae with respect to other

extant metatherians, (2) to provide new information about relationships among didelphid lineages, and (3) to explore the utility of IRBP for future analyses of marsupial phylogeny.

## MATERIALS AND METHODS

### Taxon Sampling, Phylogenetic Assumptions, and Identifications

In order to achieve a sufficiently dense sampling of didelphids for the purposes of this preliminary study, we sequenced specimens from at least one species in every currently recognized genus (see Appendix). Within genera, our choice of species was at least partly dictated by the availability of well-preserved tissues, but we made special efforts to obtain sequences from some key taxa never before represented by molecular data (e.g., *Caluromysiops irrupta*). We also sequenced more than one species from three didelphid genera whose monophyly has been questioned in the literature (*Didelphis*, *Marmosa*, *Monodelphis*), and we sequenced two or more specimens of several species to assess the variability of IRBP among conspecific individuals. In total, we sequenced 35 individuals representing 21 currently recognized didelphid species in 15 genera (see Appendix).

To test the monophyly of Didelphidae (order Didelphimorphia), we used published IRBP sequences from at least one exemplar of every other extant metatherian order except Notoryctomorpha. Springer *et al.* (1997a) reported frameshift indels and stop codons in the IRBP sequence of *Notoryctes typhlops*, clear evidence of molecular degeneracy that they interpreted as resulting from loss of protein function in this blind fossorial species. Because *Notoryctes* has never been considered as particularly close to didelphids, and because we were reluctant to introduce a potentially problematic long branch represented by characters of dubious homology, we chose to exclude this taxon from our analysis. Therefore, the nondidelphid marsupial sequences included as part of our ingroup are from Paucituberculata (*Caenolestes fuliginosus*, GenBank accession number AF025381), Microbiotheria (*Dromiciops gliroides*, AF025384), Peramelemorphia (*Echymipera kalubu*, AF025383), Dasyuromorphia (*Phascogale tapoatafa*, AF025382); and Diprotodontia (*Pseudocheirops cupreus*, AF025387); *Vombatus ursinus*, AF025386).

To root our analysis of marsupial relationships, we included sequences from several placental taxa as outgroups. Although our ingroup–outgroup distinction implicitly assumes the monophyly of Marsupialia (the metatherian crown group), a noncontroversial clade supported by numerous morphological synapomorphies (Rougier *et al.*, 1998), the inclusion of multiple outgroup taxa provides a partial test of ingroup monophyly in terms of the data analyzed herein. Our 12 placental outgroup sequences are from Insectivora (*Sorex palustris*, GenBank accession number U48587; *Erinaceus europaeus*, AF025390), Chiroptera (*Macrotus californicus*, U48585), Tubulidentata (*Orycteropus afer*, U48712), Hyracoidea (*Procavia capensis*, U8586), Proboscidea (*Loxondonta africana*, U48711), Carnivora (*Felis catus*, Z11811), Primates (*Homo sapiens*, J05253), Artiodactyla (*Sus scrofa*, U48588), Lagomorpha (*Oryctolagus cuniculus*, Z11812), and Rodentia (*Mus musculus*, AF126968; *Spalax zemni*, U48589).

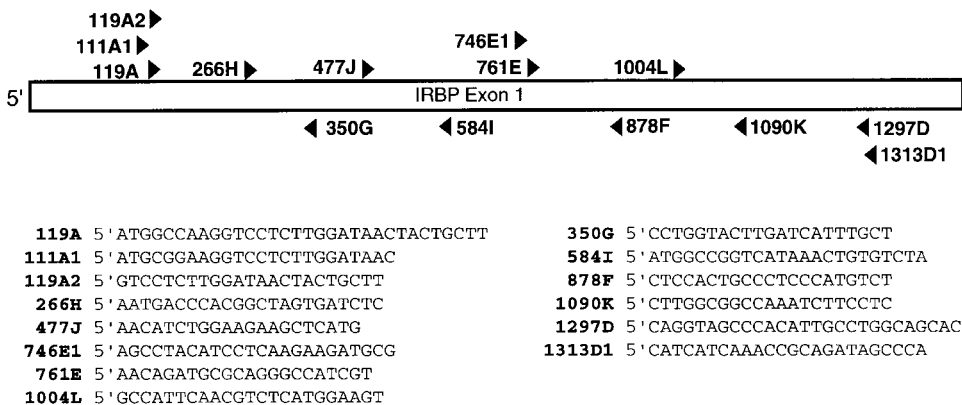
We examined morphological voucher material for every sequenced didelphid specimen except that of *Lestodelphys halli*, the identification of which was judged to be nonproblematic. To determine identifications, we compared measurements and qualita-

tive characters with published descriptions in the primary taxonomic literature. For some species, identifications were also based on comparisons with relevant type material. Our didelphid nomenclature follows Gardner (1993), with the exceptions noted in Table I. However, many didelphid species as currently recognized are obviously composite (Patton and da Silva, 1997; Patton *et al.*, 2000), so the species-level taxonomy for our sequenced material is certain to change as a consequence of future revisionary work.

### DNA Amplification and Sequencing

For most specimens, DNA was isolated from heart, liver, or kidney tissue that had been frozen or preserved in ethanol in the field. For *Caluromysiops*, DNA was extracted from small pieces of dried muscle scraped from skeletal material that had been stored in a museum cabinet since 1976. DNA was extracted from all tissues using a QiaAmp extraction kit (Qiagen Inc.). For *Lestodelphys*, sonicated DNA (extracted and purified in the laboratory of J. Kirsch) was used as a template in PCR reactions.

Extracted DNA was diluted 1 : 20 in water and used as a template in PCR reactions with combinations of primers shown in Fig. 7. For most specimens, a region approximately 1.2 kb long of IRBP exon 1 was amplified using primers A and D1. To generate fragments of suitable size for sequencing, this product was used as a template in two subsequent PCR reactions, one using primer A paired with F and one using primers E1 and D1. For some specimens, the small fragments (primer pairs A/F, and primers pairs E1/D1) were amplified directly from genomic DNA and used in subsequent sequencing reactions. DNA extracted from *Caluromysiops* dried muscle and the sonicated *Lestodelphys* DNA were sufficiently degraded that amplifications were done as a series of six overlapping 100–400 bp fragments (for *Lestodelphys* primer pairs A/G, H/I, J/K, J/F, E1/K, L/D1 were used; for *Caluromysiops* primer pairs A/I, H/I, J/I, J/F, L/D, E/D were used).



**Fig. 7.** Names and locations of primers used in PCR amplification and sequencing of IRBP exon I. Numbers correspond to where the 3' end of the primer falls on the human sequence as numbered from the initiation codon (Fong and Bridges, 1988). Primers 111A and 1297D are equivalent to Primers A and D of Stanhope *et al.* (1992).

Initial amplifications using genomic DNA as a template were performed as 20- $\mu$ L reactions using Ampli-Taq Gold polymerase (Perkin-Elmer) and recommended concentrations of primers, unincorporated nucleotides, buffer, and MgCl<sub>2</sub>. These reactions were performed on a Perkin-Elmer 9700 thermal cycler using a four-stage touchdown protocol. The first stage consisted of 5 cycles of denaturation at 95°C for 20 sec, annealing at 58°C for 15 sec, and extension at 72°C for 60 sec. The second and third stages were identical to the first except for lowered annealing temperatures of 56° and 54°C, respectively. The final stage consisted of 23 cycles with an annealing temperature of 52°C. The reaction series was preceded by an initial denaturation at 95°C for 10 min and followed by a 7-min extension at 72°C. Products were purified via electrophoresis through a 2% low melting-point agarose gel (NuSieve GTG, FMC). The appropriate band was excised from the gel using a Pasteur pipette, and the gel plug was melted in 300  $\mu$ L sterile water at 73°C for 20 min.

The resulting gel-purified product was used as a template in 30–40  $\mu$ L reamplification reactions with Ampli-Taq Gold polymerase. Reactions were subjected to 36 PCR cycles using an annealing temperature of 52°C. Aliquots (5  $\mu$ L) of the resulting products were visualized on a 2% NuSieve agarose gel. If more than one amplification product resulted, the entire reaction volume was run on a 2% NuSieve agarose gel, the appropriate band was excised and cleaned using the GeneClean II System (BIO 101, Inc.). If a single band resulted, products were cleaned directly using the GeneClean II system without gel purification.

PCR products were sequenced in both directions using amplification primers and dye-terminator chemistry (dRhodamine Ready Reaction Kit, Applied Biosystems Inc.). Reactions were cleaned using CentriSep (Princeton Separations) columns and run on an ABI 377 automated sequencer. Sequences were edited and compiled using Sequencher 3.0 software (GeneCodes, Corp.). Base-calling ambiguities between strands were resolved either by choosing the call on the cleanest strand or using the appropriate IUB ambiguity code if both strands showed the same ambiguity. All sequences have been deposited in GenBank with accession numbers AF257675 to AF257710.

### Data Analysis

We constructed multiple sequence alignments with Clustal W (Thompson *et al.*, 1994) using different gap-opening penalties (1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100) to assess alignment ambiguity among sequences that differ in length (Gatesy *et al.*, 1993). Default values were used for all other alignment parameters. All nonidentical multiple alignments discovered by this procedure, together with a nucleotide matrix in which deletions at alignment-ambiguous sites are coded as missing data (DeSalle *et al.*, 1994), can be downloaded from <ftp://ftp.amnh.org/pub/mammalogy>.

All parsimony analyses were implemented using PAUP\* 4.0b2a (D.L. Swofford) with informative characters treated as unordered and equally weighted. Each dataset (all of the nonidentical alignments and the nucleotide matrix with alignment-ambiguous sites coded as missing data) was subjected to a heuristic search of 25 random addition replicates with TBR branch swapping. Bootstrap values (Felsenstein, 1985) were calculated for each dataset using 300 bootstrap replicates with heuristic searches employed within each replicate (10 random addition replicates, TBR branch swapping). Bremer support

values (Bremer, 1994) were calculated by searching for the shortest tree(s) not consistent with a constraint tree containing a particular node. TreeRot (Sorenson, 1996) was used to create the constraint files and the PAUP commands for Bremer support calculations.

Uncorrected pairwise (“*p*”) distances, the absolute numbers of transitions and transversions, base composition, and tests of departure from stationarity were calculated using PAUP\* 4.0b2a. For all distance calculations, missing data (unsequenced regions and regions of ambiguous alignment) were ignored for the affected pairwise comparison. Amino acid composition and codon usage were analyzed using the program General Codon Usage Analysis (GCUA) ver.1.0.1b1 (written by J. McInerney). Patterns of character change were explored using MacClade 3.06 (Maddison and Maddison, 1992).

## RESULTS

### Sequence Fidelity

Contamination from extraneous DNA or PCR product can be a problem when working with degraded DNA extracted from museum specimens. We were particularly aware of the potential for contamination of our *Caluromysiops* sample (dried muscle scraped from old bones). However, results from PCR reactions suggested that we successfully amplified the target sequence in this case: for all primer pairs, a single product of the expected size resulted, the negative control yielded no amplification, and sequence for overlapping regions of all PCR fragments was identical. We further checked all resulting sequences for contamination by comparing sequence from each primer pair against all other IRBP sequences generated in the same lab. In no case were the *Caluromysiops* sequence fragments identical to any other IRBP sequence we generated.

### Sequence Alignment

All of the didelphid sequences we obtained were 1158 bp long, but all of the non-didelphid sequences obtained from GenBank were shorter than this. All sequences translate to open reading frame. The 13 different gap-opening costs we used resulted in six nonidentical alignments. Alignment I (gap cost = 1) was 1173 bp long, alignment II (gap cost = 2) was 1169 bp long, alignment III (gap cost = 5) was 1167 bp long, and alignment IV (gap costs = 10 and 20) was 1164 bp long. Alignments V (gap costs = 30–50) and VI (gap costs = 60–100) each had a total length of 1161 bp, but differed in the positioning of gaps. Only one small region of 35 nucleotides (bp 1050–1084) was affected across the entire range of alignment parameters examined, and all alignment ambiguities concern the size and placement of indels required to account for the shorter sequences of nondidelphids. Because we know of no objective criterion for choosing among these alternative hypotheses of positional homology, we scored bp 1050–1084 as missing for nondidelphids in all subsequent analyses except as noted below.

### Sequence Divergence and Saturation

The majority of conspecific didelphid specimens that we analyzed had identical sequence, including our duplicate samples of *Didelphis albiventris*, *D. marsupialis*, *Gracilinanus microtarsus*, *Lutreolina crassicaudata*, *Marmosops impavidus*, *Metachirus*

*nudicaudatus*, *Micoureus demerarae*, and *Philander mcilhennyi*. Four of our five *Marmosops noctivagus* from Peru were identical (RSV 2131, RSV 2224, RSV 2242, RSV 2294), but differed from the fifth (RSV 2225) at a single base position. The *M. noctivagus* specimen from Ecuador (ROM 105316) differed from the Peruvian specimens by an average sequence divergence (*p* distance) of 0.6%. Two *Marmosa murina* specimens from Peru diverged from each other by 0.2%, while two *Marmosops parvidens* (from different localities in Guyana) differed by 0.5%. Uncorrected pairwise sequence divergence ranged from 0.7 to 5.7% among nonconspecific didelphids, from 9.2 to 15.3% between didelphids and nondidelphid marsupials, and from 24.9 to 32.1% between marsupials and placentals.

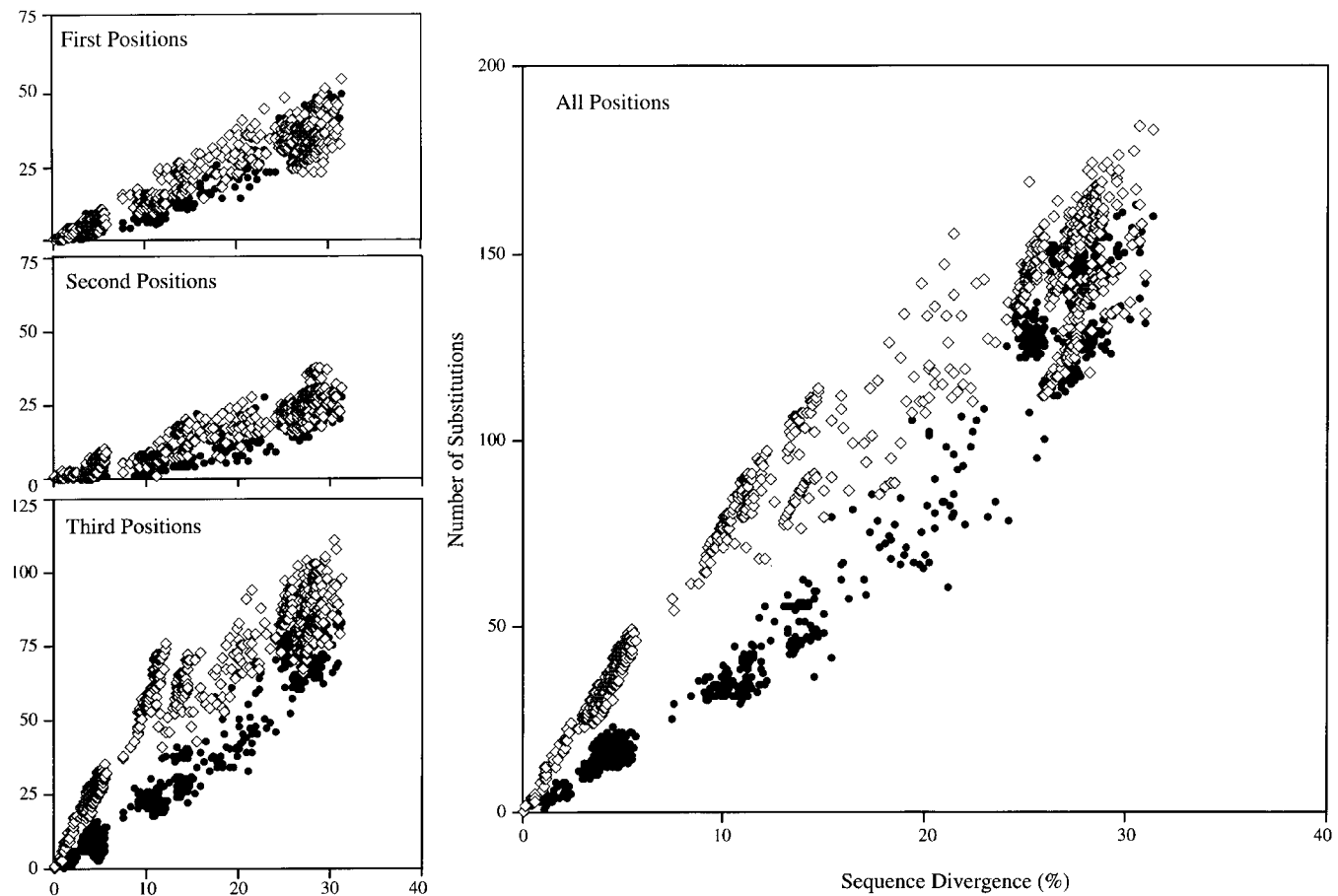
We examined sequences for saturation effects by plotting the number of transitions and transversions versus overall percent sequence divergence (Fig. 8). Across all codon positions, we found no compelling evidence for saturation in either transitions or transversions; both types of change increase linearly with increasing sequence divergence. Moreover, all three codon positions show a linear increase of transitions and transversions across the range of sequence divergence examined. The apparent lack of saturation for either transformation type at any codon position suggests that none of the usual arguments for differential character weighting apply to these data.

### Base and Amino Acid Composition

The base composition at all codon positions across all taxa and for marsupials only (Table II) is reasonably even, with only a slight bias toward C and G. However, first positions are biased toward G, second positions toward T, and third positions toward G and C. These position-specific nucleotide frequencies reflect the skewed amino acid composition of the protein as well as biased codon usage in the underlying mRNA sequence. Thus, average frequencies are high for the amino acids glutamine (GAR; 7.41%), leucine (CTN; 13.31%), and valine (GTN; 10.11%). Moreover, there is a strong bias toward CTC and CTG among the possible leucine codons, and toward GTG among the possible valine codons.

Differences in base composition among taxa (i.e., departure from stationarity) can compromise accurate phylogenetic inference if taxa with similar base compositions are not, in fact, sister taxa (Saccone *et al.*, 1989; Lockhart *et al.*, 1992). We assessed the IRBP data for stationarity of base composition using a Chi-square test as implemented in PAUP\*. When all three codon positions are considered across all taxa (marsupials and placentals), we found a statistically significant departure from stationarity ( $X^2 = 159.3$ ,  $df = 129$ ,  $P = 0.03$ ). However, this phenomenon seems to be confined to third positions, as the null hypothesis of homogeneity in base composition across taxa cannot be rejected for first and second positions (first positions:  $X^2 = 23.5$ ,  $P = 1.0$ ; second positions:  $X^2 = 24.1$ ,  $P = 1.0$ ; third positions:  $X^2 = 294.6$ ,  $P < 0.001$ ;  $df = 129$  for each test).

Although these results suggest that taxonomic variation in base composition at third positions may be problematic, significant departures from stationarity are only apparent among our placental outgroups. When the analysis is confined to just ingroup taxa (marsupials), the hypothesis of base-compositional stationarity cannot be rejected for any codon position (all positions  $X^2 = 15.5$ ,  $P = 1.0$ ; first positions  $X^2 = 7.5$ ,  $P = 1.0$ ; second



**Fig. 8.** Plots of numbers of transitions (diamonds) and transversions (filled circles) versus uncorrected pairwise sequence divergence for the 32 marsupial and 12 placental sequences included in this study.

**Table II.** Range and Average (in parentheses) of Base Composition (%) for Each Codon Position Across 32 Marsupial and 12 Placental Taxa

Position	A	C	G	T
First	20.0–25.3 (22.9)	26.5–32.0 (28.2)	34.2–38.5 (37.5)	9.4–13.6 (11.4)
Second	25.1–29.5 (28.4)	18.9–25.6 (22.9)	15.4–19.2 (16.6)	30.4–35.2 (32.1)
Third	7.3–20.1 (16.5)	28.4–42.5 (32.8)	30.3–40.9 (33.3)	9.3–21.9 (17.4)
All	17.5–24.7 (22.6)	26.0–33.0 (28.0)	27.0–33.0 (29.1)	16.7–22.3 (20.3)

positions  $X^2 = 5.1$ ,  $P = 1.0$ ; third positions  $X^2 = 22.3$ ,  $P = 1.0$ ;  $df = 93$  for each test). Therefore, base-compositional variation seems unlikely to adversely affect phylogenetic inference from marsupial IRBP sequences.

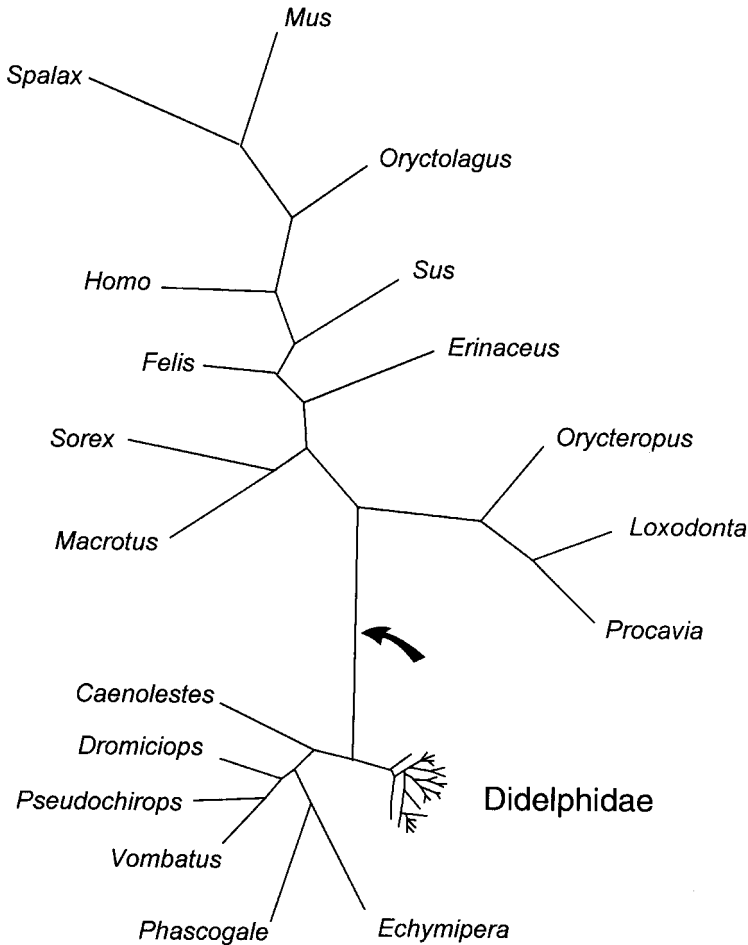
### Phylogenetic Relationships

After condensing identical conspecific didelphid sequences to single terminals, we obtained a final data matrix of 44 unique terminals scored for 1158 nucleotide positions [with the ambiguously alignable region (bp 1050–1084) coded as missing for nondidelphids]. Parsimony analysis of these data resulted in eighteen trees of minimal length, the strict consensus of which is shown as an undirected network in Fig. 9. Consistent with our ingroup–outgroup dichotomy, marsupials and placentals form discrete subtrees separated by the longest internode in this topology. Within the marsupial subtree, didelphid terminals form a cohesive and densely branched cluster.

Of the 548 parsimony-informative characters in our nucleotide matrix, 27% occur at first codon positions, 15% at second positions, and 58% at third positions. When changes in these characters among both ingroup and outgroup taxa are mapped on the topology illustrated in Fig. 9, third codon positions exhibit higher levels of inferred homoplasy than do first and second positions (RI values; Table III). However, when changes in informative characters are mapped among ingroup (marsupial) taxa only, third positions are nearly as consistent as first positions.

Rooting the maximally parsimonious network of therian relationships depicted in Fig. 9 at the marsupial–placental internode yields an almost completely resolved marsupial tree with a basal dichotomy between didelphids on the one hand and *Caenolestes*, *Dromiciops*, and Old World taxa on the other (Fig. 10). Although the latter clade is associated with only marginal bootstrap support (74%), this phylogenetic configuration is substantially more parsimonious than any alternatives we investigated. For example, constraining “Ameridelphia” [didelphids + *Caenolestes* (Szalay, 1982a,b)] to be monophyletic requires 11 extra steps, and constraining didelphids + *Caenolestes* + *Dromiciops* to be monophyletic requires 12 extra steps. Forcing *Dromiciops* to be sister to didelphids requires 13 extra steps. Therefore, any special association between didelphids and the other New World marsupials included herein is difficult to reconcile with our data.

Didelphid monophyly is impressively corroborated by very high bootstrap and Bremer support values. Among the 17 character changes that can be unambiguously mapped



**Fig. 9.** Minimal length undirected network of therian relationships based on parsimony analysis of IRBP sequences. Branch lengths (based on ACCTRAN optimization) are drawn proportional to reconstructed character changes (base substitutions). For clarity, didelphid terminals are not labeled. The inferred position of the therian root is indicated by the arrow.

onto the branch subtending the didelphid radiation, 10 are uniquely derived and unreversed (Table IV). These unequivocal synapomorphies include both transitions and transversions, changes at all three codon positions, changes that result in amino acid substitutions, and silent changes. Therefore, support for didelphid monophyly does not come from a single class of molecular events, but is provided by a variety of substitutions.

Within Didelphidae, caluromyines form a basal paraphyletic group, but two alternative branching patterns are almost equally parsimonious. In the shortest trees (Fig. 10), *Glironia* appears as the most basal didelphid clade, followed by a long branch that securely contains *Caluromys* and *Caluromysiops*. In trees only one step longer, however, *Caluromys* + *Caluromysiops* appears as the most basal didelphid lineage, and *Glironia* is

**Table III.** Tree Statistics and Homoplasy Indices for Each Codon Position of IRBP

Position	All taxa				Marsupials only			
	Number of variable characters	Number of informative characters	CI <sup>a</sup>	RI <sup>a</sup>	Number of variable characters	Number of informative characters	CI <sup>a</sup>	RI <sup>a</sup>
First	228	148	0.53	0.78	103	44	0.56	0.76
Second	140	85	0.53	0.79	57	25	0.67	0.86
Third	360	315	0.46	0.69	266	158	0.57	0.75
All	728	548	0.48	0.72	426	227	0.58	0.76

<sup>a</sup>Consistency index (CI) (considering informative characters only) and retention index (RI) are calculated for each codon position on the tree derived from simultaneous analysis of all codon positions.

sister to Didelphinae. Constraining Caluromyinae to be monophyletic requires three extra steps, so this hypothesis is less favored by our data than either paraphyletic alternative.

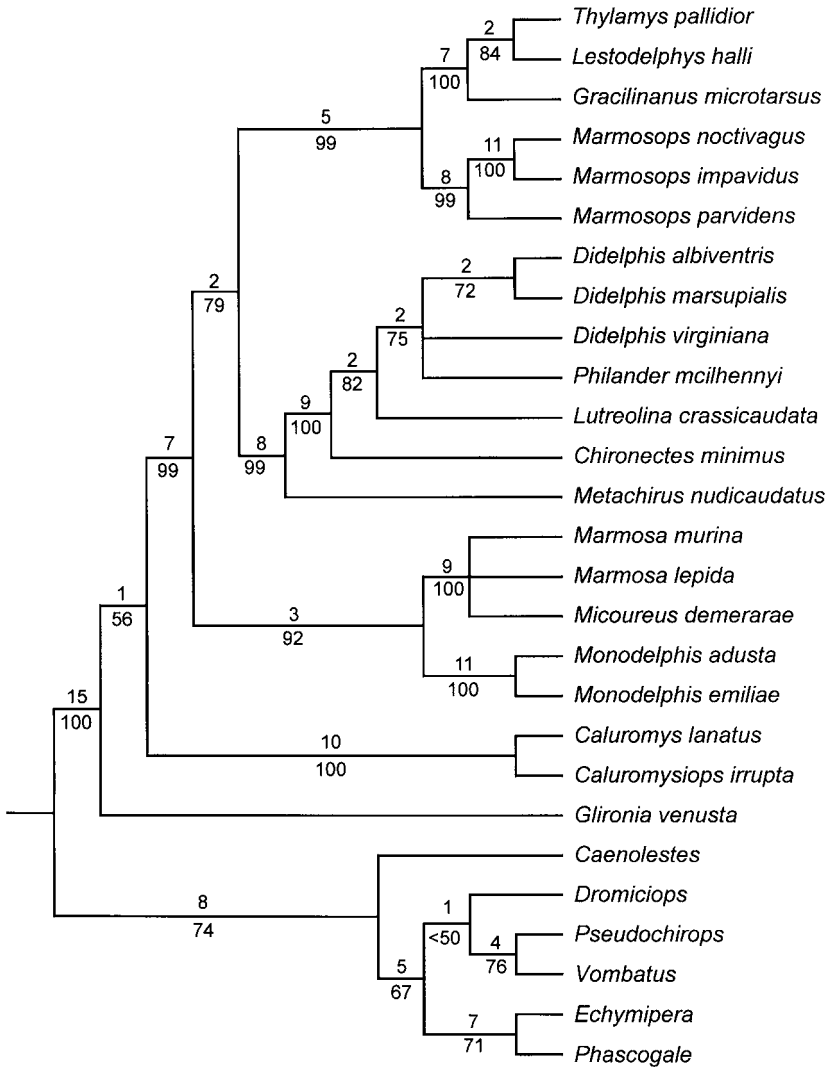
Monophyly of Didelphinae is strongly supported, and several subordinate groups are likewise associated with high bootstrap and Bremer values. The largest of these well-supported clades contains all of the large-bodied opossums in the sequence ({{(*Didelphis* + *Philander*) *Lutreolina*} *Chironectes*} *Metachirus*). However, the species of *Didelphis* that we sequenced cannot be resolved as a monophyletic group with respect to *Philander mcilhennyi*, and the branching sequence of these taxa with *Lutreolina* and *Chironectes* is associated with only marginal bootstrap and Bremer values. By contrast, the position of *Metachirus* at the base of the large opossum clade is unambiguously supported.

*Gracilinanus* appears as the well-supported sister group to *Thylamys* + *Lestodelphys*, and this triad forms another stable cluster with a monophyletic *Marmosops*. The linkage between these marmosines and the clade containing the large opossums, however, receives only marginal bootstrap and Bremer support. The last didelphine cluster—also marginally supported—consists of an unresolved grouping of *Marmosa* species with *Micoureus demerarae* joined to a monophyletic pair of *Monodelphis* species.

Because some of the basal branching structure within Didelphinae is not strongly supported, we tested our most-parsimonious topology against three alternative phylogenetic hypotheses explicit or implicit in the systematic literature. Constraining Tate's (1933) "marmosine" group (*Gracilinanus*, *Lestodelphys*, *Marmosa*, *Marmosops*, *Micoureus*, and *Thylamys*) to be monophyletic requires four extra steps, whereas constraining Reig *et al.*'s (1987) Marmosini ("marmosines" + *Monodelphis*) to be monophyletic requires only two extra steps. By contrast, constraining Hershkovitz's (1992) Marmosidae ("marmosines" + *Monodelphis* + *Metachirus*) to be monophyletic requires 10 extra steps. Of these phylogenetic alternatives, Hershkovitz's Marmosidae clearly provides the worst fit to our data.

### Sensitivity Analysis

To explore the phylogenetic consequences of scoring ambiguously alignable sites (bp 1050–1084) as missing for nondidelphids, and to assess the sensitivity (*sensu* Wheeler, 1995) of our results to alternative hypotheses of positional homology in this region, we



**Fig. 10.** Strict consensus of 18 most-parsimonious trees resulting from cladistic parsimony analysis of IRBP sequences included in this study (parsimony-informative characters = 548, tree length = 2022, CI = 0.53, RI = 0.72). The tree shows relationships among ingroup (marsupial) taxa only and is drawn as rooted with a composite outgroup containing exemplars of ten placental orders (see Fig. 9). Species are shown as terminal taxa, but several species were represented by more than one sequence in the parsimony analysis. Bremer and Bootstrap support values are shown above and below each branch, respectively.

subjected each of the six nonidentical alignments to parsimony analysis. The strict consensus of all minimum length trees resulting from all alignments (Fig. 11) is almost completely resolved and contains most of the nodes recovered when the ambiguous sites were treated as missing. The one exception is a basal trichotomy involving *Glironia*, the didelphine clade, and a clade containing *Caluromys* + *Caluromysiops*. This reflects the fact

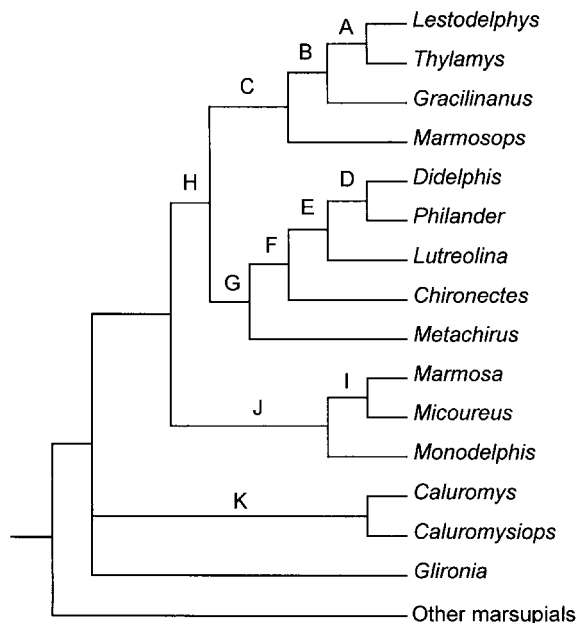
**Table IV.** Unique and Unreversed Synapomorphies Supporting Didelphid Monophyly

Character no.	Change	Type	Codon position	Effect
3 <sup>a</sup>	G → C	TV	3	Silent
14 <sup>b</sup>	C → T	TI	2	Ala → Val
103 <sup>b</sup>	T → G	TV	1	Ser → Ala
134 <sup>b</sup>	A → T	TV	2	Tyr → Phe
147 <sup>b</sup>	C → T	TI	3	Silent
313 <sup>a</sup>	C → T	TI	1	Silent
379 <sup>a</sup>	A → T	TV	1	Thr → Ser
909 <sup>a</sup>	C → G	TV	3	Silent
1097 <sup>a</sup>	C → A	TV	2	Thr → Asn
1128 <sup>a</sup>	G → A	TI	3	Silent

<sup>a</sup>Unique and unreversed within the marsupial ingroup only.

<sup>b</sup>Unique and unreversed across all ingroup and outgroup taxa.

that some of the trees resulting from analyses of different alignments have *Glironia* as the sister taxon to the didelphines, and some have *Glironia* as the sister taxon to all remaining didelphids. None of the most parsimonious trees resulting from analyses of alternative alignments places *Glironia* as sister to *Caluromys* + *Caluromysiops*.



**Fig. 11.** Results of sensitivity analysis (Wheeler, 1995) summarized as the strict consensus of all most-parsimonious trees obtained by analyzing six alternative IRBP alignments (see text). Only didelphid relationships are shown, with internal nodes labeled for cross-reference with the bootstrap and Bremer values summarized in Table V.

**Table V.** Bootstrap Values and Bremer Support (in parentheses) Associated with Clades Labeled in Fig. 11 for Six Different Alignments of IRBP Sequences (see text for alignment parameters)

	I	II	III	IV	V	VI
Ameridelphia	*	*	*	*	*	*
Didelphidae	100 (16)	100 (17)	100 (16)	100 (15)	100 (20)	100 (24)
Caluromyinae	*	*	*	*	*	*
Didelphinae	98 (7)	98 (7)	99 (7)	99 (7)	94 (7)	94 (7)
Clade A	85 (2)	82 (2)	82 (2)	85 (2)	85 (2)	85 (2)
Clade B	100 (7)	100 (7)	100 (7)	100 (7)	99 (7)	99 (7)
Clade C	98 (5)	98 (5)	97 (5)	99 (5)	96 (5)	96 (5)
Clade D	69 (2)	70 (2)	73 (2)	75 (2)	76 (2)	71 (2)
Clade E	75 (2)	76 (2)	77 (2)	79 (2)	78 (2)	78 (2)
Clade F	100 (9)	100 (9)	100 (9)	100 (9)	100 (9)	100 (9)
Clade G	99 (8)	99 (8)	99 (8)	100 (8)	99 (8)	99 (8)
Clade H	78 (2)	76 (2)	75 (2)	79 (2)	76 (2)	76 (2)
Clade I	100 (9)	100 (9)	100 (9)	100 (9)	100 (9)	100 (9)
Clade J	87 (3)	90 (3)	88 (3)	89 (3)	87 (3)	87 (3)
Clade K	100 (10)	100 (10)	100 (10)	99 (10)	99 (10)	99 (10)

\*Clade not recovered.

Alignment ambiguity does not substantially affect the evidential support for any of the nodes shown in Fig. 11. All nodes that have large bootstrap values when alignment-ambiguous sites are coded as missing (Fig. 10) have large bootstrap values under all alternative alignments (Table V). Likewise, Bremer support for all labeled relationships among didelphine genera (nodes A through J) and for the sister-group relationship between *Caluromys* and *Caluromysiops* (node K) remains the same under each alignment (Table V). The only alignment-dependent differences in Bremer values occur for Didelphidae (range = 15–24). For Bremer values as high as these, such small differences are hardly meaningful. Therefore, our principal phylogenetic results are apparently robust to alternative assessments of positional homology for ambiguously alignable sites.

## DISCUSSION

The IRBP sequences analyzed in this report display many desirable properties as data for inferring phylogenetic relationships among marsupials. First, this nuclear exon appears to be slowly evolving (e.g., by comparison with mitochondrial genes from many of the same taxa; Table VI, Fig. 12). Second, the absence of alignment ambiguities among didelphid sequences makes positional homology statements nonproblematic for the primary systematic goals of this study and suggests that alignment may not be a problem for sequence comparisons at comparable taxonomic levels within other marsupial clades. Third, the absence of saturation effects in all data partitions, even for sequence comparisons with placental outgroups, suggests that differential character weighting is unnecessary. Finally, we found no evidence for departures from base-compositional homogeneity among marsupial sequences that might cause unrelated lineages with convergent nucleotide bias to attract one another.

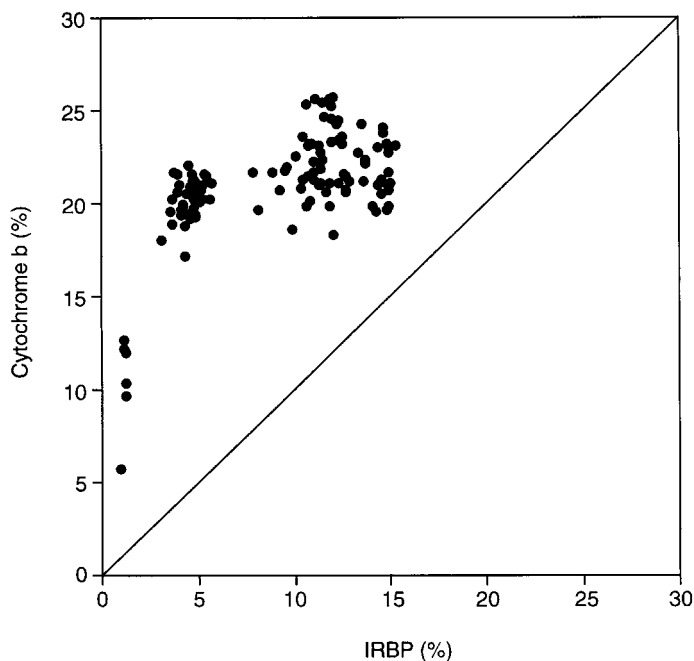
These factors encourage confidence in the principal phylogenetic results of this study, many of which corroborate hypotheses about marsupial relationships suggested by pre-

**Table VI.** Range of Uncorrected Percent Sequence Divergence (“*p*” distance) for IRBP (1158 bp) and Cytochrome *b* (1149 bp) Sequences<sup>a</sup>

Taxonomic comparisons	Percent sequence divergence	
	IRBP	Cytochrome <i>b</i>
Among <i>Didelphis</i> and <i>Philander</i> spp.	0.95–1.3	5.7–12.6
Among other didelphids	3.1–5.7	17.2–22.1
Between didelphids and other marsupials	9.3–15.3	18.6–25.7

<sup>a</sup>Data from both molecules were available for the following species: *Didelphis virginiana*, *D. marsupialis*, *D. albiventris*, *Philander mcilhennyi*, *Caluromys lanatus*, *Gliromia venusta*, *Metachirus nudicaudatus*, *Marmosops impavidus*, *Monodelphis adusta*, *Micoureus demerarae*, *Echymipera kalubu*, *Dromiciops gliroides*, *Caenolestes fuliginosus*, *Phascogale tapoatafa*, *Vombatus ursinus*, *Pseudochirops cupreus*.

vious researchers using other data and methods. In a few instances, however, our results challenge the weak evidential basis for currently accepted taxonomic groupings. Below we discuss the major points of agreement and disagreement between our analysis and earlier work, together with attendant implications for classification and evolutionary studies.



**Fig. 12.** Scatterplot of percent uncorrected pairwise sequence divergence (“*p*” distance) for IRBP versus cytochrome *b* among marsupials. The diagonal line represents the expected distribution of the data if both genes were evolving at the same rate. The species included in these comparisons are listed in the footnote to Table VI.

### Ameridelphian Paraphyly

Paraphyly of Ameridelphia (paucituberculates + didelphimorphs) is suggested by the grouping of our single paucituberculate exemplar (*Caenolestes*) with nondidelphid marsupial clades. This result was previously reported by Springer *et al.* (1997a) in their higher-order phylogenetic analysis of mammalian IRBP sequences, from which our own nondidelphid data were obtained. Our analysis suggests that ameridelphian paraphyly is robust to denser sampling of New World marsupials, although additional paucituberculate IRBP sequences (e.g., from *Lestoros* and *Rhyncholestes*) would be welcome.

Szalay (1982a,b) originally grouped paucituberculates and didelphimorphs on the basis of shared primitive tarsal characters, and did not attempt to defend the monophyly ("holophyly" in his systematic lexicon) of Ameridelphia. Indeed, Szalay's own phylogenetic diagrams (1982a, Fig. 10; 1982b, Fig. 4) appear to show caenolestids as basal to an unresolved node grouping didelphids with australidelphians. Subsequently, Luckett (1994) cited four characters as supporting ameridelphian monophyly, of which three were 12S nucleotide substitutions and the fourth was epididymal sperm pairing. However, 12S support for a sister-group relationship between caenolestids and didelphids (Gemmill and Westerman, 1994) is substantially eroded by denser taxon sampling (Springer *et al.*, 1994), and support for ameridelphian monophyly from genes encoding protamine P1 (Retief *et al.*, 1995) and phosphoglycerate kinase (Colgan, 1999) is likewise weak.

Although IRBP support for grouping *Caenolestes* with australidelphians is not as compelling as could be wished, ameridelphian monophyly is strongly contradicted by our data. This result, taken together with the weak support for ameridelphian monophyly from other sources, suggests that the evolutionary and biogeographic implications of ameridelphian paraphyly merit serious consideration. For example, the phylogenetic distribution of epididymal sperm pairing, uniquely shared by *Caenolestes* and didelphids (Biggers and DeLamater, 1965; Temple-Smith, 1987) and frequently cited in phylogenetic arguments as an ameridelphian synapomorphy (e.g., by Marshall *et al.*, 1990), could have two equally parsimonious interpretations if Ameridelphia is paraphyletic: either sperm pairing evolved independently in didelphids and caenolestids (as suggested by Springer *et al.*, 1997a), or sperm pairing evolved once in the marsupial common ancestor with a subsequent reversal in the australidelphian lineage. By contrast, the numerous anatomical resemblances between *Caenolestes* and peramelids reported by Osgood (1921) but subsequently ignored by most students of marsupial evolution merit careful evaluation as potential synapomorphies linking paucituberculates with one or more Old World clades.

### Didelphid Monophyly

IRBP sequence data provide the first compelling character-based evidence for didelphid monophyly, a result that corroborates phylogenetic interpretations of serological comparisons (Kirsch, 1977) and DNA-DNA hybridization distances (Kirsch *et al.*, 1997), but which raises questions about the apparent absence of strong nonmolecular support for this hypothesis. Is this simply a case where one dataset provides a clear phylogenetic signal that is lacking in another? Or have the morphological data bearing on this problem been incompletely or inappropriately analyzed?

As emphasized by Szalay (1982a,b, 1994), much of the literature on metatherian comparative morphology concerns variation in dental characters, particularly the molar occlusal traits frequently preserved in fossils. Although metatherian basicranial morphology is now receiving increased attention (e.g., by Archer, 1976; Wible, 1990; Wroe, 1997), much of the postcranial skeleton remains unexplored as a source of characters, and taxonomic variation in soft anatomical traits has (with a few exceptions) received only anecdotal treatment. Therefore, although didelphids do seem to lack unambiguous dental and basicranial synapomorphies, the absence of appropriately detailed comparative studies of most other organ systems prevents any general assessment of the morphological evidence at the present time.

Whereas morphological support for didelphid monophyly might be supplied by future studies of new character complexes, it is also possible that some well-known didelphid traits currently dismissed as plesiomorphies could be misinterpreted. The traditional assumption that the marsupial common ancestor was arboreal (Huxley, 1880; Winge, 1893; Dollo, 1899; Bensley, 1903), for example, has prompted many authors to interpret grasping hindfeet, prehensile tails, and other characters associated with tree-dwelling habits as primitive conditions. Yet some metatherian outgroups believed to be closely related to Marsupialia (e.g., *Pucadelphys*; see Rougier *et al.*, 1998) do not show strongly developed arboreal adaptations (Marshall *et al.*, 1995), nor do optimizations of ecology on biochemical trees suggest that a tree-dwelling lifestyle is primitive for marsupials (Springer *et al.*, 1997b). Future testing of didelphid monophyly with morphological characters should be rooted with appropriate outgroups rather than arboreal “ancestors” reconstructed to fit untested evolutionary scenarios.

### Caluromyine Paraphyly

The three genera currently placed in the subfamily Caluromyinae were first treated as a group by Reig (1955), who noted dental similarities shared with *Dromiciops* and other microbiotherians. In Reig *et al.*'s (1987) subsequent parsimony analysis, *Caluromys*, *Caluromysiops*, and *Glironia* were found to cluster together despite the fact that these taxa did not uniquely share the derived condition of any analyzed character. Instead, Reig *et al.* cited other traits to bolster their conviction that caluromyines form a natural group (op. cit., p. 72):

... significant synapomorphies linking the three genera are the broad supraoccipital [sic] process, which forms winglike ledges over the orbits; the sharpened, low, and narrow rostrum; the weakly developed and upright canines; the broad and rather inflated braincase; and the enlarged orbits. ... We have also seen that these genera are united by having big, broad palates with reduced or absent palatal vacuities, a reduced P<sup>1</sup>, dilambdodonty, a change in the proportion of the paracrista which is not united to the parastyle, trigonids longer than wide, and several other pairwise resemblances in molar teeth and the ear region. The discrepancies of *Glironia* in showing a primitive ear region and unreduced M4s, and of *Caluromysiops* in having a well-developed styler shelf and a complete set of styler cusps, as regard each other and one or the other of these two with respect to *Caluromys*, are quite swamped by the many other synapomorphies. These discrepancies may be considered good examples of heterobathmy in the evolution of molar character states, and of the polythetic nature of the concept of the taxon which unites the three genera. ... The three genera are also united in showing strictly arboreal, nocturnal, and lemur-like habits.

Unfortunately, this list of putative synapomorphies has yet to be effectively tested

in a parsimony analysis and many of the cited traits are unlikely to sustain the phylogenetic interpretation implied by this argument. Several supposedly diagnostic caluromyine character states are so ambiguously defined that homology assessment is impossible (e.g., “big, broad palates,” “enlarged orbits,” “lemur-like habits”), others could plausibly be interpreted as primitive marsupial conditions [e.g., “reduced or absent palatal vacuities” (see Marshall, 1979), some are simply incorrect (the canine fangs of *Caluromys* are not “weakly developed”), and some appear to be misconstrued (e.g., dilambdodonty, a condition that is strongly developed in many didelphines but only weakly expressed by caluromyines)]. Although a thorough reevaluation of the morphological evidence purported to support caluromyine monophyly is beyond the scope of this discussion, we agree with Szalay (1994, pp. 340–342) that the phylogenetic interpretation of these data is equivocal at best.

Molecular results supporting the monophyly of Caluromyinae consist solely of the DNA–DNA hybridization data analyzed by Kirsch and Palma (1995; see Fig. 5, above). Although neither Patton *et al.*'s (1996) cytochrome *b* comparisons (Fig. 6) nor our own data convincingly refute caluromyine monophyly, the absence of any support for this hypothesis in two independent sequencing studies invites alternative interpretations of basal didelphid relationships. In effect, our IRBP analysis securely brackets the phylogenetic position of caluromyines between two highly supported nodes: these taxa are definitely didelphids but they are clearly not didelphines. Because the sister-group relationship of *Caluromys* and *Caluromysiops* is not problematic; only the position of *Glironia* remains uncertain. Although the hypothesis that *Glironia* is sister to all extant didelphids is marginally better supported than other alternatives tested with our data, convincing phylogenetic resolution in this part of the didelphid tree must await future analyses incorporating data from other slowly evolving genes and from renewed study of morphology.

### Didelphine Monophyly

Monophyly of Didelphinae is a noncontroversial result that agrees with the conclusions of previous phylogenetic analyses of morphological data (Creighton, 1984; Reig *et al.*, 1987) and DNA–DNA hybridization results (Kirsch and Palma, 1995; Kirsch *et al.*, 1997). Despite this consensus, morphological character support for didelphine monophyly is not impressive. By comparison with caluromyines, didelphines have more carnassialized molars and more extensively fenestrated palates, but neither of these conditions is unique among marsupials and the polarity of metatherian palatal evolution is debatable (Marshall, 1979). Other didelphine synapomorphies potentially recoverable by optimizing morphological characters on most-parsimonious trees have yet to be identified, but our inspection of published data matrices suggests that additional nonmolecular support for this node is more likely to come from new anatomical sources than from the craniodental traits emphasized in past studies.

### Monophyly of a Large Opossum Group Including *Metachirus*

All previous analyses of didelphid phylogeny, morphological and molecular, have supported the monophyly of a group containing the four largest opossums (*Chironectes*, *Didelphis*, *Lutreolina*, and *Philander*), so our IRBP results simply add to the weight of

interdisciplinary evidence for this robust clade. By contrast, two alternative phylogenetic alliances of *Metachirus*—the fifth large-bodied didelphine genus—have been suggested by researchers using different datasets. Whereas our IRBP results agree with previous molecular analyses (Kirsch and Palma, 1995; Patton *et al.*, 1996) in supporting a sister-group relationship between *Metachirus* and the other large opossums, Creighton's (1984) parsimony analysis of morphological data (Fig. 3) clustered *Metachirus* with the small didelphines (*Monodelphis* + “marmosines”).

A sister-group relationship between *Metachirus* and the small didelphines was unambiguously supported in Creighton's (1984) study by just two characters, a flattened ectotympanic and absence of a pouch. However, both of these traits are variably expressed among nondidelphid polyprotodonts (Tate, 1947; Archer, 1976), so their phylogenetic interpretation within Didelphidae is crucially dependent on outgroup choice. Because Creighton's analysis was only rooted with *Dromiciops*, the robustness of his phylogenetic results to broader outgroup sampling is unknown. Reig *et al.* (1987) subsequently included presence/absence of a pouch in their morphological parsimony analyses, which more often than not suggested a sister-group relationship between *Metachirus* and the other large opossums.

Most other published compilations of potentially relevant nonmolecular character data are uninformative about the relationships of *Metachirus*. For example, Hershkovitz's (1992, p. 4) diagnosis of Marmosidae (*Metachirus* + *Monodelphis* + “marmosines”) consists almost entirely of tautologies (e.g., “tail prehensile or not”), uninterpretable descriptors (“skull light to moderately heavy”), and irrelevant plesiomorphies (“molars tritubercular”). Similarly, *Metachirus* exhibits the very widespread  $2N = 14$  karyotype currently thought to be primitive for Marsupialia, whereas all of the other large opossums exhibit the derived diploid count of 22 chromosomes (Reig *et al.*, 1977). The only exception to this discouraging absence of relevant nonmolecular data is the recent discovery that *Metachirus* shares with the other large opossums several attributes of the glans penis that are not found in caluromyines or in any of the small didelphines examined to date (Nogueira *et al.*, 1999).

Thus, although the monophyly of a large opossum group that includes *Metachirus* has not previously been espoused by morphological researchers, neither is it strongly contradicted by any nonmolecular data compiled to date. Instead, at least one recent anatomical study suggests that corroborative evidence from nontraditional character complexes may be forthcoming.

### **Relationships of *Thylamys*, *Lestodelphys*, *Gracilinanus*, and *Marmosops***

A sister-group relationship between *Thylamys* and *Lestodelphys* has been supported by most previous phylogenetic analyses of didelphid relationships and is not controversial. By contrast, the phylogenetic configuration suggested by our IRBP results for *Gracilinanus* and *Marmosops* with respect to *Thylamys* + *Lestodelphys* is unlike any previously suggested in the literature. In particular, the strong support in our data for the nested pattern  $\{[(Thylamys + Lestodelphys) Gracilinanus] Marmosops\}$  and for the monophyly of *Marmosops* is at striking odds with Kirsch and Palma's (1995) phylogenetic interpretation of their DNA–DNA hybridization experiments (Fig. 5).

The discrepancy between our results and Kirsch and Palma's (1995) is caused by

a single datum in their hybridization matrix: a very small value of  $\Delta T_m$  indicating the essential identity of DNA extracts from one specimen that they identified as *Gracilinanus agilis* and another specimen identified as *Marmosops dorothea*. As a consequence, these species clustered together in Kirsch and Palma's Fitch–Margoliash trees before joining other taxa in the sequence  $\{[(G. agilis + M. dorothea) M. parvidens] (Thylamys + Lestodelphys)\}$ . In our opinion, this result is not explicable except as a consequence of a voucher misidentification or a voucher/tissue mismatch. Because *Gracilinanus* and *Marmosops* are not difficult to distinguish morphologically (Gardner and Creighton, 1989), voucher misidentification seems unlikely. Instead, it is more probable that Kirsch and Palma's tissue sample of either *G. agilis* or *M. dorothea* was mismatched with the wrong voucher material. These species occur sympatrically (Anderson, 1997), so it is plausible that such a mismatch could have occurred in the field. In the assembly-line preparation protocols favored by many expeditions, whereby different numbers are assigned to tissue and voucher from the same specimen (Yates *et al.*, 1996), there is ample opportunity for tissue-voucher mismatches. Fortunately, hypotheses of tissue-voucher mismatch can be tested by extracting, amplifying, and sequencing DNA from both elements, a procedure that would be amply justified in the present case.

Because all of our specimens of *Marmosops* ( $N = 9$ ) and *Gracilinanus* ( $N = 2$ ) were processed one-at-a-time in the field (with tissue and voucher assigned the same number at the same time by the same person in every case), and because we personally compared each morphological voucher with types or with authoritative species descriptions (not keys) based on type material, it is unlikely that our phylogenetic results are artifacts of misidentification or mismatches.

### Node H

The node labeled H in Fig. 11 is common to our results and to some Fitch–Margoliash trees based on DNA–DNA hybridization distances (Kirsch *et al.*, 1995; Kirsch and Palma, 1995) but does not appear in any other published analyses of didelphid phylogeny. In effect, IRBP provides the first character-based support for this grouping of large and small opossums, a cluster that is strikingly inconsistent with most nonmolecular assessments of didelphine relationships. In particular, parsimony analyses of morphological characters (Creighton, 1984; Reig *et al.*, 1987) appear to support the monophyly of a clade that includes all of the small-bodied higher opossums (*Monodelphis* + “marmosines”; Figs. 3 and 4).

The morphological transformations purported to diagnose a small didelphine clade, however, are not compelling. In Creighton's (1984) analysis, for example, this group is only supported by homoplastic character transformations. Reig *et al.* (1987) scored the small didelphines as uniquely sharing a derived and unreversed morphology of the ectotympanic, but the character in question (number 31 in their matrix: “size, ectotympanic process”) is not well defined and the same feature has been differently scored by other investigators. Creighton (1984: character 28) recorded *Metachirus* as also sharing the derived morphology, whereas Goin and Rey (1997: character 11) scored *Monodelphis* as exhibiting the primitive condition. Possibly, subtle differences in defining ectotympanic states or the use of different exemplar species might explain such discrepancies, but if the underlying morphological variation is subject to alternative interpretations or intra-

generic sampling artifacts it cannot provide unequivocal support for the monophyly of small didelphines.

Thus, although Node H is not strongly supported by our data, the absence of compelling contradictory signal from published morphological data sets is noteworthy. It is also relevant that while Patton *et al.*'s (1996) analyses of cytochrome *b* sequences (Fig. 6) do not support node H, neither do they support the monophyly of a small didelphine group. The basal branching structure of the didelphine radiation, therefore, remains an open question.

### Relationships of *Marmosa*, *Micoureus*, and *Monodelphis*

A close relationship between species of *Marmosa* and *Micoureus* is common to most recent morphological and molecular analyses of didelphid relationships (e.g., Kirsch, 1977; Creighton, 1984; Kirsch *et al.*, 1995; Kirsch and Palma, 1995; Patton *et al.*, 1996). Indeed, both our study and Kirsch and Palma's (1995) suggest that *Marmosa* and *Micoureus* may not be reciprocally monophyletic groups. On the other hand, no modern phylogenetic study has included more than a few of the many species of *Marmosa* and *Micoureus* recognized by Tate (1933), so inadequate taxon sampling is a potential problem for interpreting the molecular data at hand. Because neither genus has been revised since Tate's pioneering monograph, a fresh assessment of the morphological basis for generic diagnoses in this complex is clearly overdue.

The sister-group relationship of *Monodelphis* to *Marmosa* + *Micoureus* indicated by our results is supported by all previous molecular analyses of didelphid relationships (Kirsch, 1977; Kirsch *et al.*, 1995; Kirsch and Palma, 1995; Patton *et al.*, 1996) but not by any morphological analysis published to date. Instead, both Creighton's (1984) and Reig *et al.*'s (1987) parsimony analyses of anatomical characters supported a sister-group relationship between *Monodelphis* and *Thylamys* + *Lestodelphys*. In Creighton's study, this cluster was unambiguously supported by four uniquely derived and unreversed characters (of the feet, auditory bullae, interorbital region, and gular glands). Among the didelphids scored in Reig *et al.*'s study, these three genera (together with the extinct genus *Thylatheridium*) uniquely shared a derived morphology of the last maxillary molar. By contrast, Goin and Rey (1997) tabulated morphological character data that they interpreted as supporting a sister-group relationship between *Monodelphis* + *Thylatheridium* on the one hand, and a monophyletic Marmosini (containing all other small didelphines) on the other.

These incongruent results concerning the relationships of *Monodelphis* provide the clearest example of conflict between molecular and morphological data that we have encountered in our review of didelphid systematic studies. Although some character incongruence might be resolved by a more critical approach to morphological homology assessment in future studies, and by using multiple outgroups (rather than single taxa or reconstructed ancestors) to root subsequent phylogenetic analyses, the current absence of any published morphological support for the pattern of relationships clearly indicated by all of the molecular data analyzed to date is remarkable. Possibly, adaptive convergence might explain some of the derived external and craniodental similarities among *Monodelphis*, *Thylamys*, and *Lestodelphys* (as suggested by Reig *et al.*, 1987; Goin and Rey, 1997), but very little is actually known about relevant aspects of the natural history of these taxa to substantiate such ad hoc conjectures.

### Directions for Future Research

Denser taxonomic sampling is a priority for future phylogenetic research on didelphid marsupials. Even our analysis, the most taxonomically comprehensive to date, includes only 30% of the currently recognized species, and most taxon-sampling schemes (including our own) have been based primarily on the current generic classification. Research from many sources, however, suggest that the current species-level taxonomy grossly underestimates true diversity among living didelphids (Muistrangi and Patton, 1997; Patton and da Silva, 1997; Patton *et al.*, 2000; Voss *et al.*, unpubl. observ.). Furthermore, several enigmatic taxa—including “*Gracilinanus*” *kalinowskii* with nonmolariform milk premolars (Voss *et al.*, submitted) and “*Marmosa*” *canescens* with  $2N = 22$  chromosomes (Engstrom and Gardner, 1988)—have never been represented in any phylogenetic dataset. Unique character combinations exhibited by previously unsampled didelphid taxa could significantly affect the structure of inferred relationships in future studies.

New sources of phylogenetic data is another priority. Molecules that are even more slowly evolving than IRBP could help resolve the deep-branching structure of the didelphid radiation, and more rapidly evolving molecules should contribute useful information about recent cladogenesis. Nontraditional morphological character systems—such as male reproductive anatomy, gamete ultrastructure, and postcranial osteology—may well provide support for nodes that remain weakly supported by molecular data. Even some traditional sources of morphological data have been only superficially surveyed for phylogenetically informative characters and merit renewed scrutiny.

As the preceding discussion repeatedly suggests, many of the discrepancies between molecular and morphological inferences about didelphid phylogeny are more apparent than real. Examples of hard incongruence (clear incompatibility between well-defined patterns of morphological versus molecular synapomorphy) are rare, suggesting that a fresh assessment of the anatomical evidence is in order. In particular, future phylogenetic analyses of morphological variation should be based on characters with unambiguously distinguishable states (not arbitrarily defined quantitative comparisons), and should be rooted with multiple outgroups (not singletons or reconstructed ancestors).

Finally, although many useful insights about the evolutionary process can be inferred from separate analyses of morphological and molecular datasets, we find the logic of total evidence (Eernisse and Kluge, 1993; Kluge and Wolf, 1993; Nixon and Carpenter, 1996; DeSalle and Brower, 1999) a compelling justification for combined analyses and an appropriate framework for interpreting the evolution of various character systems. In addition, fossil metatherians are more appropriate outgroups for rooting hypotheses of marsupial relationships than are the placental gene sequences usually employed for this purpose in molecular studies. Phylogenetic analyses of concatenated morphological/molecular marsupial datasets are, therefore, potentially more informative than the simple sum of their parts.

### ACKNOWLEDGMENTS

We thank George Barrowclough, Jeff Groth, Chris Norris, and Nancy Simmons for stimulating discussions and critiques of earlier drafts. David Swofford kindly made

test versions of PAUP\* available. For donations of tissue samples, we thank Jim Patton (MVZ), Bruce Patterson (FMNH), and Mark Engstrom (ROM). For assistance with laboratory work, we thank Julie Feinstein, Jeff Groth, and Marcelo Weksler. This work was supported in part by a Kalbfleisch Postdoctoral research grant to S.A.J. and by the Lewis B. and Dorothy Cullman Program for Molecular Systematic Studies. This is a contribution from the Monell Molecular Laboratory at the American Museum of Natural History.

## APPENDIX

The specimens we sequenced are listed below by Latin binomial, geographic origin (country, province/state/department, locality name), and museum catalog number (in parentheses). Other identifying numbers (if any) associated with samples preserved in institutional tissue collections are provided in square brackets. Museums are identified by their official acronyms as follows: American Museum of Natural History, New York (AMNH); Field Museum of Natural History, Chicago (FMNH); Instituto Nacional de Pesquisas da Amazônia, Manaus (INPA); Museo de Historia Natural de la Universidad Nacional Mayor de San Marcos, Lima (MUSM); Museum of Vertebrate Zoology, Berkeley (MVZ); Royal Ontario Museum, Toronto (ROM); University of Michigan Museum of Zoology, Ann Arbor (UMMZ); University of Wisconsin Museum of Zoology, Madison (UWZ).

*Caluromys lanatus*—Ecuador, Napo, Parque Nacional Yasuní (ROM 104570). *Caluromysiops irrupta*—New York Zoological Society (AMNH 244364). *Chironectes minimus*—Guyana, Barima-Waini, Waikerebi (ROM 98855 [FN 31677]). *Didelphis albiventris*—Paraguay, Canendiyu, 13.3 km N Curuguaty (UMMZ 134041 [GKC 783]); Paraguay, Presidente Hayes, 24 km NW Villa Hayes (UMMZ 134058 [GKC 816]). *Didelphis marsupialis*—Peru, Loreto, Río Gálvez (AMNH 272836 [RSV 2357], MUSM 13282 [RSV 2273]). *Didelphis virginiana*—Mexico, Yucatán, 1.5 km N Labna (ROM 96483 [FN 30300]). *Glironia venusta*—Brazil, Amazonas, alto Rio Urucu (INPA 2570 [MNFS 75]). *Gracilinanus microtarsus*—Brazil, São Paulo, Fazenda Intervalles (MVZ 182055 [MAM 38], MVZ 182056 [MAM 49]). *Lestodelphys halli*—Argentina (UWZ 22422 [Kirsch lab extract 775]). *Lutreolina crassicaudata*—Paraguay, Misiones, 2 km NE Ayolas (UMMZ 134018 [GKC 848], UMMZ 134019 [GKC 849]). *Marmosa lepida*—Guyana, Potaro-Siparuni, Iwokrama Reserve (ROM 107034 [F 38809]). *Marmosa murina*—Peru, Loreto, Río Gálvez (AMNH 272816 [RSV 2303], AMNH 272870 [RSV 2413]). *Marmosops impavidus*—Peru, Loreto, Río Gálvez (AMNH 272760 [RSV 2202], MUSM 13284 [RSV 2114]). *Marmosops noctivagus*—Ecuador, Napo, Parque Nacional Yasuní (ROM 105316 [F 37644]); Peru, Loreto, Río Gálvez (AMNH 272775 [RSV 2225], AMNH 272782 [RSV 2242], AMNH 272809 [RSV 2294], MUSM 13289 [RSV 2224], MUSM 13292 [RSV 2131]). *Marmosops parvidens*—Guyana, Potaro-Siparuni, Iwokrama Reserve (ROM 108920 [F 43900]); Guyana, Upper Takutu—Upper Essequibo, Karanambo (ROM 97938 [FN 33439]). *Metachirus nudicaudatus*—Peru, Loreto, Río Gálvez (AMNH 272780 [RSV 2236], MUSM 13293 [RSV 2329]). *Micoureus demerarae*—Peru, Loreto, Río Gálvez (AMNH 272667 [RSV 2029], MUSM 13294 [RSV 2085]). *Monodelphis adusta*—Peru, Loreto, Río Gálvez (AMNH 272695 [RSV 2086]). *Monodelphis emiliae*—Peru, Loreto, Río Gálvez (MUSM 13298 [RSV 2083]). *Phlander mcilhennyi*—Peru, Loreto, Río Gálvez (AMNH 272818 [RSV 2310], MUSM 13299

[RSV 2153]). *Thylamys pallidior*—Bolivia, Tarija, roadside above Cieneguillas (FMNH 162495 [NHB 76–97]).

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