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Molecular Phylogenetics of Australo–Papuan Possums and Gliders (Family Petauridae)

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Phylogenetic relationships within the possums of the family Petauridae, including their affinities with the family Pseudocheiridae, were inferred from DNA sequences obtained for the mitochondrial ND2 gene (1040 bp) combined with previously published partial 12S rDNA sequences. Short, deep internodes characterize some of the divergences obtained. The robustness of these nodes was assessed by several methods such as exclusion of taxa and partitioning of characters. In all analyses a monophyletic Pseudocheiridae was evident, whereas a monophyletic Petauridae was not as well supported. Within the Petauridae, *Gymnobelideus* was more closely related to *Dactylopsila–Dactylonax* than to *Petaurus*. This supports the results obtained from microcomplement fixation of albumin and DNA–DNA hybridization studies but conflicts with morphological data. © 2001 Academic Press

Key Words: **Petauridae; *Gymnobelideus*; *Petaurus*; Pseudocheiridae; ND2; phylogenetics.**

INTRODUCTION

Relationships within the diprotodontid marsupials (possums, koala, wombats, and kangaroos) are not well understood at the inter- or intrafamilial level. The only DNA sequencing study to include representatives from most diprotodontid families could not confirm monophyly for the diprotodontid families Phalangeridae, Petauridae, and Pseudocheiridae, possibly a result of the small amount of sequence that was used (395 bp of 12S rDNA) (Springer *et al.*, 1994). A suite of other data sets including morphology (e.g., Aplin and Archer 1987), DNA–DNA hybridization (e.g., Edwards and Westerman 1992; Kirsch *et al.*, 1997), serology (Kirsch, 1977), and microcomplement fixation of albumin (Baverstock *et al.*, 1990a) have also been used to resolve the affinities within the diprotodontids with varying degrees of success. Determining relationships in this group has been problematic, primarily because it is thought to have radiated over a brief period of time (Baverstock *et al.*, 1990a), possibly resulting in star-like phylogenies with short internodes. There is also extensive divergence between some taxa even within families, resulting in saturation of DNA sequences and long branches, thereby hindering phylogenetic resolution.

De Filippis and Moore (2000) found that sampling more characters improved resolution for short, deep internodes. The amount of sequence required to resolve affinities depends on several factors including the age of the internode and the rate of substitution (Saitou and Nei, 1986; De Filippis and Moore, 2000). Methods proposed for dealing with long branches and short internodes include differential weighting schemes across codons and substitution types, inclusion of additional taxa to reduce long branch lengths (Swofford *et al.*, 1996), and removal of problematic taxa from analyses (Lyons-Weiler and Hoelzer, 1997). However, in some cases it is not appropriate to exclude certain species as they may be the taxa of interest, and inclusion of additional taxa may not be possible in the case of monotypic lineages. Recently, Poe and Swofford (1999) have suggested that adding taxa could actually cause incorrect relationships to be inferred.

The Leadbeater's possum (*Gymnobelideus leadbeateri*) is an example of a monotypic lineage within the diprotodontids for which determination of its phylogenetic position has proven problematic. On the basis of serological data, Kirsch and Calaby (1976) placed *Gymnobelideus* with *Petaurus* (Petaurinae) and linked this group with the dactylopsilines (*Dactylopsila* and *Dactylonax*) and the pseudocheirines (e.g., *Pseudocheirus* and *Petauroides*). Additional support for an association between *Gymnobelideus* and *Petaurus* came from morphological (Aplin and Archer, 1987) and chromosomal (McKay, 1984) characters. Although *Gymnobelideus* and *Petaurus* both have a diploid number of $2n = 22$ (Murray *et al.*, 1990), the ancestral condition of the Phalangeroidea (McKay, 1984), the karyotypes of these species are quite different (Murray *et al.*, 1990). Kirsch and Calaby (1976) placed the petaurines, dactylopsilines, and pseudocheirines within one family (Petauridae), whereas Aplin and Archer (1987) split them into two sister families, Petauridae (comprising petaurines

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TABLE 1

Species (* Double-Stranded Sequence Was Obtained from These Individuals), Voucher Location (AM, Australian Museum; MV, Museum Victoria; DU, Deakin University; TM, Tasmanian Museum; CSIRO, Australian National Wildlife Collection), Collection Localities, and ND2 GenBank Accession Nos.

Species	Voucher	Locality	GenBank Accession No.
**Dactylonax palpator* (long-fingered triok)	EBU26447 (AM)	Tifalmin, New Guinea	AF300993
**Dactylopsila trivirgata* (common striped possum)	27902 (AM)	Boenau Village, New Guinea	AF300994
**Gymnobelideus leadbeateri* (Leadbeater's possum)	Unregistered (MV)	Unknown	AF300992
**Petauroides volans* (greater glider)	Unregistered (MV)	Unknown	AF300997
Petauroides volans (greater glider)	M16313 (CSIRO)	Coffs Harbour, Australia	—
**Petaurus norfolcensis* (squirrel glider)	SQC8 (DU)	Euroa, Victoria, Australia	AF300995
Petaurus norfolcensis (squirrel glider)	SQ1 (DU)	Euroa, Victoria, Australia	—
**Pseudocheirus peregrinus* (common ringtail possum)	Unregistered (MV)	Mornington Pen., Australia	AF300998
Pseudocheirus peregrinus (common ringtail possum)	Unregistered (MV)	Unknown	—
Petaurus breviceps (sugar glider)	Unregistered (MV)	Unknown	AF300996
**Petaurus breviceps* (sugar glider)	Unregistered (MV)	Unknown	—
Petaurus breviceps (sugar glider)	A1306 (TM)	Tasmania, Australia	—
Trichosurus vulpecula (common brushtail possum)	C30954 (MV)	Melbourne, Australia	AF300999

and dactylopsilines) and Pseudocheiridae. Alpin and Archer (1987) could identify only one synapomorphic character (the presence of a single precava) to support close links between the Petauridae and the Pseudocheiridae. However, a close relationship between these lineages has been supported by microcomplement fixation (Baverstock *et al.*, 1990a) and DNA–DNA hybridization (Kirsch *et al.*, 1997) studies. Similarly, the dactylopsilines and petaurines are also morphologically quite distinct from one another but are linked by the presence of dorsal stripes and dentition characterized by low-crowned molars with four rounded cusps and large first lower incisors (Flannery, 1994). Molecular studies (Edwards and Westerman, 1992; Baverstock *et al.*, 1990a) also indicate monophyly of *Petaurus, Gymnobelideus, Dactylopsila*, and *Dactylonax*.

Thus, the Petauridae, as currently defined, comprises four genera: *Petaurus* (five species), *Dactylonax* (monotypic), *Dactylopsila* (four species), and *Gymnobelideus* (monotypic). The Pseudocheiridae comprises two major lineages; one containing two monotypic genera (*Petauroides* and *Hemibelides*) and the other comprising four genera (*Pseudocheirus, Pseudochirops, Pseudocheirulus*, and *Petropseudes*).

The outstanding question is whether *Gymnobelideus* is most closely related to *Petaurus* or to *Dactylopsila–Dactylonax*. Biogeographically, the former appears more likely, with *Dactylopsila* and *Dactylonax* found predominantly in New Guinea, whereas *Petaurus* is more extensively distributed being found in eastern Australia and the New Guinea region. *Gymnobelideus* is found only in southeastern Australia. A close association between *Gymnobelideus* and *Petaurus* has been the traditional arrangement (Kirsch and Calaby, 1976; McKay, 1984; Aplin and Archer, 1987; Flannery, 1994). Molecular data is equivocal; both microcomplement fixation (Baverstock *et al.*, 1990a) and DNA–DNA hybridization (Edwards and Westerman, 1992) studies link *Gymnobelideus* with *Dactylopsila–Dactylonax*. Conversely, 12S ribosomal DNA sequences (Springer *et al.*, 1994) appear to support the more traditional arrangement of *Gymnobelideus* with *Petaurus*.

Here we use mitochondrial DNA sequence data from the nicotinamide dehydrogenase subunit 2 gene (ND2) and 12S ribosomal DNA (Springer *et al.*, 1994) to resolve the affinities of monotypic *Gymnobelideus*. We also consider the effects of taxon sampling and the amount of sequence data on the robustness of the tree topologies.

METHODS

Samples

Genomic DNA was isolated from tissue samples from 12 individuals representing eight species (Table 1) by the method of Gemmel and Akiyama (1996). All genera of Petauridae and both major lineages of the Pseudocheiridae were represented. *Trichosurus vulpecula* (Phalangeridae) was used as an outgroup along with the published sequence of the North American opossum, *Didelphis virginiana* (Didelphidae) (Janke *et al.*, 1994). In addition to the ND2 sequence data obtained in the present study, mitochondrial 12S rDNA sequences (Springer *et al.*, 1994) were available for four taxa examined here (representing each of the Petauridae lineages): *Gymnobelideus leadbeateri, Dactylopsila trivirgata, Petaurus breviceps*, and *Pseudocheirus peregrinus*. These were analyzed alone and in combination with ND2 (reduced to include the same taxa as were available for 12S).

Primer Design

In marsupials there has been a rearrangement of the tRNA genes that flank the 3′ end of the ND2 gene with

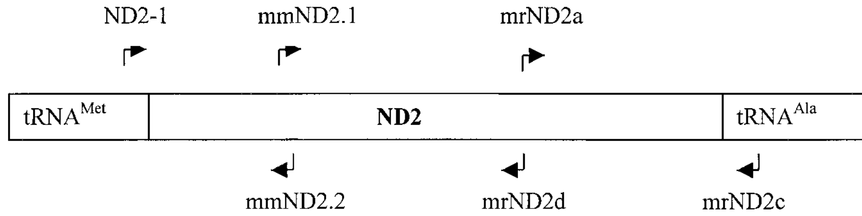

FIG. 1. Location of ND2 primers: mmND2.1 (gcaccattccacttytgagt), mrND2a (tatgaaacaaatcagctccaataa), mrND2c (gatttgcgttcgaatgatgcaag), mmND2.2 (actcaaaagtggaatggtgc), and mrND2d (ttcataggttrgtgaktgatttgatt). Primer sequences are given in parentheses (5′ to 3′).

respect to the gene order of other mammals (Pääbo *et al.*, 1991). To design a primer that permits the amplification of the entire ND2 gene, the $tRNA^{Ala}$ sequences from three marsupials, *T. vulpecula* and *Philander opossum andersoni* (Didelphidae) (Pääbo *et al.*, 1991), and *Burramys parvus* (Burramyidae) (unpublished), were aligned. A heavy-strand primer (mrND2c) was designed in a conserved region of $tRNA^{Ala}$, which, when used in conjunction with a primer located in $tRNA^{Met}$ (ND2-1; provided by the Field Museum of Natural History, Chicago), amplified the complete ND2 gene. With sequence data obtained with these primers, four additional internal primers (mmND2.1, mrND2.2, mrND2a, and mrND2d) that allowed complete, doubled-stranded sequence to be obtained (Fig. 1) were designed.

PCR amplifications were carried out in a reaction volume of 12.5 μL (25-μL reactions were used for sequencing) containing the following: 1× Bresatec reaction buffer (67 mM Tris–HCl, pH 8.8, 16.6 mM ammonium sulfate, 0.2 mg/ml gelatin, 0.45% Triton X-100), 3 mM $MgCl_2$, 0.5 units of *Taq* polymerase (Bresatec), 0.2 μM each dNTP, 0.16 μM each primer. PCR parameters for the primers ND2-1 and mrND2-c were 1 cycle at 95°C for 3 min, followed by 30 cycles of the following conditions: 93°C for 30 s, 56–58°C for 40 s, 72°C for 40 s, and then a final extension step of 72°C for 3 min.

To facilitate amplification with the remaining internal primers (mrND2.2, mrND2d, mrND2a) the complete ND2 product was diluted in sterilized water and used as a template for PCR. PCR parameters were essentially as outlined above; however, in some cases, $MgCl_2$ concentrations were reduced to 2 mM to prevent the amplification of multiple products. Extension times were also reduced to 20 s when the smaller fragments were amplified.

PCR products for sequencing were purified with the Bresatec PCR purification kit according to the manufacturer's protocol. Cycle sequencing was undertaken with the Promega *fmol* cycle-sequencing kit.

Phylogenetic Analyses

ND2 sequence and amino acid alignments were obtained with MEGA (Molecular Evolutionary Genetic Analysis) version 1.02 (Kumar *et al.*, 1993). Before phylogenetic analyses were conducted, factors that are known to effect such analyses, including base composition and transition:transversion ratios in ND2 (at each codon position) and 12S, were considered. Character changes in ND2 and 12S were plotted with MacClade version 3.08a (Maddison and Maddison, 1992). Saturation analyses were conducted for both ND2 and 12S sequences. The numbers of transitions and transversions were plotted against genetic distance for each pairwise comparison, including those with the outgroup *D. virginiana*. Comparisons between ingroup and outgroup taxa assist in identification of saturated data (Griffiths, 1997). Saturation is evident by the plateauing of the curve.

Differential weighting of substitutions and character exclusion were undertaken for ND2 in an attempt to mitigate the effects of saturation. Data partitioning schemes included weighting of third position transversions 2 and 10 times that of transitions, exclusion of third position transitions, and exclusion of all third codon changes.

Maximum-parsimony (MP) analyses were conducted with PAUP 4.0b4a (Swofford, 2000). Minimum-length trees were found with the heuristic search option (random addition, 10 replicates). Parsimony-uninformative characters were excluded. Unweighted parsimony analysis of total and partitioned data (as outlined above) was conducted. Consensus trees (50% majority rule) were computed if more than one equally parsimonious tree was found. Branch support was assessed by the decay index value (d) (Bremer, 1988) as calculated with the program Auto Decay 4.02 (Erikson, 1998).

Phylogenetic trees were also constructed with maximum-likelihood estimates (with empirical base frequencies) with the heuristic search option (with 10 random additions) under the HKY model (Hasegawa *et al.*, 1985) implemented by PAUP 4.0b4a (Swofford, 2000).

Neighbor-joining (NJ) analyses (Saitou and Nei, 1987) were conducted with Kimura's two-parameter (Kimura, 1980), Tajima–Nei (Tajima and Nei, 1984), F84 (Felsenstein, 1984), and HKY85 (Hasegawa *et al.*, 1985) distance options in PAUP 4.0b4a (Swofford, 2000).

Nodal support was assessed by bootstrapping (1000 replicates) (Felsenstein, 1985) for all analyses.

TABLE 2

Transition:Transversion Ratio for Pairwise Comparisons in ND2 (above Diagonal) and 12S (below Diagonal)

Taxon	*D. palpator*	*D. trivirgata*	*G. leadbeateri*	*P. norfolcensis*	*P. breviceps*	*P. peregrinus*	*P. volans*	*D. virginana*
D. palpator	—	1.41	0.96	0.89	0.95	0.72	0.78	0.61
D. trivirgata	ne[a]	—	0.94	1.04	1.03	0.98	0.99	0.81
G. leadbeateri	ne	1.73	—	0.94	0.99	1.05	1.18	0.81
P. norfolcensis	ne	ne	ne	—	3.95	0.94	1.01	0.68
P. breviceps	ne	1.89	2.64	ne	—	1.06	1.09	0.68
P. peregrinus	ne	2.50	2.80	ne	1.71	—	1.71	0.66
P. volans	ne	ne	ne	ne	ne	ne	—	0.71
D. virginiana	ne	0.72	0.79	ne	0.83	0.75	ne	—

[a] ne, specimen not examined.

Spectral analysis (Hendy and Penny, 1993) was conducted to permit a consideration of the sequence data independent of phylogenetic trees. Spectral analyses enable the source of the ambiguity in relationships to be assessed and allow alternative groupings, which may be overlooked if only phylogenetic trees are considered, to be evaluated. Support for a split (which is any bipartition of a pair of sequences) is a function of the number of nucleotide changes that correspond to that split. The conflict is the sum of all of the other splits that contradict the partitioning of the taxa at the first split (Lento *et al.*, 1995). This analysis was undertaken with the program Spectrum 2.0 (Charleston, 1998; Charleston and Page, 1997) with the Kimura two-parameter distance (Kimura, 1980).

RESULTS

Sequence Analyses

Sequence for 1040 bp of the ND2 gene was obtained for all 12 individuals examined (GenBank accession numbers are given in Table 1). Double-stranded sequence was obtained for a representative of each species (individuals are indicated with an asterisk in Table 1). There were 523 variable sites between ingroup taxa of which 403 were parsimony informative. The majority of these informative changes (245) occurred at the third codon, followed by 114 at the first and 44 at the second codons. For 12S there were 142 variable sites among ingroup taxa, of which 43 were parsimony informative.

ND2 was characterized by a paucity of guanine residues, occurring at an average frequency of only 0.08, whereas adenine residues were present in excess at a frequency of 0.35. Thymine and cytosine were present at frequencies of 0.31 and 0.25, respectively. The scarcity of guanines was most extreme at the first codon. Overall, purines accounted for less than half of the nucleotides (0.43). For 12S, the deficiency of guanine residues (0.19) was not as extreme as that in ND2, and purines accounted for more than half of the nucleotides (0.56).

The majority of changes in ND2 were between cytosine and thymine (from 72 to 335) and between cytosine and adenine (55–175). There were few transversional changes between guanine and cytosine (2–23) or between thymine and guanine (3–14). Transversions between adenine and thymine were more common (23–146). Transitions between guanine and adenine were underrepresented (3–98). For 12S, transitions accounted for most of the substitutions, with changes between cytosine and thymine occurring the most frequently (9–36). Transitions among guanine and adenine residues (7–26) were also common. Transversions between guanine and thymine were rare, ranging from 0 to 3 changes, as were those between cytosine and guanine (1–4) whereas those between cytosine and adenine (2–16) and between adenine and thymine (3–18) were more common.

The transition:transversion ratios for ND2 ranged from 3.95 for intrageneric comparisons to 0.72 for interfamilial comparisons (Table 2). However, for the majority of comparisons there was an equal number of transitions and transversions, indicating that transitions were approaching saturation. For 12S, transitions outnumbered transversions, with ratios ranging from 2.80 to 1.71 for interfamilial comparisons (Table 2). The transition:transversion ratio at each codon position was also calculated for ND2 (Table 3). There was a bias toward transitions at the first and second codons, which was most extreme in the comparison involving *P. breviceps* and *P. norfolcensis* (the ratio was 4.5 at the first and 8.0 at the second codons). For the third codon the ratio was generally less than 1.0 (Table 3), again suggesting that transitions were approaching saturation.

ND2 sequence divergences ranged from 1.57 to 2.47% within species and averaged 11.19% between individuals of *P. breviceps* and *P. norfolcensis* (Table 4). Intergeneric divergences within the families Petauridae and Pseudocheiridae ranged between 19.76 and 31.66%. Interfamilial divergences among the families Petauridae, Pseudocheiridae, and Phalangeridae ranged from 24.62 to 33.04% (Table 4). Sequence diver-

TABLE 3

Pairwise Comparison of Transition:Transversion Ratio at the First, Second, and Third Codon Positions of ND2

	D. palpator			*D. trivirgata*			*G. leadbeateri*			*P. norfolcensis*			*P. breviceps*			*P. peregrinus*			*P. volans*		
Taxon	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
D. palpator	—	—	—																		
D. trivirgata	1.8	2.4	1.2	—	—	—															
G. leadbeateri	0.9	2.7	0.8	0.8	2.0	0.8	—	—	—												
P. norfolcensis	1.0	2.0	0.7	1.2	1.8	0.9	0.9	3.3	0.7	—	—	—									
P. breviceps	0.9	2.2	0.8	1.3	1.8	0.8	1.0	2.9	0.8	4.5	8.0	3.6	—	—	—						
P. peregrinus	0.8	1.1	0.6	1.1	1.2	0.9	0.9	4.3	0.9	1.3	2.0	0.7	1.0	2.3	0.9	—	—	—			
P. volans	0.9	1.9	0.6	1.3	1.6	0.8	1.3	6.8	0.9	1.3	2.3	0.8	1.3	2.3	0.9	1.5	2.3	1.7			

gences in 12S were less than those for ND2, ranging from 11.46% for intergeneric comparisons to 16.71% for interfamilial comparisons (Table 4).

High levels of divergence and transition:transversion ratios close to 1.0 indicated that saturation was likely to be a factor in ND2. Saturation of transitions was evident at the third codon (Fig. 2a), suggesting that exclusion or differential weighting of third positions could increase phylogenetic resolution. Transitions at first and second codons were not saturated (Fig. 2a). Transversions were not saturated in ND2 and accumulated linearly with genetic distance (Fig. 2b). For 12S, transversions were not saturated but there was a more complex pattern for transitions, which accumulated linearly for ingroup comparisons and then plateaued in the outgroup comparisons (Fig. 2c).

Phylogenetic Analyses

Analysis of base composition indicated that nucleotides did not occur at equal frequencies in either ND2 or 12S, suggesting that a distance measure based on a model that accounts for base composition disparity would be more appropriate than the Kimura two-parameter measure (Kimura, 1980). Thus, in addition to Kimura's two-parameter measure, Tajima–Nei (Tajima and Nei, 1984), Felsenstein's F84 (Felsenstein, 1984), and Hasegawa–Kishino–Yano (HKY85) (Hasegawa *et al.*, 1985) distance measures were calculated. Neighbor-joining trees derived from each of these distances had identical topologies (Fig. 3) with only the level of bootstrap support for nodes differing between them (Fig. 4a, Table 5). *Petauroides* and *Pseudocheirus* formed one clade with high bootstrap support (91–93%). The Petauridae group (*Petaurus, Dactylopsila, Dactylonax*, and *Gymnobelideus*) comprised a second clade, although bootstrap support was lower (62–67%). Within this clade, *P. breviceps* and *P. norfolcensis* clustered together (100%), as did *Dactylopsila, Dactylonax*, and *Gymnobelideus* (54–58%). Both 12S and combined data sets produced trees with identical topologies (Fig. 4a). *Gymnobelideus* clustered with *Dactylopsila* with the highest bootstrap support (98%) derived from the combined data. This group was linked to *Petaurus* and

TABLE 4

Percentage Sequence Divergence for ND2 (above Diagonal) and 12S (below Diagonal) Pairwise Comparisons Corrected by Kimura Two-Parameter Method

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *D. palpator*	—	19.76	28.42	26.07	26.21	25.71	26.42	26.50	27.43	27.28	24.62	25.29	27.92	38.30
2. *D. trivirgata*	ne[a]	—	24.58	26.15	26.87	26.02	26.30	26.56	30.74	30.73	28.25	29.84	28.68	39.45
3. *G. leadbeateri*	11.46	ne	—	30.10	30.40	30.42	31.66	31.17	33.04	32.73	28.00	28.98	29.63	42.02
4. *P. norfolcensis* 8	ne	ne	ne	—	1.47	10.59	11.53	11.28	31.47	32.57	28.21	28.95	31.12	39.34
5. *P. norfolcensis* 1	ne	ne	ne	ne	—	10.70	11.64	11.39	31.77	32.87	29.09	29.85	31.55	39.18
6. *P. breviceps*	ne	ne	ne	ne	ne	—	1.57	2.47	30.66	31.11	30.19	31.60	28.78	37.74
7. *P. breviceps* 15079	ne	ne	ne	ne	ne	ne	—	1.67	31.33	31.79	30.23	31.78	29.37	38.55
8. *P. breviceps* TAS	14.82	ne	14.72	ne	ne	ne	ne	—	30.53	30.98	29.75	31.13	28.02	37.87
9. *P. volans* M16313	ne	ne	ne	ne	ne	ne	ne	ne	—	2.38	23.65	23.34	29.22	41.36
10. *P. volans* 193	ne	ne	ne	ne	ne	ne	ne	ne	ne	—	24.12	23.81	27.87	41.37
11. *P. peregrinus* MP	13.95	ne	16.71	ne	ne	ne	ne	16.46	ne	ne	—	2.47	27.68	36.73
12. *P. peregrinus* 98490	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	—	27.85	38.05
13. *T. vulpecula*	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne	—	36.33
14. *D. virginiana*	31.30	ne	35.86	ne	ne	ne	ne	33.73	ne	ne	30.73	ne	ne	—

[a] ne, specimen not examined.

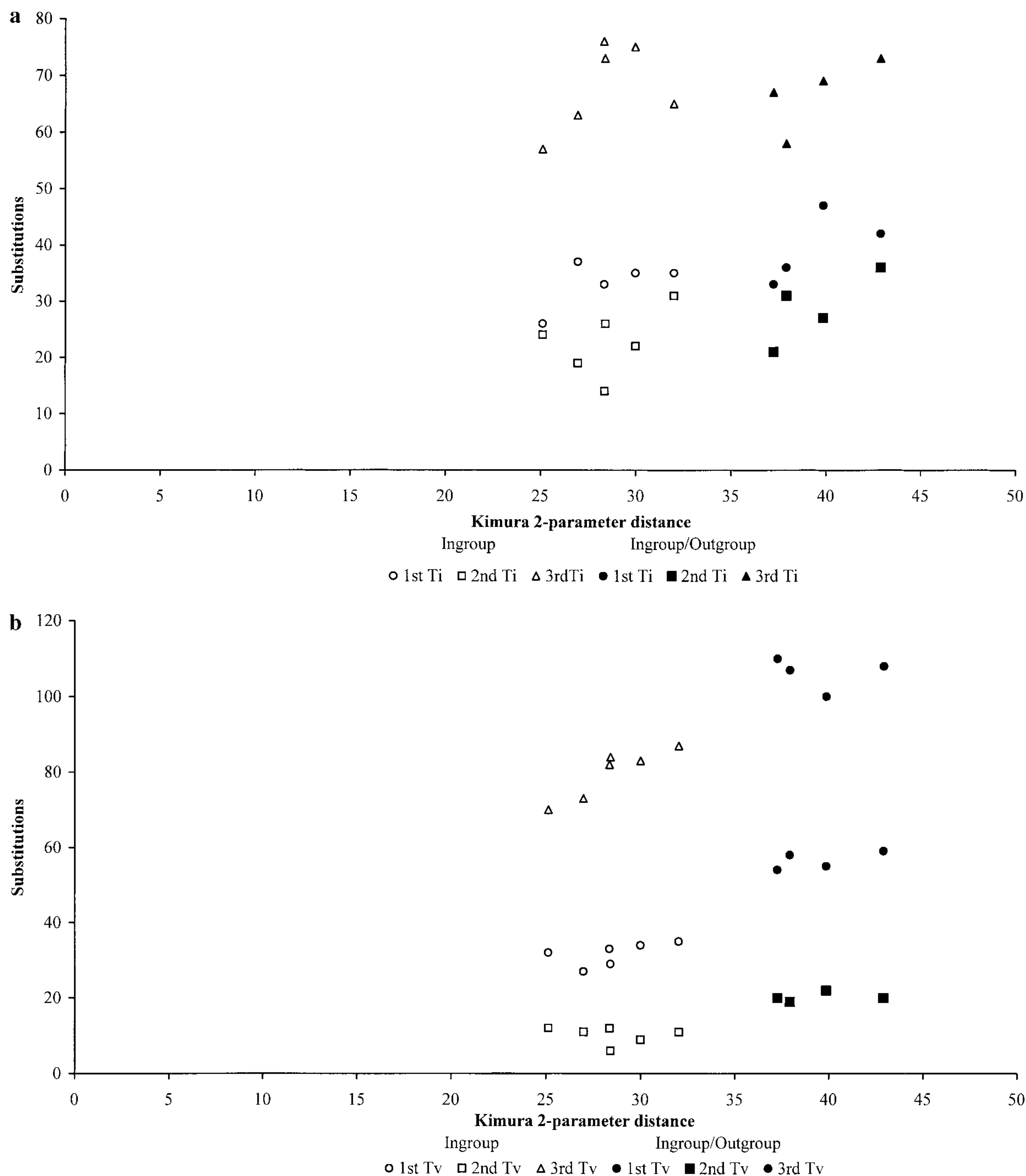

FIG. 2. Saturation plots for ND2 (a and b) and 12S (c). The number of transitions (a) and transversions (b) at each codon position of ND2 plotted against genetic distance (Kimura two-parameter distance). The total number of transitions and transversions in 12S plotted against genetic distance. Ingroups are Petauridae and Pseudocheiridae and the outgroup is *Didelphis virginiana*.

there was moderate support for this arrangement (Table 5). When the ND2 data was limited to include only those taxa available for 12S, the positions of *Petaurus* and *Pseudocheirus* were interchanged.

The results from maximum-parsimony analyses of ND2 were considerably less consistent, with nine different topologies resulting from the various weighting schemes and data partitions (Fig. 4, Tables 5 and 6). All analyses identified a sister relationship between *Pseudocheirus* and *Petauroides*, with the highest bootstrap support (98%) when either all transitions or just third position transitions were excluded and the lowest bootstrap support (73%) when all third position changes were removed. There was 100% bootstrap sup-

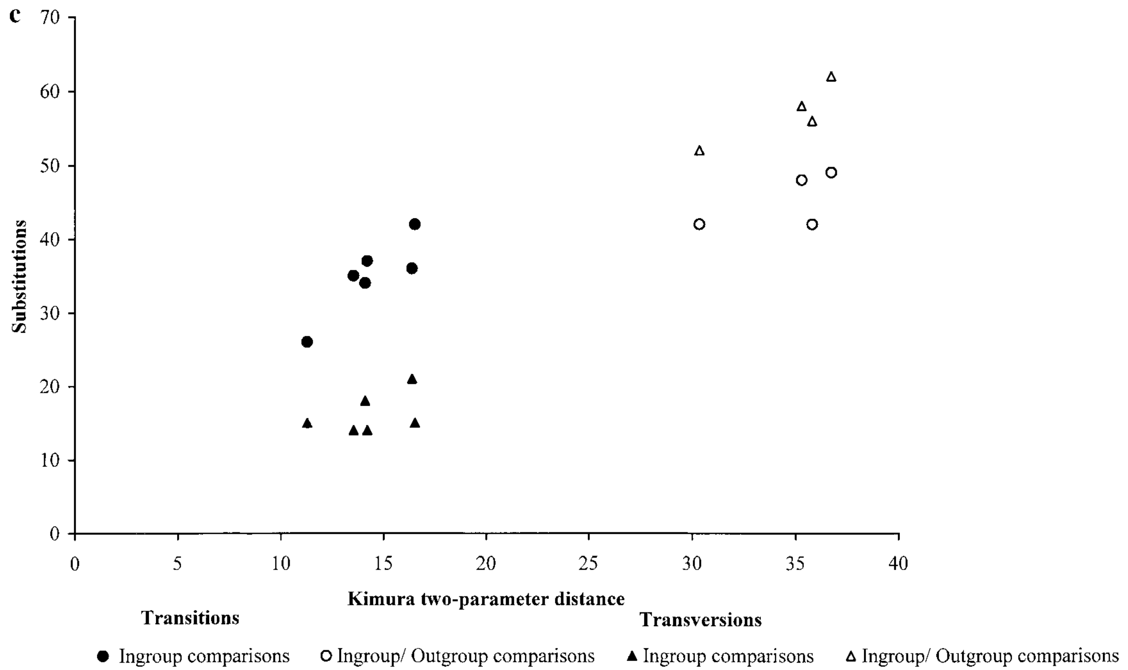

FIG. 2—*Continued*

port and high Bremer support (d = 45) for the association of *P. breviceps* and *P. norfolcensis* in all analyses. Most other clades had lower Bremer support (d = 1 to d = 8). *Dactylopsila* and *Dactylonax* clustered together in most analyses, with the amount of bootstrap support highest when transitions were excluded (94%). Analyses based only on third positions clustered *Dactylopsila* with *Gymnobelideus* and clustered *Dactylonax* with *Pseudocheirus–Petauroides* (Fig. 4i).

Unweighted parsimony clustered *Gymnobelideus* with *Pseudocheirus–Petauroides*; these were in turn linked to *Dactylopsila–Dactylonax*, with *Petaurus* basal to this group (Fig. 4e). These associations, however, lacked nodal support (Table 5). When third position changes were excluded, 11 trees of equal length were produced (Table 6) and in the majority-rule consensus tree the position of *Gymnobelideus* was unresolved. *Dactylopsila–Dactylonax* clustered with *Petaurus* and *Gymnobelideus* clustered with *Pseudocheirus–Petauroides* in transversion parsimony (Fig. 4c) but with low bootstrap support.

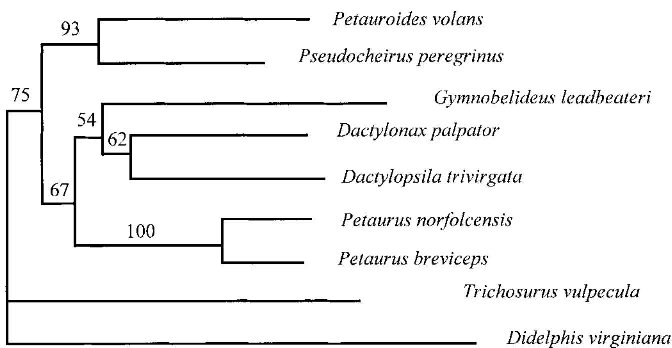

FIG. 3. ND2 neighbor-joining tree (Kimura two-parameter) with bootstrap values shown above branches. *Didelphis virginiana* and *Trichosurus vulpecula* are the outgroups.

Combined ND2–12S and 12S data analyses produced the same topology (Fig. 4a) in which *Gymnobelideus* and *Dactylopsila* were sister taxa (85 and 56% bootstrap support, respectively). *Petaurus* was linked to this group (57 and 74%, respectively) (Fig. 4a, Table 5). Exclusion of third positions also produced this topology. The *Gymnobelideus–Dactylopsila* clade was well supported (91%, Bremer support d = 9) when only third position changes were included for the combined data (Fig. 4b, Table 5). Transversion parsimony of the combined sequences produced a single tree in which *Gymnobelideus* was the sister taxon of *Pseudocheirus* and these were linked to *Dactylopsila*. *Petaurus* was basal to this group. There was, however, no significant bootstrap support for these associations (Table 5). Transversion parsimony of 12S sequences produced two trees of equal length (Table 6); *Gymnobelideus* and *Petaurus* formed a clade linked to either *Pseudocheirus* or *Dactylopsila* (Figs. 4f and 4g).

Maximum-likelihood analysis of total ND2 clustered *Gymnobelideus* with *Pseudocheirus* and clustered *Petaurus* with *Dactylopsila–Dactylonax* but with less than 50% bootstrap support (Fig. 4c). The 12S tree placed *Gymnobelideus* with *Petaurus* (Fig. 4f), whereas in the combined data *Gymnobelideus* clustered with *Dactylopsila* (Fig. 4a, Table 5).

To assess the impact that taxon sampling had on

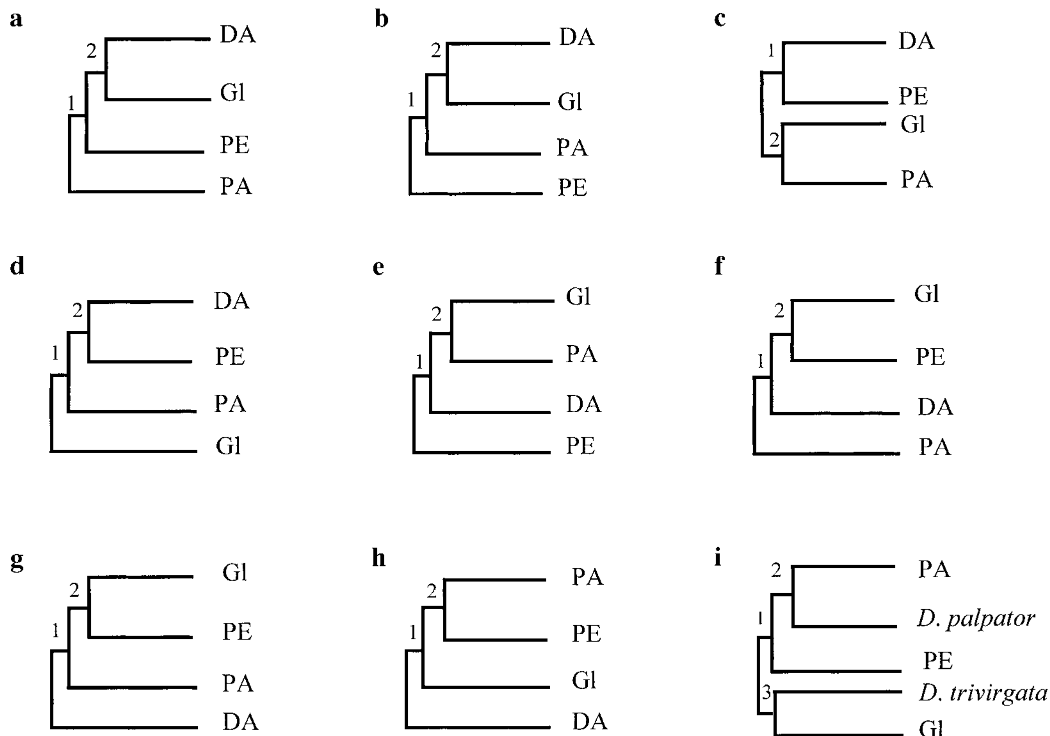

FIG. 4. Phylogenetic tree topologies (a–i). Nodes are numbered, and corresponding bootstrap and decay values are shown in Table 5; see Table 5 for abbreviations.

phylogenetic resolution, *Dactylopsila, Dactylonax, Gymnobelideus*, and *Petaurus* were excluded in turn from the total ND2 analysis. Exclusion of *Gymnobelideus* from the analyses increased bootstrap support for the node defining the Petauridae (*Petaurus, Dactylopsila*, and *Dactylonax*), from less than 50% bootstrap support to 63% in maximum-parsimony and from ca. 60 to 80% in neighbor-joining. Interestingly, the removal of *Dactylonax* and *Gymnobelideus* improved support for the node defining the Petauridae from 80 to 90% in the NJ tree, whereas the removal of *Dactylopsila* instead reduced nodal support to 51%. When both *Dactylopsila* and *Dactylonax* were removed, *Gymnobelideus* was linked to *Pseudocheirus–Petauroides* with bootstrap support of 51% in MP. The NJ tree placed *Gymnobelideus* with *Petaurus* (with less than 50% bootstrap support). When *Petaurus* was removed, bootstrap support for the node linking the remaining Petauridae (*Gymnobelideus, Dactylopsila*, and *Dactylonax*) was 90% in the NJ tree and 52% in the MP tree.

Spectral analyses of the ND2 data revealed strong support for the associations of *P. norfolcensis* with *P. breviceps* and *Petauroides* with *Pseudocheirus* (Fig. 5). Both clades had high support and minimal conflict. In contrast, the affinities of *Gymnobelideus* were less resolved. There were equal amounts of support and conflict for *Gymnobelideus* grouping with *Dactylopsila* or *Dactylonax*. There was also limited support for a group containing these three taxa. There was, however, no support for any arrangement that placed *Gymnobelideus* with *Petaurus*. Spectral analyses of the 12S and combined data also showed some support for the placement of *Dactylopsila* with *Gymnobelideus* but there was more conflict than support for this arrangement (Fig. 5). 12S provided minimal support and considerable conflict for the placement of *Gymnobelideus* with *Petaurus*.

DISCUSSION

Phylogenetic Resolution

The ability of phylogenetic analyses to resolve relationships is influenced by the degree to which the data fit the assumptions of the model being employed. Heterogeneity in base composition, transition:transversion ratios, and saturation can all affect resolution. For the ND2 data it was apparent that base composition was not equal, thereby violating one of the assumptions required for the Kimura two-parameter model. Nevertheless, for the neighbor-joining analyses, identical topologies were obtained regardless of the model employed to determine distance.

The effects of differential weighting schemes on the topologies obtained from parsimony analyses were

TABLE 5

Bootstrap Support for Nodes 1 and 2 (as Defined in Figs. 4a–4i) for Parsimony (Bremer Support Is Given in Parentheses), Neighbor-Joining, and Maximum–likelihood Trees

Tree topology	Gene	Parsimony							ML	Neighbor-joining			
		MP	Ex 3rd	Only 3rd	1,0	2,1	10,1	Tv		K2P	TN	F84	HKY
DA	ND2	*	68 (3)	—	77 (18)	— (3)	72 (27)	94 (8)	84	67	55	54	56
PE	ND2	100 (45)	100 (26)	100 (19)	100 (108)	100 (61)	100 (189)	100 (29)	100	100	100	100	100
PA	ND2	92 (5)	73 (0)	98 (5)	98 (49)	95 (23)	97 (93)	98 (14)	95	93	92	92	91
a	ND2									67,54	62,58	66,56	66,56
	NDT		59,* (1,1)		*,*								
	12S	74,56 (3,1)								82,63	83,63	82,59	82,62
	Com	57,85 (2,8)	77,68 (7,6)		68,61 (5,6)	*,74 (1,7)			54,60	64,98	65,97	66,98	70,98
b	NDT	55,53 (1,3)		*,83 (0,6)		52,62 (2,3)			*,55	58,88	56,90	52,89	57,91
	Com			*,91 (1,9)									
c	ND2		*,*			*,* (2,0)		54,* (3,1)	*,*				
d	ND2				*,*	*,* (0,2)	*,* (2,1)						
e	ND2	*,*	*,*										
	NDT						*,*						
	Com							52,* (3,3)					
f	12S							*,61 (0,1)	66,60				
g	12S							*,61 (0,1)					
h	NDT				*,*		*,*						
	Com						*,*						
i	ND2			*,*,* (2,3,3)									

Note. Tree topologies are referred to with the letters a–i. Clades DA (*D. trivrigata* and *D. palpator*), PE (*P. breviceps* and *P. norfolcensis*), and PA (*P. peregrinus* and *P. volans*) are represented by both taxa in parentheses for the full ND2 data set and by the first taxon only in the truncated ND2 (NDT), 12S, and combined ND2–12S (Com) data sets. Nodes with less than 50% bootstrap support are marked with an asterisk. MP, maximum parsimony; Ex 3rd, excluding third position changes; Only 3rd, including only third position changes; 1, 0, excluding third position transitions; 2, 1 and 10, 1, weighting third position transversions 2 times and 10 times that of transitions; Tv, transversions only; ML, maximum-likelihood. Neighbor-joining methods, Kimura two-parameter (K2P), Tajima–Nei (TN), Felsenstein (F84), Hasegawa–Kishino–Yano (HKY) models.

more pronounced. Lyons-Weiler and Hoelzer (1997) suggested that application of less weight to more rapidly evolving sites could not only alleviate the effects of homoplasy but could also assist in dealing with long branches. The indication that third position transitions in ND2 were saturated suggested that application of less weight to these sites might improve the resolution of deeper nodes; however, this was not the case and none of the topologies produced had significant bootstrap support. Elimination of transitions from the analyses also caused a loss of resolution. By the exclusion of characters, potentially informative data are also lost. For example, in ND2 more than half of the parsimony-informative sites are at the third position; exclusion of these sites or even just elimination of transitions effectively removes between one half and one quarter of the data. Björklund (1999) and Källersjo *et al.* (1999) have argued that third position changes, although highly homoplasic, contain the most phylogenetic structure. When only third position changes were considered from the combined ND2 and 12S data there was in fact high bootstrap support for the association of *Gymnobelideus* with *Dactylopsila*.

Baverstock *et al.* (1990a) reported the presence of short, deep internodes in the diprotodontids. Such internodes were also apparent in the present study, particularly the nodes involving *Gymnobelideus, Dactylopsila–Dactylonax*, and *Petaurus*. The amount of sequence that is required to resolve relationships among three species is dependent upon the length of the internode between the first and the second speciation event (T2) and the amount of evolution beyond the second speciation event (T1) (Moore and DeFilippis, 1997). Saitou and Nei (1986) expressed this relationship as R, which is the fraction of the total evolutionary time between the first and the second speciation event, where $R = T2/(T1+T2)$. Accordingly, 1000 bp of sequence would be required to recover the correct topology (with 0.95 probability) when $R = 0.2$ and 4200 bp would be needed when $R = 0.1$. In the NJ tree from the combined data (Fig. 4a), in which *Gymnobelideus* and *Dactylopsila* are sister taxa and are linked to *Petaurus*, R is 0.19. This suggests that the 1040 bp of ND2 sequence were adequate to resolve this node. DeFilippis and Moore (2000) demonstrated empirically that increasing the amount of sequence was also useful in increasing bootstrap support for short, deep internodes. In the present study, as the amount of sequence was increased, support for the node linking *Gymnobelideus* with *Dactylopsila* improved from 63% for 395 bp of 12S rDNA to 88% for 1040 bp of ND2, to 98% for 1435 bp of combined 12S rDNA and ND2.

TABLE 6

Tree Statistics: Number of Informative Characters, Length (Number of Trees), Consistency Index (CI), Retention Index (RI), and Rescaled Consistency Index (RC) for Each Data Set (ND2, All Taxa; NDT, Truncated Taxa Set, 12S, all taxa; Com, combined ND2 and 12S)

	Measure	Maximum parsimony	Ex 3rd	Differential weighting (1,0)	Differential weighting (2,1)	Differential weighting (10,1)	Transitions only	Only 3rd
ND2	Inform	423	165	400	427	258	427	224
	Length (trees)	1182 (2)	365 (11)	736 (1)	1573 (2)	787 (1)	4597 (1)	463 (1)
	CI	0.569	0.603	0.537	0.554	0.559	0.520	0.484
	RI	0.647	0.710	0.682	0.649	0.621	0.653	0.448
	RC	0.344	0.428	0.366	0.360	0.347	0.340	0.217
NDT	Inform	186	138	209	219	114	219	137
	Length (trees)	373 (1)	72 (1)	306 (2)	624 (1)	231 (2)	2033 (1)	240 (1)
	CI	0.660	0.659	0.605	0.679	0.671	0.618	0.571
	RI	0.317	0.347	0.271	0.386	0.333	0.247	0.248
	RC	0.209	0.229	0.164	0.194	0.224	0.618	0.142
12S	Inform	55	—	—	—	—	—	16
	Length (trees)	83 (1)	—	—	—	—	—	26 (2)
	CI	0.687	—	—	—	—	—	0.615
	RI	0.395	—	—	—	—	—	0.375
	RC	0.272	—	—	—	—	—	0.231
Com	Inform	229	115	252	262	157	262	153
	Length (trees)	457 (1)	221 (1)	389 (1)	709 (1)	317 (1)	2123 (1)	268 (1)
	CI	0.663	0.670	0.622	0.678	0.669	0.618	0.571
	RI	0.328	0.365	0.297	0.294	0.331	0.247	0.248
	RC	0.217	0.245	0.185	0.200	0.222	0.152	0.142

Note. Maximum parsimony analyses; Ex 3rd, excluding third position changes; Only 3rd, including only third position changes; 1,0, excluding third position transitions; 2,1 and 3,1 weighting third position transversions 2 times and 10 times that of transitions; and including transversions only (transversion parsimony).

Lyons-Weiler and Hoelzer (1997) and Graybeal (1998) found that the addition of more taxa in cases in which there are long branches could increase the accuracy of phylogenetic trees by the reduction of long branch lengths. By limitation of the total ND2 data set to include only single species of *Petaurus, Dactylopsila, Gymnobelideus*, and *Pseudocheirus*, monophyly of the Petauridae was not obtained in either NJ or MP analyses. However, by inclusion of an additional species of *Petaurus*, the Petauridae (*Petaurus, Dactylopsila, Gymnobelideus*) was identified as monophyletic. Poe and Swofford (1999) found that the addition of some taxa could lead to the inference of spurious relationships. This effect was also apparent from the results of the present study. Removal of *Gymnobelideus* from analyses improved the support for monophyly of the remainder of the Petauridae, suggesting that *Gymnobelideus* was creating instability in the phylogeny. Removal of *Dactylonax* increased confidence in the NJ tree but reduced resolution in the MP tree.

Phylogenetic Relationships

Although mindful of the limitations of resolution described in the previous section, the present study did identify several well-supported relationships. Monophyly of Pseudocheiridae as represented by *Petauroides* and *Pseudocheirus* was strongly supported by ND2 in accordance with microcomplement fixation (Baverstock *et al.*, 1990b) and DNA–DNA hybridization (Westerman *et al.*, 1990) studies. A close association between *Dactylopsila* and *Dactylonax* was also confirmed, although the levels of divergence between them indicates that they are quite distinct genera (see also Edwards and Westerman, 1992). Previous molecular studies indicated that Petauridae and Pseudocheiridae are among the most closely related diprotodontid families (Edwards and Westerman, 1992; Kirsch, 1977; Baverstock *et al.*, 1990a). In the present study, divergence levels involving the Phalangeridae (as represented by *Trichosurus*) were sometimes lower than those involving comparisons between the Petauridae and the Pseudocheiridae. Nevertheless, the Petauridae and Pseudocheiridae were consistently identified as sister lineages with respect to the Phalangeridae in all phylogenetic analyses. This suggests that degree of sequence divergence alone is not enough to determine relationships or taxonomic rank.

Genetic distances between sister taxa did not appear to be directly correlated with the level of bootstrap support obtained for the linking node. High bootstrap support was obtained for the node linking *P. breviceps* and *P. norfolcensis* (ca. 11% divergence, 100% bootstrap support), but this was also true for some more

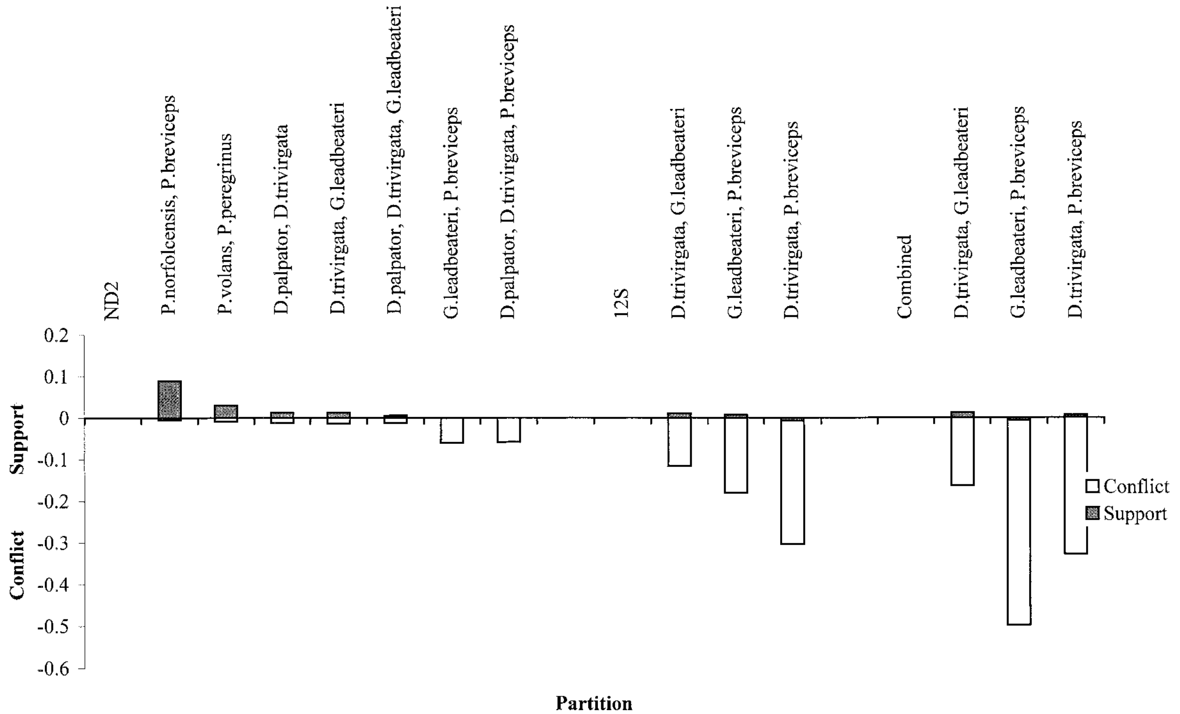

FIG. 5. Spectral analyses of ND2, 12S, and combined data sets. The amount of conflict and support is given for species comparisons. Comparisons with negative support values are not shown.

divergent sister taxa, such as *Petauroides* and *Pseudocheirus* (ca. 24% divergence, 98% bootstrap support). Conversely, sister taxa that were less divergent, such as *Dactylopsila* and *Dactylonax* (ca. 20% divergence), had lower bootstrap support (ca. 60%) for the node linking them.

Although a monophyletic Pseudocheiridae was strongly supported, there was less support for a monophyletic Petauridae. Monophyly of the group was dependent on the type of analysis, data partitioning, and taxon sampling. Bootstrap support was only moderate for a monophyletic Petauridae. Results were more conclusive, however, on the position of *Gymnobelideus*.

There was virtually no support for a sister relationship between *Gymnobelideus* and *Petaurus*, contrary to morphological (Aplin and Archer, 1987) and 12S sequence (Springer *et al.*, 1994) studies. The present study indicated that *Gymnobelideus* was more closely related to *Dactylopsila–Dactylonax* than to *Petaurus*, as also indicated by microcomplement fixation (Baverstock *et al.*, 1990a) and DNA–DNA hybridization (Edwards and Westerman, 1992) studies. Furthermore, when the 12S sequence data of Springer *et al.* (1994) was reanalyzed by inclusion of only diprotodontid taxa, *Gymnobelideus* in fact clustered with *Dactylopsila* and not with *Petaurus*. Given the broad agreement among the various molecular data sets, it can be concluded that *Gymnobelideus* is most closely related to *Dactylopsila–Dactylonax*.

Morphological characters that unite *Gymnobelideus* with *Petaurus* include possession of a reduced hypocone of M\5, a laterally compressed P\3, lack of a prominent parastyle of M\2, and a reduced squamosal wing (Archer, 1984). Conversely, *Gymnobelideus* and *Dactylopsila–Dactylonax* both share a reduced P\3 (Archer, 1984). Given the phylogenetic relationships inferred from the various molecular studies, we would consider the former characters plesiomorphic for the Petauridae. Aplin and Archer (1987) also suggested that the craniodental features that typify *Dactylopsila–Dactylonax* are more extreme versions of typical petaurine features.

The present study identified three major lineages within the Petauridae–Pseudocheiridae assemblage: *Gymnobelideus–Dactylopsila–Dactylonax, Petaurus*, and *Pseudocheirus–Petauroides*. The question remains: how should these groups be classified? There are no clear-cut levels of genetic divergence that define the subfamilies and families. The level of divergence recorded between *Gymnobelideus* and *Petaurus* (ca. 30%) is equivalent to that distinguishing the families Pseudocheiridae and Petauridae. This suggests that either *Gymnobelideus* represents a third family (including *Dactylopsila–Dactylonax*) or each of the three major lineages should be recognized as subfamilies within the one family. The latter treatment would be more consistent with the levels of divergence recorded be-

tween the Petauridae–Pseudocheiridae assemblage and the Phalangeridae.

Biogeography and the Fossil Record

A sister relationship between *Gymnobelideus* and *Dactylopsila–Dactylonax* appears curious at first, given the highly disjunct distribution of these two lineages. *Gymnobelideus* is found only in southeastern Australia, whereas *Dactylopsila* and *Dactylonax* are found predominantly in New Guinea (only one species reaches northern Australia). Geographically fragmented lineages that are shared between New Guinea and southeastern Australia have been reported in other marsupials and birds (Schodde and Calaby, 1972).

Schodde and Calaby (1972) suggested that the occurrence of shared lineages in the rainforests of montane New Guinea and eastern Australia is a consequence of changing climatic conditions. In the Miocene, rainforests extended from northern to southeastern Australia (Galloway and Kemp, 1981). Flannery (1994) has suggested that the dactylopsilines evolved in New Guinea from a *Gymnobelideus*-like ancestor, during the time when New Guinea and Australia were connected. As conditions became colder and drier, rainforests contracted until only pockets remained along the eastern coast of Australia and in New Guinea at the end of the Miocene (Galloway and Kemp, 1981). This would have resulted in geographically disjunct rainforest lineages.

The Pleistocene period was also characterized by cyclical climatic oscillations (Galloway and Kemp, 1981). Glacial phases would have caused further range contractions of rainforest-adapted species, whereas the interglacial phases would have favored range expansion, which may have intermittently reconnected some refugia. *Gymnobelideus* is restricted to mountain ash forests, so its range would have contracted as conditions became more arid. Remains of *Gymnobelideus* have been found in cave deposits at Wombeyan in New South Wales and Buchan in northeastern Victoria (Wakefield, 1972), indicating a more extensive distribution for *Gymnobelideus* during the Pleistocene. In more recent deposits *Gymnobelideus* bones are absent, reflecting the range contraction of this species.

Although there continues to be debate regarding the validity of use of DNA sequence data to estimate divergence dates (reviewed in Avise, 1994), it is useful in providing broad estimates for closely related lineages. The general mammalian rate of 2% sequence divergence per million years (Brown *et al.*, 1979) has been applied previously to estimate divergence times in marsupials (e.g., Krajewski *et al.*, 1997). With this estimate, divergence times among the major lineages including Petauridae, Pseudocheiridae, and Phalangeridae fall within a period of 16–12 million years ago. *Dactylopsila* and *Dactylonax* diverged around 10 million years ago and *P. breviceps* and *P. norfolcensis* around 6 million years ago.

These estimates are slightly earlier than those obtained by Aplin *et al.* (1993) from microcomplement fixation data that placed the divergence of *Gymnobelideus* and *Dactylopsila* at 10 million years ago. The estimates of both the present study and that of Aplin *et al.* (1993) are vastly different from those obtained from the DNA–DNA hybridization study of Edwards and Westerman (1992), in which the divergence of *Gymnobelideus* and *Dactylopsila* was estimated at around 32 million years ago.

The oldest fossil sites containing possums are from central Australia in the Lake Eyre and Lake Frome basins (Woodburne *et al.*, 1987) and are estimated to be 25–30 million years old (late Oligocene–early Miocene). The fauna there is quite different from that of today, with Pseudocheiridae and Burramyidae being the only extant possum lineage represented (Flannery, 1989). The earliest fossils recognizable as Petauridae are estimated to be around 23 million years old from Geilston Bay in Tasmania (Tedford *et al.*, 1975). Petauridae fossils (slightly more plesiomorphic than modern genera) have also been collected from Riversleigh in northwestern Queensland (Archer *et al.*, 1989) and recently *Dactylopsila* fossils have been recovered (Brammall and Archer, 1999). These deposits are dated between 23 and 16 million years old and are thought to represent the time when possums reached their greatest diversity (Archer *et al.*, 1999), when much of Australia was covered in rainforest.

The Miocene Riversleigh deposits include the extant possum families Pseudocheiridae, Petauridae, Phalangeridae, Burramyidae, and Acrobatidae (Archer *et al.*, 1989). Although mindful that divergence times may be underestimated for more ancient lineages (as multiple hits at the same codon position will have no impact on the genetic distance between sequences), the divergence times estimated here correspond roughly to the period represented by the Riversleigh assemblage.

The earliest fossils of *Petaurus* are 4.5 million years old (Pliocene epoch) (Flannery, 1992). Archer (1984) has argued that *Petaurus*, with its ability to glide, did not proliferate prior to the Pliocene because open forests were not widespread before this time. The ability to glide is not advantageous in closed rainforests. Estimates of divergence obtained from the present study for the two species of *Petaurus* support a Pliocene radiation for the genus. The DNA–DNA hybridization dates (Edwards and Westerman, 1992) are also consistent with a Pliocene radiation. The DNA–DNA hybridization dates appear to be more consistent with those of other molecular studies and the fossil record for the more recent divergences. As more sequence data on marsupials accumulates it will be possible to better assess congruence and reliability of divergence estimates across different molecular markers.

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