Phylogenetic relationships of the thylacine (Mammalia: Thylacinidae) among dasyuroid marsupials: evidence from cytochrome *b* DNA sequences

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SUMMARY

DNA sequences from the mitochondrial cytochrome *b* gene were obtained from a museum specimen of the presumed extinct thylacine (*Thylacinus cynocephalus*) and were compared with homologous sequences from 13 representatives of the Australian marsupial family Dasyuridae. The relationship of the thylacine to dasyurids has been suggested by previous anatomical and molecular studies, but its position within the dasyuroid radiation has not been addressed with genetic data. Phylogenetic analysis of the sequences reported here suggests that the thylacine is a sister group to Dasyuridae and lends support to the hypothesis that *Thylacinus* represents an ancient Australian marsupial lineage. Relationships with Dasyuridae support the results of other recent molecular studies, particularly in showing the affinities of endemic New Guinean subfamilies with larger Australian clades.

1. INTRODUCTION

The thylacine (*Thylacinus cynocephalus*), presumed extinct since 1936, was among the most enigmatic species of Australia's marsupial fauna. Congeneric fossils show that the lineage arose at least 15 Ma ago, but its mosaic of primitive and derived anatomical features make determination of a close relative extremely problematic (Archer 1982*b*). Bensley (1903) was the first to point out that, in terms of dental and pedal morphology, the thylacine did not resemble other Australian marsupials. Sinclair (1906) suggested that the thylacine closely resembled mid-Miocene borhyaenids, an extinct marsupial lineage from South America. For example, borhyaenids and thylacines are unusual among marsupials in sharing reduced or absent epipùbic bones. More support for this alliance came from Woods' (1924) detailed study of dentition. Simpson (1941), however, concluded that the dental resemblances between the two groups are convergent adaptations to carnivory, and that thylacines were specialized members of the Australian polyprotodont family Dasyuridae. Simpson's views were supported by Marshall (1977).

In a study of basicranial morphology, Archer (1976*a*) concluded that thylacines shared no derived features with borhyaenids or dasyuroids. Rather, thylacines possessed several features considered primitive for marsupials (e.g. a foramen ovale). After reconsidering dentition, however, Archer (1976*b*) concluded that thylacines and borhyaenids might have shared a common ancestor with specialized teeth and reduced epipubics, but retaining a primitive basicranium.

Two studies published in 1982 seemed to put the thylacine issue to rest. Szalay (1982) found that thylacines resemble all other Australian marsupials (and the South American microbiotheriid *Dromiciops*) in tarsal morphology, a character set that, in Szalay's view, provides a fundamental distinction between South American and Australian clades. In one of the earliest attempts of molecular analysis on museum-preserved material, Sarich *et al.* (1982) used comparative serology to show that thylacines are most similar to dasyurids with respect to albumin. Thomas *et al.* (1989) subsequently obtained short DNA sequences from the cytochrome *b* and 12S rRNA genes from thylacine skins. The rRNA gene sequences, when compared with homologues from other marsupial orders, also suggested a close relationship between the thylacine and Dasyuridae.

Archer (1982*b*) accepted the balance of evidence for dasyuroid affinities of the thylacine and attempted to integrate it into a coherent phylogenetic hypothesis. Archer proposed that thylacines were the remnants of an 'old dasyuroid radiation' in Australia. The stock from which they arose shared many primitive features in common with their own didelphoid (or micro-

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biotheriid) ancestors and subsequently gave rise to modern dasyuroids. Interestingly, Kirsch & Archer (1982), in a numerical cladistic study of dental and other anatomical characters of carnivorous marsupials, found that thylacines were closely associated with the Tasmanian devil (*Sarcophilus harrisii*, a dasyurid) and did not form the earliest branch of the dasyuroid tree. In his last review of the issue, Archer (1984) did not emphasize these numerical results and continued to treat *Thylacinus* as a separate family (Thylacinidae) within Dasyuroidea.

The central purpose of this study is to bring DNA sequence data to bear on the phylogenetic position of *Thylacinus* with respect to dasyurid marsupials. No molecular study to date has addressed this issue and hence no genetic data have been available to evaluate the validity of Thylacinidae as a separate family within Dasyuroidea. If thylacines are only 'specialized' dasyurids, then they should have a close genetic relative within the family (e.g. *Sarcophilus*, as suggested by Kirsch & Archer (1982)). If, however, they are an ancient lineage, they should appear as a sister group to all dasyurids. To discriminate between these hypotheses, some resolution of phylogeny within Dasyuridae is also required.

The phylogenetic structure of Dasyuridae has been studied intensively by morphologists for several decades. This work is summarized by Archer (1982*a*, 1984), who proposed the subfamilial and tribal classification in table 1. The 17 extant dasyurid genera are organized into five subfamilies, two of which are further subdivided into tribes. The nominate subfamily, Dasyurinae, includes the quolls (*Dasyurus*) and devil (*Sarcophilus*), as well as smaller forms (*Dasyuroides*, *Dasycercus*, *Myoictis*) in the tribe Dasyurini. Tribe Parantechini includes three genera of 'false antechinuses' reassigned from *Antechinus* (Phascogalinae) on the basis of phallic morphology (Archer 1982*a*; Woolley 1982). True antechinuses and phascogales comprise Phascogalinae. Muricinae and Phascolosoricinae are New Guinean endemics of uncertain affinities. Sminthopsinae includes the dunnarts (*Sminthopsis*), kultarr (*Antechinomys*) and ningauis (*Ningaui*) in Sminthopsini and planigales in Planigalini.

Table 1. *Dasyuroid classification from Archer (1982a)*

Superfamily Dasyuroidea
Family Thylacinindae: *Thylacinus*
Family Myrmecobiidae: *Myrmecobius*
Family Dasyuridae
Subfamily Dasyurinae
Tribe Dasyurini: *Dasyurus*[a], *Sarcophilus*, *Dasyuroides*, *Dasycercus*, *Myoictis*
Tribe Parantechini: *Parantechinus*, *Pseudantechinus*, *Dasykaluta*
Subfamily Phascogalinae: *Phascogale*, *Antechinus*
Subfamily Muricinae: *Murexia*
Subfamily Phascolosoricinae: *Phascolosorex*, *Neophascogale*
Subfamily Sminthopsinae
Tribe Sminthopsini: *Sminthopsis*, *Antechinomys*, *Ningaui*
Tribe Planigalini: *Planigale*

[a] Includes Archer's (1982*a*) *Satanellus*.

With the exception of the New Guinean endemics, Archer's classification has been largely supported by molecular systematic studies such as the allozyme work of Baverstock *et al.* (1982). Several recent molecular analyses, however, have suggested that *Murexia* is closely related to New Guinean *Antechinus* species and could be considered part of Phascogalinae (Baverstock *et al.* 1990; Kirsch *et al.* 1991). Similarly, the microcomplement fixation (MCF) data of Baverstock *et al.* (1990) suggested that *Phascolosorex* and *Neophascogale* are closely related to members of Dasyurini. No molecular data to date have been able to demonstrate the monophyly of Sminthopsinae, although most agree that its constituent genera are quite divergent from a clade that includes the other subfamilies (Baverstock *et al.* 1990; Kirsch *et al.* 1991).

Thomas *et al.* (1989) showed that cytochrome *b* sequence divergence among marsupial orders was too large to provide resolution of phylogenetic branching order. Divergence among dasyurids, however, has probably occurred in the past 20 Ma (Archer 1982*a*), and cytochrome *b* should provide better resolution over this shorter time span.

2. MATERIALS AND METHODS

(*a*) *Exemplar species and specimens*

DNAs from the following species, representing all dasyurid subfamilies and tribes, were used in this study (specimen information is given in the appendix): *Thylacinus cynocephalus*, *Dasyurus maculatus*, *Dasyurus hallucatus*, *Sarcophilus harrisii*, *Parantechinus apicalis*, *Pseudantechinus macdonnellensis*, *Dasykaluta rosamondae*, *Phascogale tapoatafa*, *Antechinus stuartii*, *Antechinus swainsonii*, *Murexia longicaudata*, *Phascolosorex dorsalis*, *Sminthopsis crassicaudata*, and *Planigale maculata*. A bandicoot, *Perameles nasuta* (Perameloidea), was included as an unambiguous outgroup to dasyurids.

(*b*) *DNA extraction and purification*

For extant species, DNA was isolated from ethanol-preserved liver tissue by using standard methods of cell lysis, organic extraction, enzymic digestion, and ethanol precipitation (Sambrook *et al.* 1989). DNA from the thylacine was recovered from 0.6 g of dried muscle and cartilage removed from a prepared skeleton. Ancient DNA extraction followed the protocol of Pääbo (1990). The particular specimen chosen was obtained by the Smithsonian Institution in 1904; the precise time of death is not known.

(*c*) PCR *amplification and direct sequencing*

Polymerase chain reactions (PCR) were done in 100 μl volumes using 1.5 mM Mg^{2+}, 1.0 μM concentrations of each primer, and 1.0 U of Taq polymerase (supplied by Perkin–Elmer Cetus or Promega). A thermal cycle began with 2.5 min at 94 °C for initial denaturation, followed by 35 cycles of denaturation (94 °C, 40 s), primer annealing (48–50 °C, 1 min), and polymerase extension (68–72 °C, 3.5 min). A final extension for 7 min, was included to minimize the number of partial strands.

For extant species, the 656 base pair (b.p.) fragment between positions 14841 and 15498 was amplified as a

double-stranded product and sequenced by using both internal and terminal primers. Thylacine DNA was found to be too degraded for amplification of the 656 b.p. fragment, and so sequence was obtained from two smaller and overlapping regions between positions 14841 and 15149, and 15087 and 15498. Primers and their sources are:

L14841, 5′-CCATCCAACATCTCAGCATGATGAAA-3′ (Kocher *et al.* 1989);
L15087, 5′-TACTTAAACAAAGAAACCTGAAA-3′ (Edwards *et al.* 1991);
L15136, 5′-ATAGCAACAGCATTTGTAGG-3′ (this study);
H14958, 5′-CTGCAGTCAGCCGTAATTTACGTCTC-3′ (Thomas *et al.* 1989);
H15149, 5′-CCCCTCAGAATGATATTTGTCCTCA-3′ (Kocher *et al.* 1989);
H15498, 5′-GGAATAAGTTATCTGGGTCTC-3′ (P. Arctander, personal communication).

H and L refer to heavy and light strands of mitochondrial DNA, respectively, and primer numbers correspond to their 3′ base position in the human cytochrome *b* sequence (Anderson *et al.* 1981).

Balanced-primer reaction products were purified by electrophoresis through a 2.5% low-melting agarose gel, stained with ethidium bromide, excised, and stored in 250–1000 μl of water. These gel slices were melted at 65 °C for 3–5 min and 5–10 μl was removed for asymmetric PCR reactions (McCabe 1990). Reaction mixtures and thermal cycles for asymmetric amplification were identical to those given above, except that primer amounts were set to 0.5 μM (excess primer) and 0.01 μM (limiting primer). A 9 μl portion of each asymmetric reaction product was electrophoresed through 2.5% agarose and visualized by ethidium bromide staining to check for the presence of a visible single-stranded DNA band (usually the leading band). Successful reactions were precipitated in 2.5 M ammonium acetate and 2 volumes of cold 95% ethanol, dried, and rehydrated in 10 μl of water for sequencing. Dideoxy sequencing followed the protocol for the Sequenase enzyme system (United States Biochemical; Sambrook *et al.* 1989) using [^{35}S]dATP.

Because many dasyurid cytochrome *b* sequences contain a region of secondary structure near position 14841, this primer was often unsuccessful for direct sequencing. To obtain sequences in this region, some PCR products were cloned into pUC 18 plasmid vectors and sequenced with an M13 forward primer (Sambrook *et al.* 1989).

(*d*) *Alignment*

DNA sequences obtained from each autoradiograph were aligned manually with previous overlapping sequences from the same sample to check for accuracy. After resolution of base-calling errors and sequencing artefacts, sequences for each species were aligned with one another. Manual alignment was straightforward, as no gaps were required to maintain the reading frame.

(*e*) *Phylogenetic analysis*

Distance and parsimony methods were used to estimate phylogenetic relationships among sequences (Swofford & Olsen 1990). Pairwise sequence distances were estimated by Kimura's (1980) two-parameter method, which attempts to correct observed dissimilarities for multiple substitutions in sequences evolving with a transition bias. Distances were calculated by the DNADIST program of J. Felsenstein's PHYLIP package (version 3.3). A weighted least-squares tree was estimated for the distance matrix by using the Fitch–Margoliash (1967) algorithm as implemented by the FITCH program of PHYLIP. This algorithm attempts to find a topology and assignment of branch lengths that require a minimum distortion of observed distances (Springer & Krajewski 1989). The resolving power of sequence distances was assayed by bootstrap resampling of sites (Felsenstein 1985). Bootstrap pseudoreplicate distance matrices were constructed from 100 resamplings of the original alignment, best-fit trees were constructed for each, and the results summarized as a majority-rule consensus tree with node frequencies suggesting levels of resolution for each implied clade. Because some species pairs were very divergent (sequences less than 80% identical), this procedure was repeated using only transversion differences. Transversions are known to occur more rarely than transitions in cytochrome *b* evolution (Irwin *et al.* 1991), and so transversion distances might be less distorted by multiple substitutions.

The DNAPARS program of PHYLIP was used to construct minimum-length trees following the method of Fitch (1971). The resolving power of parsimony was assayed by bootstrap resampling of sites, using the DNABOOT program of PHYLIP. Because codon positions evolve at unequal rates (with third positions more variable than first or second positions), several weighting schemes were applied in parsimony analyses: (i) all positions with equal weights; (ii) first and second positions with equal weight and third positions with zero weight; (iii) each position weighted as the inverse of its variability in the global alignment and standardized against the third position value (3:6:1 for first, second, and third positions, respectively); and (iv) each position weighted as the inverse of its mean variability over all pairwise alignments (6:15:1, again standardized to third positions). In addition, a tree was constructed from transversion differences alone to remove the influence of potentially homoplastic transitions.

3. RESULTS

Figure 1 shows the global alignment of 574 b.p. of cytochrome *b* for all 15 species included in this study. The sequence obtained from the thylacine specimen is identical to that in Thomas *et al.* (1989) except for two mismatches near the 5′ end. It differs from dasyurid sequences by 13–20% and from the human sequence by 20.4%. These comparisons demonstrate the authenticity of the thylacine DNA.

Dasyuroid cytochrome *b* sequences show patterns of variability typical of mammalian mitochondrial protein-coding genes. As expected, third position transitions occur with the highest frequency and second position transversions with the lowest. A moderate transition bias is also evident between the most similar pairs (maximum 3.5-fold among dasyuroids), but decays considerably for more divergent sequences (bias is 1.3-fold, on average, for dasyurids against the bandicoot). The mean relative variabilities at codon positions over all pairwise comparisons are 2.6:1:14.7 for first:second:third positions.

Table 2 is a matrix of sequence distances showing levels of difference (*p*, above diagonal) and estimated divergence (two-parameter *d*, below diagonal). Values of *p* range from 0.04 to 0.221 among dasyuroids and from 0.16 to 0.22 between dasyuroids and the bandicoot, suggesting that the larger values are close to saturation.

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                14842    14850    14860    14870    14880    14890    14900    14910    14920    14930
                 |        |        |        |        |        |        |        |        |        |
                 PheGlySerLeuLeuGlyIleCysLeuValIleGlnIleLeuThrGlyLeuPheValAlaMetHisTyrThrSerAspThrSerThrAlaPheSe
Thylacinus       CTTCGGGTCCTTACTAGGAATCTGCCTAGTCATTCAAATCTTAACAGGCCTATTTCTAGCAATACATTATACATCAGACACATCAACTGCCTTCTC
A. swainsonii    ...T..A...C........G.A.....GA....C.....TC........T.C.....C........C..C..C.....T.TGCTC........T..
A. stuartii      ...T..A..AC........G.A.....TA....C.....C........T.C..C..C..C.....C..C..C.....T..GCTC...........
Murexia          ......A......T....CGCAC.....A....C..............T.C..C..T...........C..T.....T...CT...C........
Dasykaluta       ......A...C.G........T.....TA....C......C.G........T..C...........C..C..T.........CTT..A........
Parantechinus    ...T..A..............T......A.T..C...G..C.C........C..C...........C..C..C.....T..TCT...A........
Pseudantechinus  .C.T..A.T.....................A.T.........C.C...........C.........C..C..C........CCT...C..T.....
Phascogale       ...T..A......T..............A.T.A..C....C........T.C..CT.G........C..C..C........TCTT..C......A.
D. hallucatus    ...T..A..TC..........A......A.T..C......C.T......T.G..C.....T.....C..C..T.....T...CTT..C........
D. maculatus     ...T..A..AC..T.....G.T......A.T.........TC.C...........C.....T.....T...CTT..C..T.....
Phascolosorex    ......C..T...........A...T..A....C......C.C.....A.....C...........C..C..C.....T...CTC.........A.
Sminthopsis      ...T..C.....GT.G...G.T......A.A.................GT.........T..G..C........T..T..TCTC...........
Planigale        ...T..A..TC.............................C.......AT....C.........G..C..C........T..T.T...A........
Sarcophilus      .........T...T.......A......A.T........TC.C..C..A.....C.........C..C..T.........CTT..C........
Perameles        ...T..A..AC..T.....G.....................TC.T...........C.....C.....................CTC..A..T.....

                14940    14950    14960    14970    14980    14990    15000    15010    15020    15030
                 |        |        |        |        |        |        |        |        |        |
                rSerValAlaHisIleCysArgAspValAsnTyrGlyTrpLeuIleArgAsnLeuHisAlaAsnGlyAlaSerMetPhePheMetCysLeuPheLe
Thylacinus       CTCAGTAGCACATATCTGCCGAGACGTAAATTATGGATGACTTATTCGTAACCTCCATGCCAATGGAGCCTCCATATTCTTCATATGCTTATTTCT
A. swainsonii    ...C.....C..C.....T.A......C..C.....C.....AG.C..C..T..T.........C........A.....T.........C.T..C..
A. stuartii      ...C.....C..C.....TA.......C..C..G..C.....A..C.AC.G...T.................A.....T.........C.T..C..
Murexia          T..T.....C..C..T..T........C..C..C..C.....A..C..C.....T.....T.........T..A..............C.G..C..
Dasykaluta       .........C.....T.....T..T.....C..C........CT.A..C.....A..C..............A..G............C.G..CT.
Parantechinus    T..C.....T..C..T.....C..T..T..CC.C..C.....CC.C..C..T..A.....T...........A.....................C..
Pseudantechinus  .........C..C..T.....T.....C..CC.C..C.....CC.C..C.....A..C..T..C..G.....A..G.............C....C..
Phascogale       ...C.....C.........T.....T..C..C..C..C.....GC.C.....T.........T........T..A..............C.T..C..
D. hallucatus    ...C.....C..............T..G...C.C..T...T.AC....C.....A..C..T..C..G..T...........T......C....CT.
D. maculatus     ...C.....C..C..T.....T..T..T..CC.C..C.....CC..........G..A..T..C..G..T..A..............C.G.....
Phascolosorex    ...C.....C..C.........T..T..T..C..C..T.....AC....C..TT.A.....T..C.....T..A..............C.T..C..
Sminthopsis      T........C..C........C..T..T..C..C..C..G..A..C..A.....T..C.....C..G..T...........T......C.....T.
Planigale        ...C.....C..C..T..T........C..C..C..C..GT.A..C..C.....T..C..T..C.....A...........T......C....CT.
Sarcophilus      ...C.....C..C..T..T..T..T..T..CC.C..C.....AC..........G..C..T.....G..T..A........T......C.G.....
Perameles        A........C..C.....................C..C.....G..C..C..T..A.....A..C........A........T...........C..

                      15040    15050    15060    15070    15080    15090    15100    15110    15120
                       |        |        |        |        |        |        |        |        |
                      uHisValGlyArgGlyIleTyrTyrGlySerTyrLeuTyrLysGluThrTrpAsnIleGlyValIleLeuLeuLeuThrValMetAlaThrAlaPh
Thylacinus       TCATGTAGGACGAGGTATCTACTACGGATCATACCTGTACAAAGAAACATGAAACATTGGAGTTATCCTCCTACTAACAGTAATAGCAACTGCATT
A. swainsonii    C.....C..CT....G..............T..T..C..............T..C.....A..T..G..T..G..C.A.........C.....
A. stuartii      C.....C..C.....................T.....C..................C.....A..T..G..T..G..C.A.........CA....
Murexia          C.....C..C.....G..T.....T.....T...T.A.................T..C.....A...T.A..T.....C..G..............
Dasykaluta       A.....T..T.....G..T.....T.....T..T..C.....................T..A..T..A..T..T..T..C.........C.....
Parantechinus    A........C.....A..T........T..C..T..C.....................C........T.....T..T..C.....C.........
Pseudantechinus  G..C.....C.....A..T...........C.....T.................T..C..C..G..T..T..T..C..T..C.....T.TA.....
Phascogale       C........T.T...A.....T........C........T.....................C........C.....C...........A.....
D. hallucatus    A........C..............T........T..C..T.....................T.....C..A..T.....T..T..C..C.....A.....
D. maculatus     A........C.....G..............T..T..C.........................A........T..C..T..C........A.....
Phascolosorex    A...A.C..C.....A..T.....T.....C.....C.....................C..A.....A.....CG.C..C.....T..A.....
Sminthopsis      A..C.....C.....A..T........C..T.....C..T........C..............AG.A..T.....C..C..T.....C.....G..
Planigale        A..C.....C.T...A..T.....T..G....CTTCA..T.....................T..G.....A..T.....T..T.....G..A.....
Sarcophilus      A.....G..C.....A..............T..T..C.....................C..A..T.....T..C..T..C........A.....
Perameles        G..C...........A..T..T........C..T..A.T.....................A.....A...T....T..C.....T..A..C..
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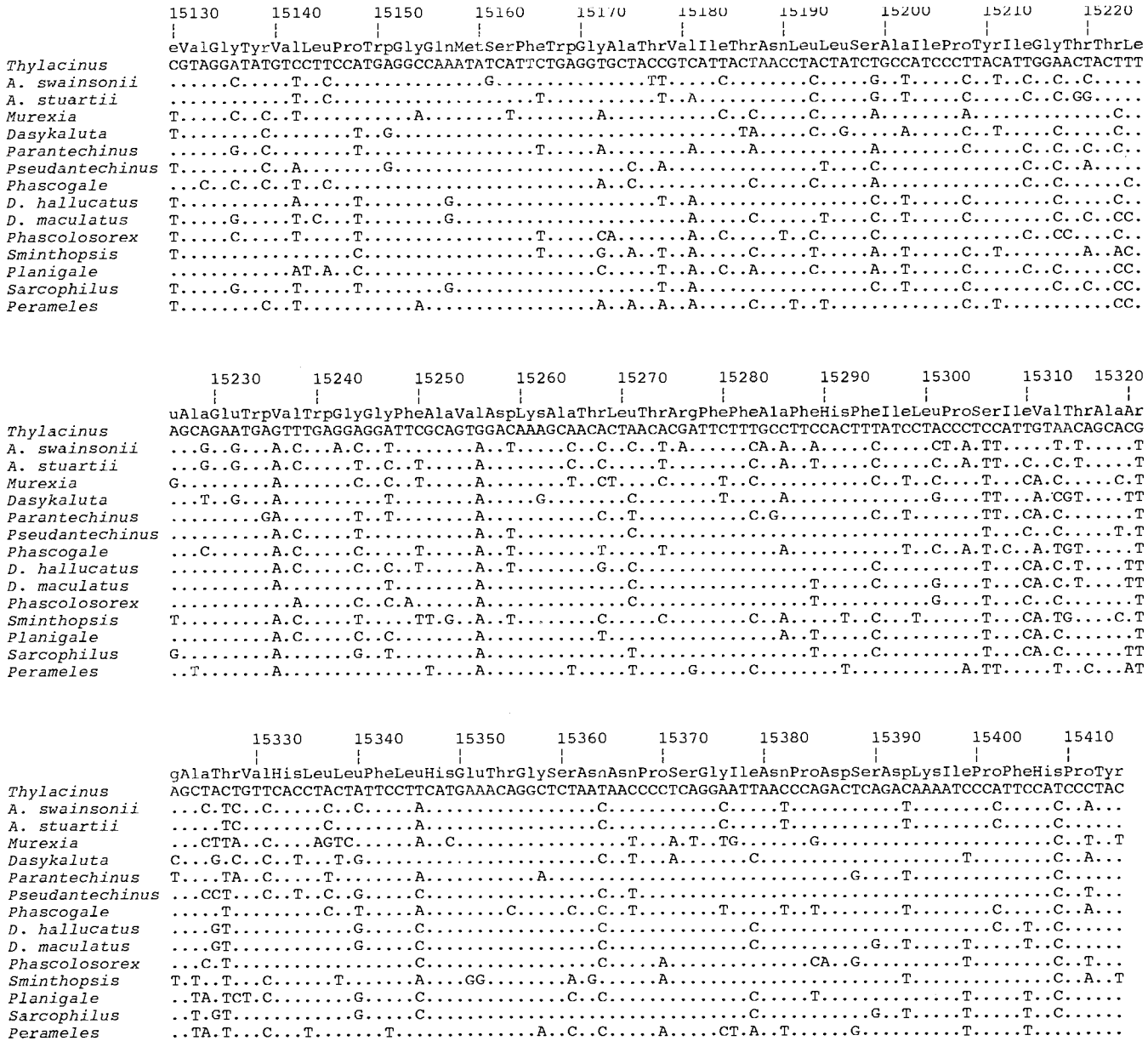

Figure 1. Aligned cytochrome *b* sequences between positions 14841 and 15416 for 15 species of marsupials. Positions are numbered according to the human mitochondrial scale (Anderson *et al.* 1981). The sequence for *Thylacinus* is translated at the top. Dots indicate a match to the *Thylacinus* sequence. Genus abbreviations: *A.* = *Antechinus*; *D.* = *Dasyurus*. These sequences have been deposited in the GENBANK database.

Table 2. *Matrix of sequence distances for cytochrome b between positions 14841 and 15416*

(Values above diagonal are percent mismatch; values below diagonal are distances calculated with Kimura's two-parameter method. Genus abbreviations: *A.* = *Antechinus*; *D.* = *Dasyurus*.)

	Murexia	*Dasykaluta*	*Parantechinus*	*Pseudantechinus*	*Phascogale*	*A. swainsonii*	*A. stuartii*	*D. hallucatus*	*D. maculatus*	*Phascolosorex*	*Sminthopsis*	*Planigale*	*Sarcophilus*	*Thylacinus*	*Perameles*
Murexia	0.0000	0.185	0.145	0.172	0.159	0.179	0.157	0.174	0.181	0.172	0.199	0.183	0.176	0.164	0.209
Dasykaluta	0.2244	0.0000	0.159	0.145	0.195	0.188	0.174	0.132	0.124	0.172	0.221	0.181	0.146	0.157	0.197
Parantechinus	0.1864	0.1822	0.0000	0.125	0.169	0.181	0.159	0.143	0.125	0.134	0.181	0.162	0.131	0.152	0.172
Pseudantechinus	0.2084	0.1682	0.1417	0.0000	0.157	0.186	0.167	0.117	0.110	0.139	0.190	0.159	0.127	0.153	0.171
Phascogale	0.1901	0.2357	0.2002	0.1854	0.0000	0.162	0.138	0.148	0.162	0.171	0.202	0.176	0.174	0.160	0.169
A. swainsonii	0.2102	0.2267	0.2167	0.2185	0.1853	0.0000	0.068	0.174	0.178	0.190	0.216	0.197	0.190	0.200	0.220
A. stuartii	0.1855	0.2086	0.1854	0.2005	0.1675	0.0731	0.0000	0.141	0.150	0.157	0.188	0.166	0.150	0.162	0.199
D. hallucatus	0.2122	0.1556	0.1618	0.1336	0.1837	0.2035	0.1698	0.0000	0.075	0.127	0.176	0.124	0.085	0.141	0.183
D. maculatus	0.2192	0.1403	0.1405	0.1276	0.1964	0.2094	0.1801	0.0846	0.0000	0.127	0.174	0.141	0.040	0.134	0.153
Phascolosorex	0.2072	0.1999	0.1605	0.1587	0.2046	0.2217	0.1893	0.1435	0.1442	0.0000	0.206	0.160	0.124	0.148	0.190
Sminthopsis	0.2460	0.2724	0.2111	0.2359	0.2529	0.2725	0.2325	0.2155	0.2142	0.2315	0.0000	0.174	0.176	0.183	0.204
Planigale	0.2213	0.2137	0.1902	0.1847	0.2079	0.2330	0.1990	0.1446	0.1635	0.1857	0.2088	0.0000	0.141	0.171	0.179
Sarcophilus	0.2148	0.1675	0.1484	0.1436	0.2084	0.2233	0.1882	0.0940	0.0447	0.1445	0.2131	0.1618	0.0000	0.145	0.186
Thylacinus	0.1969	0.1835	0.1786	0.1753	0.1904	0.2405	0.1967	0.1667	0.1609	0.1855	0.2238	0.2058	0.1692	0.0000	0.160
Perameles	0.2397	0.2430	0.2065	0.2093	0.2454	0.2731	0.2485	0.2260	0.1752	0.2310	0.2475	0.2194	0.2241	0.1877	0.0000

Best-fit analysis of two-parameter distances yields the tree in figure 2, which also shows the results of bootstrapping. On this tree, *Thylacinus* appears as the first branch after the bandicoot outgroup, with dasyurids forming a monophyletic lineage in 75% of the bootstrap trees. This position for *Thylacinus* is exactly that predicted by Archer (1982*b*). Other aspects of the tree in figure 2 are interesting with respect to relationships among dasyurids. Most of Archer's (1982*a*) subfamilies appear as monophyletic groups, again excepting the New Guinean endemics. *Murexia* is clearly allied with the Phascogalinae, confirming results from MCF analysis (Baverstock *et al.* 1990) and DNA hybridization (Kirsch *et al.* 1991). *Pascolosorex* appears to be part of Dasyurinae, although here the bootstrap resolution is much poorer (Dasyurinae, including *Phascolosorex*, is monophyletic in only 37% of pseudoreplicate trees). This result lends some support to the same finding by Baverstock *et al.* (1990). Within Dasyurinae, monophyly of the large-bodied forms (*Dasyurus* and *Sarcophilus*) is suggested, although further resolution of tribal relationships is not possible. The alliance of *Dasyurus maculatus* with *Sarcophilus* and apart from *D. hallucatus* is significant and supports Archer's (1982*a*) species-level hypothesis of relationships within the tribe. *Planigale* and *Sminthopsis* form a clade, but with only a 36% bootstrap frequency. Low bootstrap values also suggest that there is virtually no resolution of relationships between Dasyurinae, Phascogalinae, and Sminthopsinae.

It is important to point out that bootstrap values, as interpreted here, are intended to show only relative degrees of resolution on each tree topology and should not be regarded as statistical confidence intervals. This has been discussed by Felsenstein (1985) and many subsequent authors. As noted by Krajewski & Dickerman (1990), bootstrap values indicate only the consistency with which particular groups are identified as presumptive clades. This measure is conditional on the data set, the method of analysis, and the proviso that sampling error is the only way in which the data deviate from the assumptions of the analytical method. In particular, we do not specify an 'alpha-level' for bootstrap values, above which nodes are considered unconditionally significant.

Bootstrap analysis of transversion distances results in a topology similar to that in figure 2, with *Thylacinus* as sister group to the dasyurids. Bootstrap values on this tree were substantially lower than in figure 2, however, and only the branching order within Phascogalinae and Dasyurini showed resolution above 50%.

Bootstrapped parsimony analysis of unweighted sites produces the tree shown in figure 3. Again, *Thylacinus* appears as the sister group to dasyurids at a bootstrap frequency of 76%. The principal difference between parsimony and distance trees is the placement of *Parantechinus* and *Pseudantechinus*. Parsimony places both taxa near the base of the dasyurid tree, although bootstrap values show that the positions of these genera are completely unresolved. *Murexia* is resolved on the same branch with Phascogalinae, and *Phascolosorex* associates weakly with dasyurines.

Figure 4 shows a bootstrapped parsimony analysis of

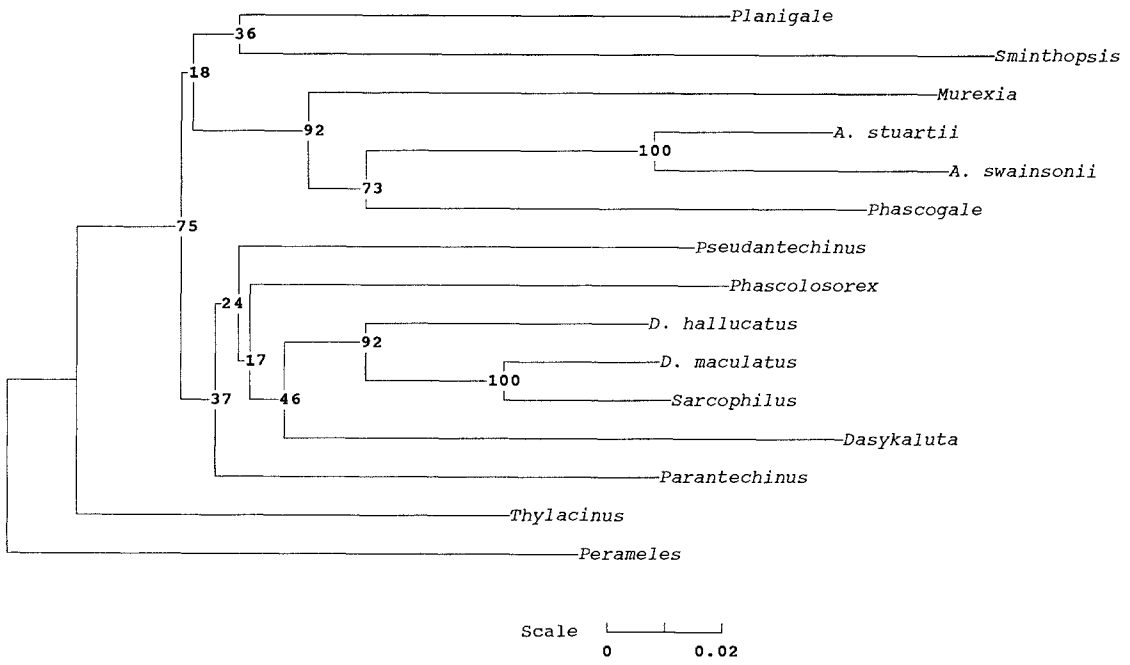

Figure 2. Best-fit tree for the two-parameter sequence distances in table 2. The weighted sum of squares is 0.53390. The outgroup branch to *Perameles* is folded arbitrarily near its midpoint. Tree was generated by the FITCH program of PHYLIP 3.3. Nodal values are the number of clade occurrences out of 100 bootstrap resamplings.

sites weighted by codon position variability in the global alignment (weights are 3:6:1). On this tree, *Pseudantechinus* and *Parantechinus* have moved back within the Dasyurinae, but other branching patterns and resolution levels are similar to those for unweighted parsimony. More extreme weighting of codon positions (6:15:1, based on average variability over all pairwise alignments) produces a tree on which *Thylacinus* appears as sister-group to Dasyurinae. Bootstrap frequencies in this analysis are substantially lower than those on previous trees, suggesting that although the best topology has changed after weighting, the resolving power of the sequences has not improved. Parsimony analysis with only first and second position sites produces a drastically altered tree in which none of the subfamilies remain intact (*Thylacinus* appears as sister-group to *Phascolosorex*). Transversion parsimony yields a topology similar to those in figures 2 and 4 (except for branching patterns with Dasyurinae) but with generally lower bootstrap frequencies.

To make more specific comparisons of alternative phylogenetic hypotheses for *Thylacinus*, the length of the most parsimonious tree for unweighted sites was compared with those for two alternative topologies. The first is identical to figure 3 except that *Thylacinus*

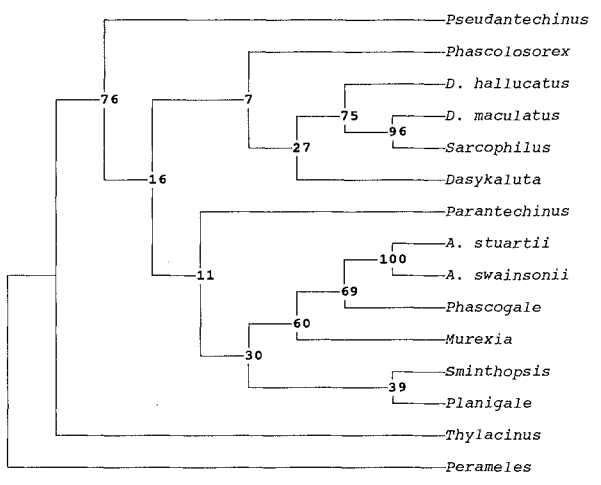

Figure 3. Bootstrapped parsimony analysis of sites in figure 2. All sites have equal weight. Nodal values are the number of clade occurrences out of 100 bootstrap resamplings. Tree was generated by the DNABOOT program of PHYLIP 3.3 and has length 775 steps.

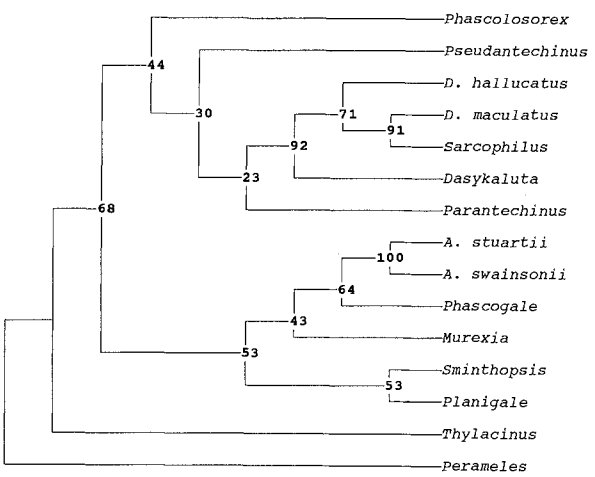

Figure 4. Bootstrapped parsimony analysis of sites in figure 2, with sites weighted by codon position. Weights are 3, 6, and 1 for first, second, and third positions, respectively. Weights are inversely proportional to the relative fractions of variable positions in the global alignment. Bootstrap analysis was done by duplicating each informative first- and second-position site three and six times, respectively, in the input matrix for DNABOOT (PHYLIP 3.3).

is the sister group to *Sarcophilus* as in the results of Kirsch & Archer (1982). The second places *Thylacinus* as sister group to to Dasyurinae (including *Phascolosorex*). These alternative trees have lengths of 811 steps and 788 steps, respectively, and are significantly longer than the best tree (775 steps) as suggested by Templeton's (1983) test (implemented by DNAPARS).

4. DISCUSSION

Phylogenetic analysis of dasyuroid cytochrome *b* sequences demonstrates that *Thylacinus* is quite divergent from members of Dasyuridae and probably represents an earlier and separate lineage. Although the cytochrome *b* sequences gathered to date do not completely resolve deep branches of the dasyuroid tree, it is clear that *Thylacinus* lacks a close relative among dasyurids. This view of the thylacine's position is consistent with Archer's (1982*b*) suggestion that the thylacine represents an old dasyuroid lineage and possibly the remnant of an Oligocene radiation (Archer 1984). Whatever the historical details, we can tentatively reject Simpson's (1941) view that the thylacine is a specialized (in the sense of being recently derived) dasyurid.

Equally interesting are the relationships within Dasyuridae implied by cytochrome *b*. Three major lineages are apparent that largely correspond to recognized subfamilies. Most analyses place *Sminthopsis* and *Planigale* on the same branch, although with low bootstrap frequencies, lending support to Archer's Sminthopsinae. This agrees with the DNA hybridization data of Kirsch *et al.* (1991), although Baverstock *et al.* (1982, 1990) could not resolve these deep branches with allozyme or MCF data.

Most analyses of cytochrome *b* sequences resolve the subfamily Phascogalinae (*Phascogale* and *Antechinus*) and show that the New Guinean *Murexia* is a close relative. Again, this relationship was detected by both MCF and DNA hybridization studies (Baverstock *et al.* 1990; Kirsch *et al.* 1991). Because the position of *Phascogale* with this clade is not highly resolved, comments on the validity of Muricinae would be premature.

Cytochrome *b* sequences support the MCF data of Baverstock *et al.* (1990) in placing Phascolosoricinae as a relative of Dasyurinae. Resolution of branching order within Dasyurinae is rather poor, although the data resolve a close relationship between *Sarcophilus* and *Dasyurus maculatus*. That the latter is not sister group to its congener *D. hallucatus* is consistent with Archer's (1982*a*) alliance of *hallucatus* and *D. albopunctatus* in *Satanellus*. Cytochrome *b*, like allozymes (Baverstock *et al.* 1982), is unable to resolve tribes Parantechini and Dasyurini as monophyletic, although branch lengths on the tree in figure 2 suggest a rapid separation of dasyurine lineages.

The 5′ portion of cytochrome *b* is quite divergent between dasyurid subfamilies and is unable to resolve relationships among Sminthopsinae, Phascogalinae, and Dasyurinae. Virtually every other molecular systematic study of dasyurids (Baverstock *et al.* 1982, 1990; Kirsch *et al.* 1991) has shown that Sminthopsinae is quite divergent from the other subfamilies. Further studies are in progress that include representatives of all dasyurid genera, the thylacine, and the numbat, as well as additional sequence from the 3′ portion of cytochrome *b*. This larger data set should provide a clearer picture of dasyuroid relationships and, thereby, some of the earliest events in the evolution of Australia's marsupial fauna.

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APPENDIX 1

Not all specimens used in this study have been lodged as museum vouchers. Collector and field data are provided for these samples. *Murexia longicaudata* (male): collector P. A. Woolley; 26 January 1983; Mount Missim (vicinity Waw), Papua New Guinea; reference specimen La Trobe University no. 19. *Parantechinus apicalis*: collector P. A. Woolley. *Phascogale tapoatafa*: collector P. R. Baverstock; 20 November 1979; Jim Jim Creek, Northern Territories, Australia; field no. 1. *Phascolosorex dorsalis* (female): collector P. A. Woolley, 29 September 1982; Mount Kaindi (vicinity Waw), Papua New Guinea; reference specimen La Trobe University no. 9. *Antechinus swainsonii*: collector Anthony Smith; 14 December 1980; Otways lighthouse, Cape Otway, Victoria; field no. ASO-1. *Sminthopsis crassicaudata* (female): collector Julien Reid; 2 December 1986; Coongia Lakes, South Australia; field no. C003. *Planigale maculata* (female): collector Dennis King; 6 May 1986; Millstream, Western Australia (21° 35′, 117° 04′); field no. F-94; reference specimen Western Australia Museum no. M26905 (species identification of this specimen is tentative). *Dasyurus hallucatus*: one of three specimens collected by Dennis King; 3 May 1986; Dolphin Island, Western Australia (20° 29′, 116° 50′); field no. F-57, F-59, or F-60; reference specimens Western Australia Museum no. M26196, M25195, or M26197. *Sarcophilus harrisii* (male): collector Steve Nicol; 26 June 1979; University of Tasmania campus; field no. 1. *Pseudantechinus macdonnellensis* (female): one of two individuals collected by P. A. Woolley; 7 November 1976; Abydos and Woodstock Stations, Western Australia (21° 37′ S, 118° 57′ E). *Dasykaluta rosamondae* (female): lab-reared offspring (1976–1977) of stock collected by P. A. Woolley at Abydos and Woodstock Stations, Western Australia. *Perameles nasuta*: University of Wisconsin Zoological Museum tissue specimen 375. *Dasyurus maculatus*: University of Wisconsin Zoological Museum tissue specimen 15. *Antechinus stuartii*: University of Wisconsin Zoological Museum tissue specimen 396. *Thylacinus cynocephalus*: USNM 49723, Smithsonian Institution, Washington, D.C.