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DNA phylogeny of the extinct marsupial wolf

Richard H. Thomas*†, Walter Schaffner‡, Allan C. Wilson* & Svante Pääbo*

* Department of Biochemistry, University of California, Berkeley, California 94720, USA
‡ Institut für Molekularbiologie II der Universität Zürich, Hönggerberg, CH-8093 Zürich, Switzerland

THE phylogenetic affiliation of the extinct marsupial wolf (*Thylacinus cynocephalus*), which once was widespread in Australia, has been uncertain. On the basis of morphology, some systematists argue that the thylacine was most closely related to an extinct group of South American carnivorous marsupials, the borhyaenids[1-3], whereas others consider it to be closer to Australian carnivorous marsupials[4]. Here we use direct sequencing by means of the polymerase chain reaction (PCR) to compare 219 bases of mitochondrial (mt) DNA from museum specimens of the marsupial wolf and representatives of six genera of extant marsupials. In agreement with the results of an antigenic study of albumin[5], our genetic data suggest that the marsupial wolf was more closely related to other Australian marsupial carnivores than to those of South America. Thus, the marsupial wolf represents an example of convergent morphological evolution to South American carnivorous marsupials as well as to true wolves.

Direct sequencing by means of the PCR[6,7] has enabled the retrieval of DNA sequences from museum specimens and archaeological remains[8-11]. This is because the PCR can synthesize many copies of a small number of intact DNA molecules in the presence of a vast excess of damaged molecules[9]. To perform the PCR on thylacine DNA, we designed primer pairs that matched sequences in the genes for cytochrome *b* and the 12S ribosomal (r) RNA in the mtDNA of other vertebrates[12]. Each primer pair was selected so that it would amplify a fragment of, at most, 160 base pairs (bp), because longer sequences of old DNA have generally proved impossible to amplify[8,9]. We performed extractions of thylacine DNA from several samples (0.1 g) of tissue. One hide sample yielded a human mtDNA sequence, whereas a muscle sample gave a double sequence of both marsupial and human origin. Amplification of shorter sequences (~80 bp), however, from the muscle sample gave exclusively marsupial sequence. Human contamination is a frequent problem when amplifying sequences from old specimens, particularly if they have been tended for a long time by museum curators. Furthermore, DNA stemming from the specimen itself is usually more degraded and/or damaged than the contaminating DNA. This results in the specimen sequence becoming more predominant in the amplification products as the amplified sequences become shorter[10]. The preparation from a second hide sample gave unambiguous marsupial sequences of up to 160 bp, whereas amplifications of longer sequences yielded no specific products.

Figure 1 shows the sequences of the 12S rRNA and cytochrome *b* gene segments for the thylacine and six other marsupials. Through alignment by eye we found 94 bp of each of the 12S rRNA sequences that could be used for phylogenetic analyses. Table 1 shows the numbers of transversion and transition differences for each pair of species. For the 12S sequence the numbers of transversions separating the tested marsupials and the placental mammal (cow) exceed those separating the marsupials from one another. Transitions in the 12S gene show a less pronounced tendency for an increase correlated with increasing phylogenetic distance. This implies that saturation (due to multiple substitutions at the same site) is being approached for transitions in the 12S rRNA gene[13,14]. For the cytochrome *b* sequences most of the changes are at silent sites, where transversions as well as transitions seem to have reached saturation. Therefore, the cytochrome *b* sequence seems to evolve faster than the 12S rRNA sequence in marsupials. This is in agreement with our observations for mtDNA of placental mammals, where the segment of cytochrome *b* analysed evolves at least five times as fast as this 12S rRNA gene segment (data not shown). For this reason, only the 12S rRNA sequences were used for the phylogenetic tree analysis.

The tree shown in Fig. 2 relates the marsupials to one another based on the most parsimonious accounting for the differences observed in the 12S rRNA sequences. A tree in which the thylacine represents an early radiation with respect to Australian marsupials, comparable to that of the South American opossum (*Philander*), would require five more evolutionary substitutions. In a statistical test of the branching order of the tree shown in Fig. 2 (see legend), the thylacine forms a group with the other Australian carnivorous marsupials 98% of the time. Thus, the marsupial wolf falls well within the radiation of Australian marsupials, where it is closely related to the dasyurids, a group that includes the Tasmanian devil and Australian tiger cat.

The close relationship of the 12S rRNA gene in the marsupial wolf to that in dasyurids receives support from the sequences of the cytochrome *b* gene. Among these three species the transition bias typical of mtDNA can be discerned (Table 1), whereas for most of the other species the record of the transition bias has been erased by multiple substitutions. This indicates that the dasyurids and the thylacine diverged more recently than the other species studied[13,15].

A rough estimate of the time of divergence of the thylacine lineage from that leading to the dasyurids can be made from the average percentage of substitutions that are transitions (Table 1). This value (~70%) is similar for both the cytochrome *b* and the 12S rRNA genes and places the divergence of these species at 10-20 million years (Myr) ago, which is the time required to drop halfway to the plateau value of transitions (45%)[13,15]. Such a date is earlier than that inferred from

TABLE 1 Pairwise comparisons of transversions/transitions in marsupial mtDNAs

mtDNAs compared	*Thyl.*	*Sarc.*	*Dasy.*	*Echy.*	*Tric.*	*Phal.*	*Phil.*	*Bos*
Thylacinus	—	2/4	1/3	6/5	4/7	5/6	5/6	14/11
Sarcophilus	9/16	—	1/1	6/4	4/8	5/7	5/5	14/11
Dasyurus	6/16	3/11	—	5/5	3/7	4/7	4/6	13/12
Echymipera	10/14	11/15	10/11	—	2/5	1/6	5/1	10/9
Trichosurus	11/14	12/15	11/14	11/17	—	1/3	3/5	12/12
Phalanger	11/13	14/16	13/12	13/18	8/13	—	4/7	11/13
Philander	9/13	12/13	9/10	7/16	12/15	14/13	—	9/10
Bos	12/11	13/15	12/11	12/14	11/15	15/15	11/11	—

The numbers of transversion differences followed by the numbers of transition differences are given above the diagonal for the 94 bp of the 12S rRNA gene and below the diagonal for the 116 bp segment of the cytochrome *b* gene. A transversion is the replacement of a pyrimidine by a purine or vice versa; a transition is the replacement of a purine by a different purine or a pyrimidine by a different pyrimidine.

† Present address: Department of Genetics, University of Nottingham, Nottingham NG7 2UH, UK.

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a 12S rRNA

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Thylacinus   AAAT-TAGAACATAAC----GAATTATCTATTGAAAC-AAAGATA---TGAAGGAGGATTTAGTAGTAAATTAAGAATAGAGAGCTTAATTGAAAAAGGCAATGGGGTGCGT
Sarcophilus  .GTAC....G...............G..A.C.....T......C.................................................T.......A........
Dasyurus     CT.......................G....C............C.................................................T.......A........
Echymipera   ..C......GA.............C....TA.....-TT... ATGCC...............................T............T.......A...C...C
Trichosurus  ..........T............C..C..TA......CT... ATAC..............................................T.......A..AC...C
Phalanger    ..TA......A............C....CTA.....-.T...GATAC.C...-........................................C.......A..AC...C
Philander        ....G..........R...C.CA.TA......T....--TGCT............................................TT.......A...C...C
Bos          .C.CCA...GA..C.AGCAC...AGT.A.TA......C...A---..CCA............C......C...........T.....G.....TT....C...AA.CA..C
```

b cytochrome b

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Thylacinus   C TTT GGA TCC TTA CTA GGA ATC TGC CTA GTC ATT CAA ATC TTA ACA GGC TTA TTT CTA GCA ATA CAT TAC ACA TCA GAC ACA TCA ACT GCC TTC TCC TCA GTA GCA CAT ATC TGT C
               Phe Gly Ser Leu Leu Gly Ile Cys Leu Val Ile Gln Ile Leu Thr Gly Leu Phe Leu Ala Met His Tyr Thr Ser Asp Thr Ser Thr Ala Phe Ser Ser Val Ala His Ile Cys
Sarcophilus  . ..C ..G ..T ... T.. ... ..A ... ... A.T ... ... ..T C.C ..C ..A C.. ..C ... ... ... ..C ... ..C ..T ... ... .TC ..C ... ..T ... ..C ... ..C ..C ..T ... .
               ... ... ... ... ... ... Met ... ... Ile ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... Phe ... ... ... ... ... ... ... ... ... ...
Dasyurus     . ... ... ..Y C.. T.. ... G.A ... ... A.T ... ... ... C.C ... ... C.. ..C ... ... ... ..C ... ..T ... ... ... CTT ..C ..T ... ... ..C ... ..C ..C ..T ..C .
               ... ... ... ... ... ... Val ... ... Ile ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... Leu ... ... ... ... ... ... ... ... ... ...
Echymipera   T ... ..C ..A ... ... ... ... ... T.. ... ..C ... ... C.C ... ..A C.. ..C ... ..C ... ... ... ..G ... ... ..C CT. ..A ..T ... ..A ..C ..G ..T ..C ..T ..C .
               ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... Leu ... ... ... ... ... ... ... ... ... ...
Trichosurus  . ..C ... ..A C.. ... ..C ... ... T.. ACT ..A ... ... C.T ... ... ... ..C T.. ... ..G ..C ... ..C G.T ..T ... G.. ..A ..T ... ..A ... ... ..C ..C ... ... .
               ... ... ... ... ... ... ... ... ... Thr Met ... ... ... ... ... ... ... ... ... ... ... ... ... Ala ... ... Ala ... ... ... ... ... ... ... ... ... ...
Phalanger    . ... ... ..A C.. T.. ..T T.. ... ... ACA ..C ... ... ... ... ... C.. ... T.. ... ... ... ..C ..T C.C ... ... ... ..A ..T ..T ..A ... A.C ..C ..C ... .C. .
               ... ... ... ... ... ... Phe ... ... Thr ... ... ... ... ... ... ... ... ... ... ... ... ... ... Pro ... ... ... ... ... ... ... ... Ile ... ... ... Ser
Philander    T ... ..T ..A C.T ... ... ..A ... ... A.T ... ... ... C.C ... ... C.. ... T.. ..T ... ... ..T ... ... ... ... CT. ..C ..A ..T ..A ... ... ..C ... ... ..C .
               ... ... ... ... ... ... Met ... ... Ile ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... Leu ... ... ... ... ... ... ... ... ... ...
Bos          T ..C ..T ... C.C ..G ... ... ... ... A.. C.A ... ... C.C ... ... C.. ..C ... ... ... ..C ... ... ..C ... ... A.. ..A ..A ... ... ..T ..T A.C ... ... ..C .
               ... ... ... ... ... ... ... ... ... Ile Leu ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... Thr ... ... ... ... ... ... Thr ... ... ...
```

FIG. 1 mtDNA sequences from seven marsupial species, and cow. Dots indicate sequence identity with *Thylacinus* mtDNA. Dashes indicate deletions. *a*, Part of the 12S rRNA gene (corresponding to positions 1,154–1,261 in the cow mtDNA sequence, see ref. 17); the overlined positions were used for analyses. *b*, Part of the cytochrome *b* gene (corresponding to positions 14,609–14,724 in the cow mtDNA sequence, see ref. 17).

METHODS. Ancient DNA was extracted as described in ref. 8. The thylacine material consisted of two small pieces of untanned hide with attached hair collected in the early twentieth century and a piece of dried muscle (<1 g) adhering to a bone collected before 1893 in Tasmania. The sample of a Tasmanian devil, *Sarcophilus harrisii* (*Dasyuridae*), consisted of a few square millimetres of tissue from a 35-year-old museum skin (Museum of Vertebrate Zoology, UC Berkeley (MVZ), No. 127035). Frozen or ethanol-preserved liver or kidney samples were used for the remaining marsupials: *Dasyurus maculatus* (tiger cat, *Dasyuridae*, Australia, from J. A. W. Kirsch, Madison, WI), *Echymipera kalubu* (bandicoot, *Peramelidae*, New Guinea, MVZ No. FC871), *Trichosurus vulpecula* (possum, *Phalangeridae*, Australia, MVZ No. FC1968), *Phalanger sericeus* (possum, *Phalangeridae*, New Guinea, MVZ No. FC843), and *Philander opossum andersoni* (opossum, *Didelphidae*, South America, MVZ No. FC4901). Extractions with no tissue present were done to control for contamination by extraneous DNA. Initial studies on the state of preservation of DNA extracted from an untanned skin of a thylacine in the Zoological Museum of the University, Zürich, Switzerland, have been reported[9]. Amplifications were performed in 25 μl Tris (67 mM, pH 8.8) containing $MgCl_2$ (2 mM), bovine serum albumin (20 $\mu g\ ml^{-1}$), 1 mM of each dNTP, 1 μM of each primer, template DNA (10–1000 ng) and *Taq* polymerase (2 units, Cetus). Primers used for 12S rRNA gene sequences were L1373 (5′-CGCTGCAGAGAAATGGGCTACATTTTCT-3′) and H1478 (5′-TGACTGCAGAGGGTGACGGGCGGTGTGT-3′); for cytochrome *b* gene sequences they were L14841 (5′-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3′) and H14958 (5′-CTGCAGTCAGCCGTAATTTACGTCTC-3′). L and H refer to the light and heavy strands, respectively, and numbers to the base most 3′ of the primer in human mtDNA[18]. The temperature profile for 40 cycles of amplification was 40 s at 92 °C (denaturation), 1 min at 55 °C (annealing of primers), 2 min at 72 °C (polymerization). Purification in agarose (4%) of the initial amplification product, single-strand amplification of each strand and dideoxy chain termination sequencing with the limiting primer from the second amplification were as described[8,9].

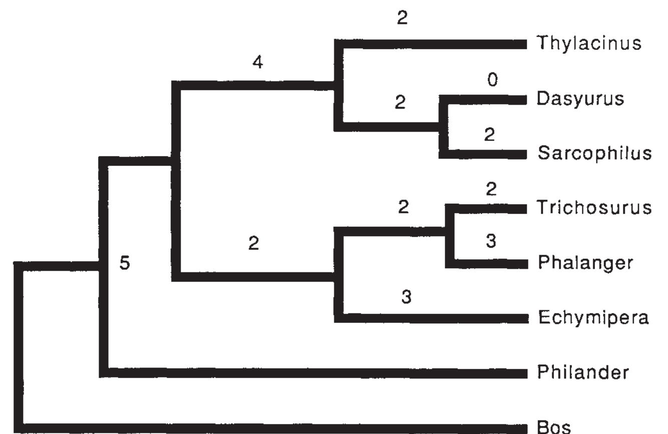

FIG. 2 Evolutionary tree for the marsupial wolf (*Thylacinus*) and six other marsupials based on 12S rRNA gene sequences. The numbers of events assigned to each segment of the tree are indicated. The tree is a majority-rule consensus tree obtained by boot-strapping[19] using the computer program PAUP[20]. It accounts for sequence differences at 19 positions with 27 changes. In the boot-strap analysis where subsets of the variable sites were resampled at random 100 times with replacement and the most parsimonious trees constructed in each case, the thylacine forms a monophyletic group with *Dasyurus* and *Sarcophilus* in 98% of the cases. If attention is restricted to the taxa *Philander*, *Echymipera*, *Thylacinus* and *Dasyurus* (or *Sarcophilus*), there are five (or four, respectively) informative substitutions grouping *Thylacinus* with the dasyurids and none grouping *Thylacinus* with *Philander*. When the cow (*Bos taurus*) sequence is added to the analysis, it connects to the *Philander* lineage.

immunological distances for albumin (6–10 Myr)[5,16], but agrees with what is known of the thylacine fossil record, which began 12–15 Myr ago and is confined to Australia[4]. In any event, the divergence of the thylacinids from dasyurids clearly postdates the Palaeocene separation of Australia from Antarctica and South America by tens of millions of years, thus ruling out the possibility that South American marsupials are closely related to the thylacine or represent its direct ancestors.

Although these results and immunological distances for albumin[5,16] strongly support an Australian origin for the thylacine, the morphological evidence does not. There are three tooth characters and a pelvic trait that the thylacine shares uniquely with the South American borhyaenids, as opposed to two features of the hind limb that are shared uniquely with dasyurids[4]. The morphological score of four to two favouring the hypothesis that the thylacine lies outside the Australian

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cluster of marsupial lineages contrasts with the score of zero to five against it derived from the molecular results[5] (Fig. 1). This statistically significant discrepancy justifies the proposal that the dental and pelvic traits shared uniquely by thylacines and borhyaenids suggest a remarkable amount of convergent or parallel evolution, resulting in the resemblance between these species. The marsupial wolf is thus a striking example of morphological convergence not only to placental wolves but also to South American carnivorous marsupials. This is likely to have been caused by parallel adaptations to similar modes of predation on different continents. The study of the molecular basis for such convergence at the level of the organism is a challenge for biology. □

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High abundance of viruses found in aquatic environments

Øivind Bergh, Knut Yngve Børsheim*, Gunnar Bratbak† & Mikal Heldal

Department of Microbiology and Plant Physiology, University of Bergen, Jahnebakken 5, N-5007 Bergen, Norway

THE concentration of bacteriophages in natural unpolluted waters is in general believed to be low[1,2], and they have therefore been considered ecologically unimportant[3]. Using a new method for quantitative enumeration, we have found up to 2.5×10^8 virus particles per millilitre in natural waters. These concentrations indicate that virus infection may be an important factor in the ecological control of planktonic micro-organisms, and that viruses might mediate genetic exchange among bacteria in natural aquatic environments.

The highest total counts of viruses and bacteria were found in samples from the eutrophic lake Plussee (Table 1). We found total counts of viruses of between 5×10^6 and 15×10^6 per ml in marine samples taken during the productive part of the year. Marine samples taken in winter, however, were found to have very low numbers of viruses (Table 1), thus indicating a seasonal variation in the concentration of viruses in natural waters. Our virus counts are 10^3-10^7 times higher than previous reports on virus numbers in natural aquatic environments, which are based on counts of plaque-forming units using various host bacteria[1,2]. Previously, most marine bacteriophages that have been isolated and described have had a head size larger than 60 nm (ref. 4). We have found, however, that smaller viruses with head size less than 60 nm seem to dominate natural populations (Table 1).

* Present address: The Biological Station, University of Trondheim, Bynesveien 46, N-7018 Trondheim, Norway.
† To whom correspondence should be addressed.

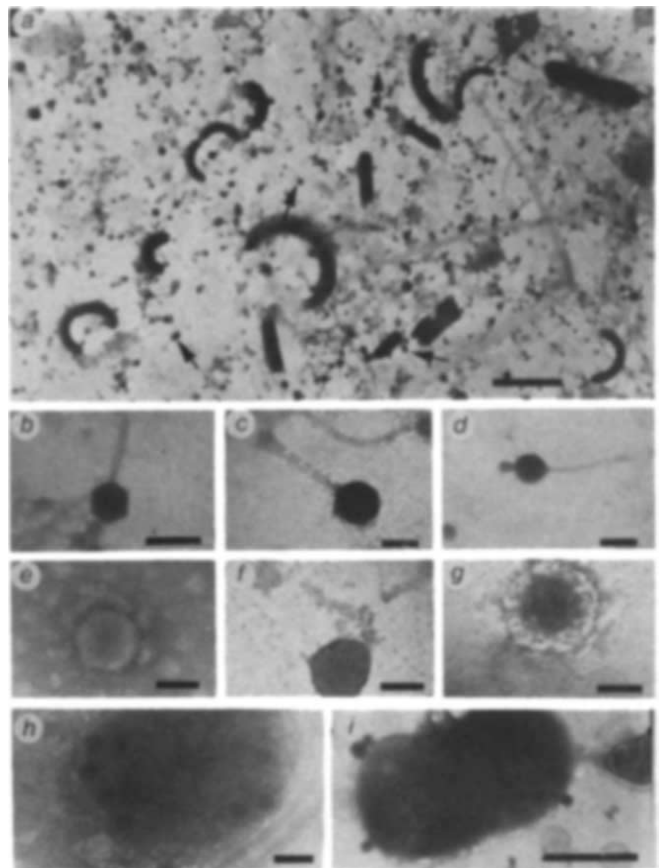

FIG. 1 Phages and bacteria observed in water samples from various natural marine and limnic ecosystems. *a*, Overview of bacteria and virus-sized particles from Lake Plussee. *b–e*, Particles classified as bacteriophages according to size and morphology. *f–g*, Particles of uncertain affiliation, but classified as viral particles in total counts. *h*, Bacterium with virus-like particles inside. *i*, Bacterium with phages attached to its surface. The particles, including bacteria, were collected by centrifugation of fixed water samples, 2% glutaraldehyde (Plussee, Chesapeake Bay, North Atlantic), 2% formaldehyde (Barents Sea), or from unfixed samples (Korsfjorden, Raunefjorden). Water samples were filled in centrifuge tubes with plastic-moulded flat bottoms. Grids with carbon-coated formvar fim were then dropped onto the flat bottom and the samples were centrifuged (swing-out rotor) at 100,000*g* for 90 min. After removal of water, the grids were air-dried, stained with uranyl acetate (2%) and total bacteria and virus particles counted at high magnification (×20,000–100,000) in a Jeol 100-CX transmission electron microscope. Scale bars: *a*, 1 μm; *b–d* and *h*, 0.1 μm; *e–g*, 0.05 μm; *i*, 0.5 μm.

Bacteriophages that can be assigned to the Bradley groups A or B[5] are easily recognized by their tail structures (Fig. 1*a* (arrowheads), *b*, *c* and *d*). Some examples of virus-like particles, apparently without any tail structure but otherwise of similar size and morphology, are shown in Fig. 1*e*, *f* and *g*. These particles may be bacteriophages of Bradley groups C, D or E; or they may be phages of group A or B that have lost their tail. They may also be viruses relating to eukaryotic hosts such as microalgae.

Most of the virus particles we observed appeared to be free in the water, but some were also associated with bacteria. The bacterium shown in Fig. 1*h* seems to be in a phase of lytic disruption with numerous virus-like particles inside. Figure 1*i*

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