MBE Advance Access published January 8, 2015

Late Pleistocene Australian Marsupial DNA Clarifies the Affinities of Extinct Megafaunal Kangaroos and Wallabies

Bastien Llamas,*[,†,1] Paul Brotherton,[†,‡,1] Kieren J. Mitchell,[1] Jennifer E.L. Templeton,[1,2] Vicki A. Thomson,[1] Jessica L. Metcalf,[§,1] Kyle N. Armstrong,[1] Marta Kasper,[¶,1] Stephen M. Richards,[1] Aaron B. Camens,[2,3] Michael S.Y. Lee,[3,4] and Alan Cooper*[,1]

[1]Australian Centre for Ancient DNA, School of Earth and Environmental Sciences, University of Adelaide, SA, Australia

[2]School of Biological Sciences, Flinders University, SA, Australia

[3]School of Earth and Environmental Sciences, University of Adelaide, SA, Australia

[4]Earth Sciences Section, The South Australian Museum, SA, Australia

[†]These authors contributed equally to this work.

[‡]Present address: BiSN Laboratory Services, Runcorn, United Kingdom

[§]Present address: BioFrontiers Institute, University of Colorado, Boulder, CO.

[¶]Present address: Forensic Science SA, Adelaide, SA, Australia.

***Corresponding author:** E-mail: bastien.llamas@adelaide.edu.au; alan.cooper@adelaide.edu.au.

Associate editor: Michael Rosenberg

Abstract

Understanding the evolution of Australia's extinct marsupial megafauna has been hindered by a relatively incomplete fossil record and convergent or highly specialized morphology, which confound phylogenetic analyses. Further, the harsh Australian climate and early date of most megafaunal extinctions (39–52 ka) means that the vast majority of fossil remains are unsuitable for ancient DNA analyses. Here, we apply cross-species DNA capture to fossils from relatively high latitude, high altitude caves in Tasmania. Using low-stringency hybridization and high-throughput sequencing, we were able to retrieve mitochondrial sequences from two extinct megafaunal macropodid species. The two specimens, *Simosthenurus occidentalis* (giant short-faced kangaroo) and *Protemnodon anak* (giant wallaby), have been radiocarbon dated to 46–50 and 40–45 ka, respectively. This is significantly older than any Australian fossil that has previously yielded DNA sequence information. Processing the raw sequence data from these samples posed a bioinformatic challenge due to the poor preservation of DNA. We explored several approaches in order to maximize the signal-to-noise ratio in retained sequencing reads. Our findings demonstrate the critical importance of adopting stringent processing criteria when distant outgroups are used as references for mapping highly fragmented DNA. Based on the most stringent nucleotide data sets (879 bp for *S. occidentalis* and 2,383 bp for *P. anak*), total-evidence phylogenetic analyses confirm that macropodids consist of three primary lineages: Sthenurines such as *Simosthenurus* (extinct short-faced kangaroos), the macropodines (all other wallabies and kangaroos), and the enigmatic living banded hare-wallaby *Lagostrophus fasciatus* (Lagostrophinae). *Protemnodon* emerges as a close relative of *Macropus* (large living kangaroos), a position not supported by recent morphological phylogenetic analyses.

Key words: **ancient DNA, phylogenetics, Sthenurinae, Lagostrophinae, Macropodinae.**

Introduction

The Late Pleistocene was marked by the extinction of many large terrestrial vertebrates (megafauna) around the world. One of the most remarkable and least understood of these extinction events occurred in Australia, where diverse marsupial, avian, and reptile megafauna dominated a uniquely isolated continent. Further, the relative antiquity of the Australian extinctions (39–52 ka) (Roberts et al. 2001; Price et al. 2011; Gillespie et al. 2012) compared with Late Pleistocene extinctions on other continents (ca. 10-30 ka) (Guthrie 2006) has meant that their drivers remain controversial. Among the many species lost during the Late Pleistocene were enigmatic animals such as the huge "marsupial rhinoceros" *Diprotodon*, the "marsupial lion" *Thylacoleo*, the giant short-faced sthenurine kangaroos, the 5-m-long monitor lizard *Varanus priscus*, and the 200 kg flightless bird *Genyornis*.

Among Australia's extinct megafauna, the short-faced browsing sthenurine kangaroos are a group of particular interest because their phylogenetic relationship to extant macropodines (kangaroos, wallabies, and relatives within the family Macropodidae) is unclear. The Sthenurinae, with 6 genera and 26 species described to date (Prideaux 2004), were an ecologically distinct Miocene radiation that parallel extant macropodines. Some studies of craniodental morphology suggest that the endangered banded hare-wallaby (*Lagostrophus fasciatus*), currently restricted to a relictual

Article

distribution on three small islands off Western Australia, might represent the sole remaining extant sthenurine lineage (Flannery 1983, 1989; Murray 1995). However, more recent morphological studies placed the banded hare-wallaby as an isolated lineage either sister to all modern macropodines, to the exclusion of sthenurines (Prideaux 2004), or sister to a sthenurine/macropodine clade (Prideaux and Warburton 2010; Prideaux and Tedford 2012). Genetic studies have confirmed that *L. fasciatus* is indeed the sole living representative of an ancient kangaroo lineage (Westerman et al. 2002), but cannot determine how it is related to sthenurines due to the lack of molecular data for the latter, or indeed any Late Pleistocene extinct Australian megafaunal taxa.

The problems encountered in determining the phylogenetic relationships of the sthenurine kangaroos are typical of Australia's extinct megafauna. Firstly, the pre-Pleistocene Australian fossil record is quite poor compared with other continents (Archer et al. 1999). Secondly, many extinct Australian marsupial megafauna were morphologically highly divergent, evolving into extreme forms unlike any living species and thereby hindering phylogenetic inference. Finally, unlike recently extinct megafauna from other continents, the antiquity and taphonomy of Australian megafaunal fossil deposits have usually precluded the retrieval of ancient DNA (aDNA). Molecular data have been instrumental in resolving the phylogenetic relationships of many living and extinct species (Bunce et al. 2009; Green et al. 2010; Reich et al. 2010; Rohland et al. 2010; Meyer et al. 2012; Miller et al. 2012, 2013; Mitchell, Llamas, et al. 2014; Mitchell, Wood, et al. 2014), and have the potential to elucidate phylogenetic relationships when morphological data are equivocal or misleading (Springer et al. 2007; Lee and Camens 2009). Although great strides have been made in the molecular analysis of ancient remains from high altitudes/latitudes (where low temperatures permit the survival of aDNA), retrieval of DNA from lower altitudes/latitudes (the usual situation in Australia) remains problematic due to the tight correlation between high temperatures and elevated rates of DNA decay (Smith et al. 2003; Allentoft et al. 2012). As a result, Late Pleistocene Australian megafaunal fossils represent a challenge for current aDNA methodologies and there have been relatively few successful studies: The isolation of emu DNA from eggshells (19 ka) (Oskam et al. 2010); plant and murid DNA from a midden (30.5 ka) (Murray et al. 2012); and bird, reptile, and mammal DNA from bulk extracts of highly fragmented bones (4.3–45.6 uncalibrated ^{14}C ka) (Murray et al. 2013). In the latter study, putative macropodid sequences (<36 uncalibrated ^{14}C ka) were identified that may have been contributed by extinct megafaunal taxa. However, a limitation of bulk DNA extractions is that unequivocal identification of the remains is difficult, as individual sequences are not directly associated with individual fossils.

Recent studies have described methods for selectively capturing and sequencing short, low-concentration endogenous DNA fragments using primer extension (Briggs et al. 2009; Krause et al. 2010), microarrays (Burbano et al. 2010), in-solution molecular baits (Avila-Arcos et al. 2011; Fu, Meyer, et al. 2013; Mitchell, Llamas, et al. 2014; Mitchell, Wood, et al. 2014), and custom polymerase chain reaction (PCR) products (Maricic et al. 2010; Sanchez-Quinto et al. 2012; Brotherton et al. 2013; Fu, Mittnik, et al. 2013; Der Sarkissian et al. 2014). Although these methods appear well suited for Australian megafaunal material, a current limitation for studying extinct species is the need for molecular information from a close phylogenetic relative to design primers or baits for hybridization enrichment. This is a considerable problem in the study of Australian megafauna, as many recently extinct forms are only distantly related to their closest living relatives (e.g., sthenurine kangaroos diverged from kangaroos and wallabies prior to the Middle Miocene approximately 16 Ma [Prideaux and Warburton 2010; Prideaux and Tedford 2012]). However, recent molecular hybridization studies have shown that DNA samples can be successfully enriched using molecular baits designed from quite divergent species (Mason et al. 2011; Li et al. 2013; Mitchell, Llamas, et al. 2014; Mitchell, Wood, et al. 2014). This suggests that hybridization capture might be a viable method for studying the genetics of Australian megafauna.

Here, we report the use of multispecies DNA hybridization capture to characterize partial mitochondrial genomes (879 and 2,383 bp, respectively) from two extinct Australian megafaunal taxa found at Mt Cripps, Tasmania: *Simosthenurus occidentalis* (giant short-faced kangaroo, hereafter referred to as *Simosthenurus*) and *Protemnodon anak* (giant wallaby, hereafter *Protemnodon*). Radiocarbon dating of the *Simosthenurus* and *Protemnodon* material (46–50 and 40–45 ka, respectively) (Gillespie et al. 2012) indicates that these samples are significantly older than any extinct Australian megafaunal remains that have previously yielded DNA. We combine our new DNA sequence data with an existing morphological character matrix to create a total-evidence phylogeny that clarifies the phylogenetic position of these enigmatic megafauna.

Results

aDNA Analysis

Biochemical analyses of the specimens during radiocarbon dating analyses suggested poor preservation of organic material, with very low nitrogen content (0.44–0.45%). As a consequence, only one of three attempts to radiocarbon-date the *Simosthenurus* sample was successful (reference OxA-17143) (Gillespie et al. 2012). Preliminary PCR tests for the presence of marsupial DNA indicated that the DNA was highly fragmented (supplementary note S2, Supplementary Material online), meaning that a phylogenetic study would be unlikely to be successful using a PCR approach. Although molecular hybridization followed by high-throughput Next Generation Sequencing (NGS) seemed appropriate, the lack of a close relative complicated bait design. Osteological analyses suggest that both *Simosthenurus* and *Protemnodon* are part of the same family as modern kangaroos (Macropodidae) (Prideaux and Warburton 2010), so we designed hybridization baits from five divergent macropodid taxa (asterisked in fig. 1A) to enrich the DNA extracts for macropodid DNA prior to

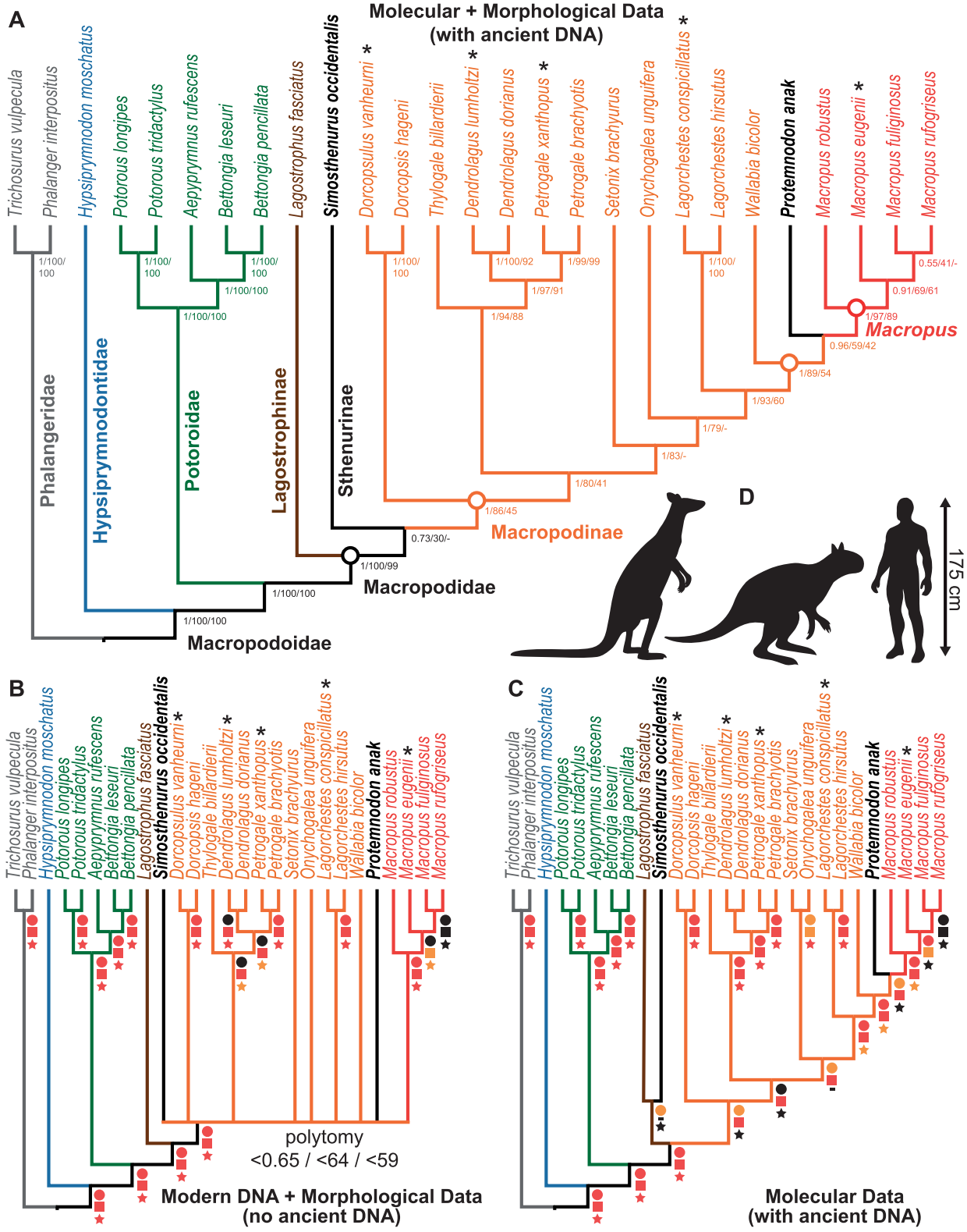

FIG. 1. Phylogenetic relationships of Australian macropodoids (including extinct megafauna) using molecular and morphological data. (*A*) Phylogenetic analyses using all molecular (including aDNA) and morphological data. Taxa used for bait design are marked with an asterisk. Clade support is shown as PP/Maximum Likelihood Bootstrap (ML)/Maximum Parsimony Bootstrap (MP). The Bayesian consensus topology is shown; The "-" sign denotes clade not found in ML and/or MP trees. Open circles denote robustly supported clades that tightly constrain affinities of extinct taxa. (*B*) Phylogenetic analyses using only modern molecular data (excluding aDNA) and morphological data for all taxa, including the extinct megafauna. Support values are given as follows: Filled circles are for PP, filled squares are for ML, and filled stars are for MP; Red shows robust support (>0.9/>70/>70), orange shows weak support (>0.8/>50/>50), and black shows little or no support (<0.8/<50/<50). (*C*) Phylogenetic analyses using only molecular data (including aDNA). Symbols and colors as in (*B*). (*D*) Adult size of *Protemnodon anak* (left silhouette) and *Simosthenurus occidentalis* (middle silhouette) relative to a 175-cm-tall human.

sequencing (see Materials and Methods and supplementary note S3, Supplementary Material online).

We obtained 5,723,580 raw sequencing reads for *Protemnodon* (Ion Torrent PGM only) and 1,039,524 for *Simosthenurus* (Ion Torrent PGM: 678,102; Roche 454 GS-FLX: 286,711; Pacific Biosciences RS: 74,711). Reads were trimmed for residual adapter sequences and low-quality sequences. Reads shorter than 25 nucleotides were filtered out as part of the trimming process (supplementary note S4, Supplementary Material online). The resulting data set had an average read size of 37.1 ± 8.9 nt for *Protemnodon* and 40.8 ± 15.8 nt for *Simosthenurus* (PGM: 47.6 ± 21.9 nt; 454 GS-FLX: 38.6 ± 10.2 nt; RS: 41.6 ± 12.1 nt). The overall short length of the reads confirmed the highly degraded nature of the DNA. Moreover, the taxonomic distribution of the filtered reads showed the large majority of reads were either assigned to prokaryotes or could not be assigned to any known taxonomic group, suggesting a high level of background, exogenous DNA (supplementary fig. S3, Supplementary Material online).

To retrieve the endogenous reads belonging to *Simosthenurus* and *Protemnodon*, we used the short reads mapper BWA (Li and Durbin 2009) following published guidelines for aDNA analysis (Orlando et al. 2011; Kircher 2012; Schubert et al. 2012). As no reference data were available for *Simosthenurus* and *Protemnodon*, we used the five mitochondrial genomes used to design molecular baits as mapping references. We explored the parameter space for mismatches and gap openings to account for the resulting phylogenetic distance between the references and the target organism (supplementary note S4, Supplementary Material online). Analyses using the most stringent parameters resulted in 58 mapped reads for *Simosthenurus* and 161 reads for *Protemnodon* (supplementary table S5, Supplementary Material online). Neither data set contained reads of likely human origin (Kircher 2012). Progressively relaxing the mapping parameters increased the number of mapped reads (up to an order of magnitude), but this was accompanied by the incorporation of a small proportion of potential human DNA contaminants (0.6–3.2%) (supplementary table S5, Supplementary Material online).

Monitoring for likely human reads does not preclude the presence of other contaminating environmental DNA sequences, especially when using Ion Torrent data, which include frequent indel errors (Loman et al. 2012; Quail et al. 2012; Bragg et al. 2013), and when mapping is performed using phylogenetically distant reference genomes. Thus, we further refined the most stringent mapping analysis using BLASTN and discarded all reads that did not readily align to a known marsupial sequence (see supplementary note S4, Supplementary Material online). The final *Simosthenurus* data set included 37 unique reads covering 879 bp of the mitochondrial genome, whereas the final *Protemnodon* data set included 121 unique reads covering 2,383 bp of the mitochondrial genome. Unfortunately, the low number of reads did not allow us to assess the DNA damage pattern characteristic of aDNA reads, that is, accumulation of 5′ C-to-T misincorporations, and presence of purines at the position immediately before the start and after the end of the reads (Briggs et al. 2007; Orlando et al. 2011; Sawyer et al. 2012).

Phylogenetic Analyses

We added our final aDNA sequence data set to a data matrix comprising mitochondrial genomes from 23 extant macropodoids, and two phalangerid possum outgroups (supplementary table S2, Supplementary Material online). We augmented this nucleotide matrix with morphological data (83 characters) obtained from a previous study (Prideaux and Warburton 2010; Prideaux and Tedford 2012). To examine the effect of our new aDNA sequences on phylogenetic resolution among macropodids, we considered three separate data sets: 1) All morphological and all nucleotide data including aDNA (total-evidence), 2) all morphological data plus nucleotide data from extant taxa only (no aDNA), and 3) all nucleotide data without any morphological characters. Bayesian, Maximum Likelihood (ML), and Maximum Parsimony analyses were performed on each data set (see supplementary note S6, Supplementary Material online). The discussion below focuses on the Bayesian and likelihood results, but parsimony also gave consistent trees although sometimes with weaker support.

The total-evidence analyses, which used all morphological and molecular (including aDNA) data, resolved the positions of both *Simosthenurus* and *Protemnodon* (fig. 1A). *Simosthenurus* was robustly placed within the family Macropodidae (Bayesian Posterior Probability [PP] = 1, ML bootstrap = 100%) but outside of the subfamily Macropodinae (PP = 1, ML = 86%). However, the branching order between Lagostrophinae, Sthenurinae, and Macropodinae could not be robustly resolved. We attempted to improve topological support for the branching among Lagostrophinae, Macropodinae, and Sthenurinae by performing a Bayesian clock-based analysis. The resulting phylogeny was largely consistent with the results of undated analyses (supplementary fig. S12, Supplementary Material online). Node age estimates were extremely wide due to difficulties in accurately constraining the age of the root caused by gaps in the Australian stratigraphic record. Consequently, we focused only on undated analyses. In a further attempt to improve phylogenetic resolution, we repeated the dated Bayesian analysis with the addition of morphological characters for four fossil taxa (*Wanburoo*, *Dorcopsoides*, *Hadronomas*, and *Ngamaroo*) from Prideaux and Warburton (2010). The resulting tree was largely consistent with the undated analyses of the core taxa (supplementary fig. S13, Supplementary Material online). The addition of two fossil sthenurine taxa (*Wanburoo* and *Hadronomas*) did not improve topological support for the branching order among the three macropodid subfamilies. Consequently, in all further analyses we excluded taxa for which no molecular data were available.

Protemnodon formed a robust clade with *Wallabia* and *Macropus* (PP = 1, ML = 89%), and within this clade had moderate support as sister to the sampled *Macropus* (PP = 0.96, ML = 59%). The wider phylogenetic relationships of extant taxa were highly consistent with recent trees based on

larger combined mitochondrial and nuclear data sets (e.g., Meredith et al. 2009a; Mitchell, Pratt, et al. 2014). In contrast to some studies (Meredith et al. 2009b), our results support the monophyly of *Macropus* with respect to *Wallabia* (Phillips et al. 2013).

When the aDNA data were excluded from our analyses, resolution was much poorer. Support for both *Simosthenurus* and *Protemnodon* falling within Macropodidae remained (fig. 1*B*), but their positions within Macropodidae were unresolved, with the basal lineages of the family forming a polytomy. Using only nucleotide data (including aDNA) produced a well-resolved tree similar to the total-evidence analysis (fig. 1*C*). *Simosthenurus* was again placed within Macropodidae but outside Macropodinae, and *Protemnodon* was again placed in a clade with *Wallabia* and *Macropus* as a sister lineage to sampled *Macropus* species. However, support for these groupings was generally weaker than in the total-evidence analysis.

Impact of Sequence Data Processing on Phylogenetic Results

The bulk of the sequencing data was generated using an Ion Torrent PGM, a platform known to generate homopolymer-associated indel errors (Loman et al. 2012; Quail et al. 2012; Bragg et al. 2013). The short reads mapper TMAP (https://github.com/nh13/TMAP) has been optimized for Ion Torrent data and previous studies have demonstrated that it can successfully reconstruct the mitochondrial genomes of extinct organisms de novo (Mitchell, Llamas, et al. 2014; Mitchell, Wood, et al. 2014) using an iterative mapping approach (Green et al. 2008; Hahn et al. 2013). In this approach, the information from newly mapped reads is used as a new reference to seed the mapping of reads in more divergent regions during the next mapping iteration. Iterative mapping continues by growing the assembly from existing seeds, until either the mitochondrial genome has been completed or the number of reads added stops increasing.

We used TMAP to perform iterative mapping on the *Protemnodon* and *Simosthenurus* data sets. As expected, we observed an increasing number of reads mapped following each iteration (fig. 2), leading to an increased coverage of the mitochondrial DNA (mtDNA) but still failing to reconstruct complete mitogenomes (supplementary table S6, Supplementary Material online). Misincorporation patterns and purine frequency before the start of the reads were as expected for aDNA, although there was a relatively elevated background noise due to the relatively low number of reads (supplementary figs. S4–S7, Supplementary Material online).

We investigated the taxonomic distribution of the reads incorporated during the iterative mapping by aligning reads from each iteration against the GenBank nucleotide database using BLASTN. No prokaryote reads were detected after the first iteration of mapping. However, we observed an increase in the number of nonmarsupial reads after each subsequent mapping iteration, whereas the number of marsupial reads remained stable after the second iteration (line graphs in fig. 2; supplementary tables S8 and S9, Supplementary Material online). Proportionally, only reads of prokaryote origin increased significantly over successive iterations (bar graphs in fig. 2 and supplementary figs. S9 and S10, Supplementary Material online).

We further explored the impact of the increased incorporation of contaminant reads during the mapping iterations on the phylogenetic analyses. The alignments obtained after the initial and the last iterations were used for ML and Bayesian phylogenetic analyses as described above and in supplementary note S6, Supplementary Material online, using the combined morphological and molecular data sets (supplementary fig. S8, Supplementary Material online). The topologies were strongly supported and remained unchanged from figure 1. However, the terminal branch length for both extinct marsupials increased significantly between the initial and the final mapping iterations (supplementary table S7, Supplementary Material online).

Discussion

Phylogenetic Affinities of Extinct Australian Megafauna

Homoplasy in macropodid morphology means that analyses of morphological data alone have been unable to conclusively resolve the phylogenetic relationships of *Simosthenurus* and *Protemnodon* (Flannery 1989). The addition of ancient mtDNA provides vital independent evidence for the precise affinities of these taxa, although the limited information contained in the short sequences means that the use of both molecular and morphological data resulted in stronger results than molecular data alone. The new aDNA substantially revised the phylogenetic position of *Protemnodon*, and confirmed the position of sthenurines as a distinct lineage, separate from living kangaroos and wallabies, within Macropodidae. *Protemnodon* has long been of contentious and unstable affinities (Prideaux and Warburton 2010). The most recent morphological analysis placed *Protemnodon* as a sister member of the Macropodinae (outside of a clade comprising *Lagorchestes*, *Onychogalea*, *Wallabia*, and *Macropus* [Prideaux and Warburton 2010; Prideaux and Tedford 2012]), whereas the molecular and combined data suggest that *Protemnodon* is more closely related to *Macropus*. Sthenurine kangaroos (as represented by *Simosthenurus*) are identified as the third major lineage of the Macropodidae, along with macropodines and lagostrophines. This is supported by both analyses of the molecular data alone (fig. 1C) and recent morphological studies (Prideaux and Warburton 2010; Prideaux and Tedford 2012), strengthening support for the results of our total-evidence analyses (fig. 1*A*). Although relationships between sthenurines, macropodines, and lagostrophines are not robustly resolved in our analyses, the sthenurine–macropodine clade found in the Bayesian and likelihood analyses (fig. 1*A*) is consistent with certain dental and upper appendicular characters, and an inferred increased emphasis on bipedal hopping (Prideaux and Warburton 2010; Prideaux and Tedford 2012)—even if recent evidence argues against hopping as default locomotion for the large Pleistocene sthenurines (Janis et al. 2014).

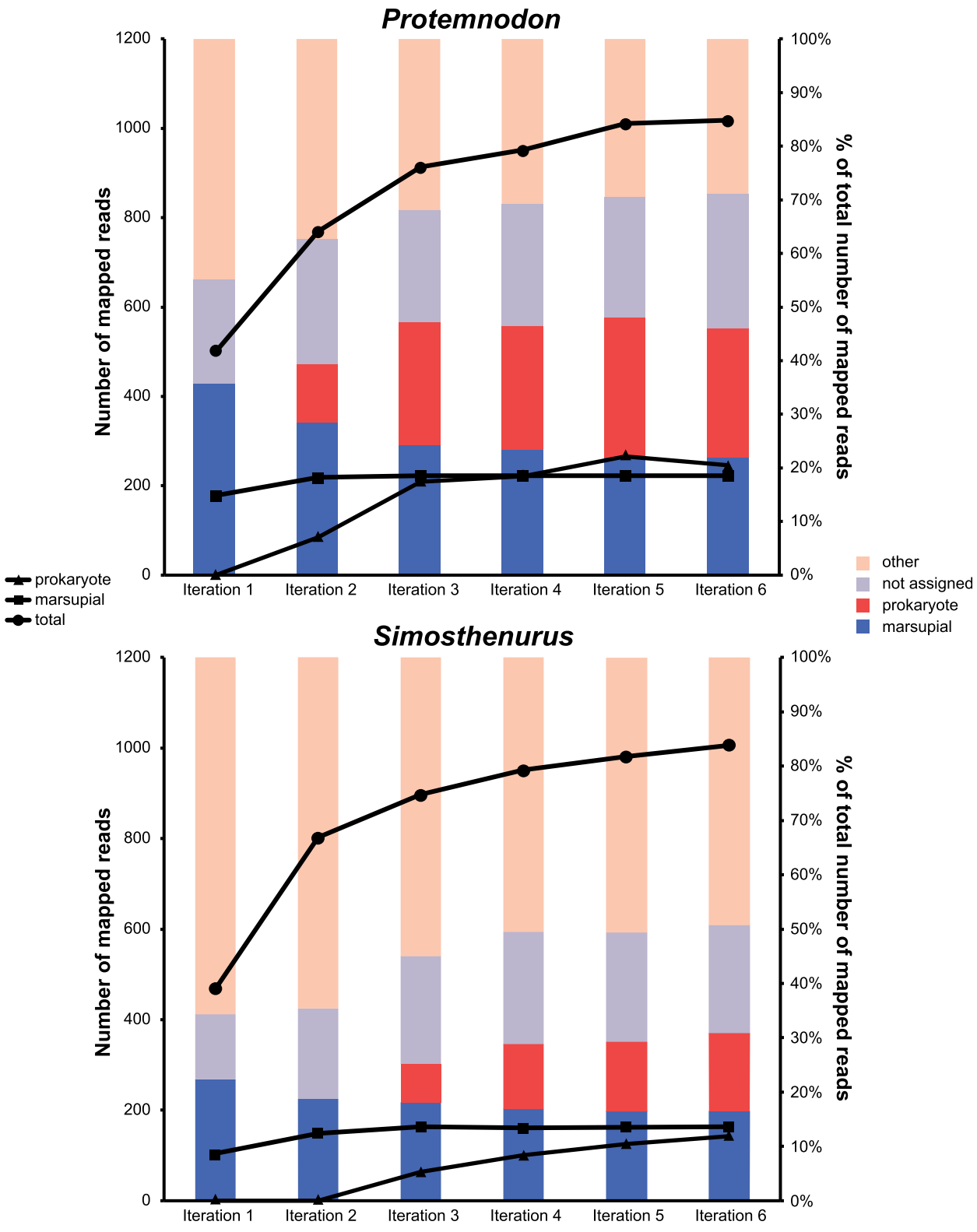

FIG. 2. Taxonomic classification of mapped reads after each mapping iteration. The line graphs (*y* axis and legend on the left) represent the number of mapped reads: Total number (filled circles), reads assigned to marsupials (filled squares), and reads assigned to prokaryotes (filled triangles). The bar graphs (*y* axis and legend on the right) represent the percentage of the total number of mapped reads assigned to the different taxonomic groups. The "not assigned" group is reads that could not be assigned to any organism for which data are available in the GenBank database ("not assigned" and "no hits" reads in supplementary tables S8 and S9, Supplementary Material online).

Limitations of Hybridization Enrichment for Analyzing Upper Pleistocene Australian Remains

Cross-species hybridization capture has previously been applied to specimens for which reference sequences from closely related outgroups are available (Mason et al. 2011; Mitchell, Llamas, et al. 2014; Mitchell, Wood, et al. 2014), but the technique is potentially efficient even across highly phylogenetically divergent taxa (Li et al. 2013). As a result, cross-species hybridization capture holds great potential for the aDNA field, where many extinct species lack close living outgroups. In this study we retrieved only a limited number of unique mtDNA reads, resulting in coverage of a small portion of the mitochondrial genome. However, this is more likely to be due to the poor preservation of DNA than phylogenetic distance: The *Simosthenurus* and *Protemnodon* specimens used in this study were collected as cave floor surface finds, and were consequently exposed to changes in the cave environment without protection from a soil matrix. The overall short length of the DNA reads retrieved for both specimens confirms that the templates have undergone substantial decay (Smith et al. 2003; Allentoft et al. 2012). In summary, Late Pleistocene Australian megafaunal fossils will continue to represent a challenge for aDNA studies due to poor preservation; however, the advent of hybridization capture has made molecular analysis of such specimens possible.

Impact of Contaminant Reads in Phylogenetic Analyses

The low endogenous DNA content in the extinct marsupial DNA extracts seemed to impair our iterative mapping approach. In the first iteration of mapping, only reads from conserved regions were mapped successfully due to the divergence between the extinct taxon and the reference (supplementary fig. S11, Supplementary Material online). A potential limitation is that these newly mapped reads may not lead to a robust consensus sequence due to the presence of artefactual substitutions typical of aDNA data. If the number of reads from authentic endogenous DNA is too low, seeds are more likely to grow through the addition of damaged or contaminant reads and the resulting degenerated consensus seed will then serve as a template for contaminant reads in the next iteration.

Phylogenetic analyses of consensus sequences obtained using multiple rounds of iterative mapping resulted in robustly supported trees with topologies identical to figure 1*A*. However, the incorporation of an increasing number of prokaryote reads during iterative mapping resulted in a substantial increase in branch length for the extinct taxa in the final mapping iteration, whereas branch length remained unchanged for all other taxa in the phylogeny.

Theoretically, contaminant sequencing reads could be mapped to any target genome if the mapping parameters were too relaxed. However, the examination of damage patterns would reveal a distribution of substitutions along the reads instead of an accumulation of 5′ C-to-T transitions. However in this case, iterative mapping resulted in the incorporation of contaminant sequences into the consensus sequence and these regions were also used as a reference during the DNA damage analyses. As a result, the contaminant reads could not be detected through standard examination of damage patterns.

The Importance of Mapping Methods for the Analysis of Phylogenetically Divergent Taxa

The iterative mapping approach was first used in the aDNA field to reconstruct a Neanderthal mitochondrial genome (Green et al. 2008). Most subsequent aDNA studies focused on taxa for which closely related reference genomes were available (i.e., early humans, extinct hominins, or horses). It is only recently that mitochondrial genomes from phylogenetically divergent extinct taxa, such as the elephant bird, have been successfully reconstructed from distantly related reference genomes using iterative mapping (Mitchell, Llamas, et al. 2014; Mitchell, Wood, et al. 2014).

The iterative mapping approach clearly reached operational limits in this study. The first two iterations for *Simosthenurus* and only the first iteration for *Protemnodon* produced data sets apparently free of bacterial DNA contaminants. Then without stringent filtering, the combination of low sequence coverage, DNA damage, and presence of contaminant environmental DNA rapidly led to the reconstruction of a chimeric consensus containing bacterial sequences, although this still produced a similar resulting tree. However, iterative mapping is likely to be efficient with molecular data sets where preservation is reasonably good (Green et al. 2008; Mitchell, Llamas, et al. 2014; Mitchell, Wood, et al. 2014) and should be considered when studying extinct taxa. It is also likely to be relevant in the analysis of ancient pathogen genomes, where variable genomic structure and horizontal gene transfer limit the potential of direct mapping against modern references.

Conclusion

Cross-species DNA capture by hybridization combined with NGS is a promising method to decipher the evolutionary history of the extinct Australian megafauna, and other taxa that are similarly phylogenetically distinct. The analyses of partial mtDNA sequences retrieved from both *Simosthenurus* and *Protemnodon* are broadly consistent with morphological data, but provide clarification of their phylogenetic positions. Sthenurines form one of the three primary lineages of the marsupial family Macropodidae, along with lagostrophines (represented today solely by the banded hare-wallaby *Lagostrophus fasciatus*) and macropodines (all other extant kangaroos and wallabies). These results support the hypothesis that the endangered banded hare wallaby (*L. fasciatus*) is the last surviving member of a distinct macropodid lineage, but is not a sthenurine. In contrast, our results suggest that *Protemnodon* represents a much more recent lineage closely related to *Macropus*, a position not predicted from morphology alone. Further aDNA analysis of enigmatic taxa such as *Thylacoleo* or *Diprotodon* will be required to fully resolve the evolutionary history of the

Australian megafauna, and cross-species DNA capture provides an important new approach for this endeavor.

Materials and Methods

All aDNA work was performed at the Australian Centre for Ancient DNA (ACAD, in Adelaide), a purpose-built laboratory dedicated to aDNA studies using established protocols for aDNA work.

Extinct Marsupial Samples

Skeletal remains of the extinct *S. occidentalis* and *P. anak* specimens were collected at Mt Cripps, Tasmania, in caves CP222 (Calcite Column Chasm) and CP213 (Bone Aven), respectively. (See supplementary note S1, Supplementary Material online, for specimen identification, dating information, sample preparation, and DNA extraction.)

aDNA Libraries Preparation

The *Simosthenurus* DNA extract (ACAD3501B) and *Protemnodon* DNA extract (ACAD9010A) were processed as described previously (Brotherton et al. 2013) to generate aDNA libraries.

Molecular Baits Design Strategy and Preparation

Although no genetic information is available for either extinct marsupial, the latest paleontological studies place *Simosthenurus* and *Protemnodon* within Macropodidae (Prideaux and Warburton 2010). Therefore, we applied a cross-species hybridization strategy to capture mtDNA fragments from the aDNA libraries using complete mitochondrial genomes from five extant macropodids: *Dendrolagus lumholtzi*, *Dorcopsulus vanheurni*, *Lagorchestes conspicillatus*, *Macropus eugenii*, and *Petrogale xanthopus* (supplementary table S2, Supplementary Material online). The preparation of molecular baits was adapted from Brotherton et al. (2013). Details can be found in supplementary note S3, Supplementary Material online.

Hybridization Capture Assay

DNA capture by hybridization was performed following Brotherton et al. (2013), using 100 ng of biotinylated DNA baits (see also supplementary fig. S1, Supplementary Material online). As in Brotherton et al., we selectively enriched for marsupial DNA of significant homology to bait sequence by performing increasingly stringent washes, which involved increasing the temperature and decreasing the salt concentration during washing rounds. The main innovation of the DNA capture technique was the use of strand-displacing polymerases to detach the target DNA fragments from the biotinylated baits to ensure that DNA baits would not contaminate the mtDNA-enriched libraries. We used DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs [NEB]) for *Protemnodon* (Brotherton et al. 2013), and the *Bst* DNA Polymerase, Large Fragment (NEB) for *Simosthenurus*.

Next Generation Sequencing

The short adapters of the mtDNA-enriched *Simosthenurus* DNA library were converted by PCR (see Brotherton et al. 2013) into full-length adapters that included sequencing primers for each of the following sequencing platforms: Roche 454 GS-FLX, Ion Torrent PGM, and Pacific Biosciences RS. Likewise, the mtDNA-enriched *Protemnodon* DNA library was converted into an Ion Torrent PGM sequencing library. Sequencing was performed at Pacific Biosciences (Pacific Biosciences RS), the Australian Genome Research Facility (Roche 454 GS-FLX), and the Australian Cancer Research Foundation Cancer Genomics Facility (Ion Torrent PGM).

Mapping of NGS Reads

After adapter and quality trimming, and filtering of reads shorter than 25 nt, we aligned the filtered data sets against the GenBank nr database (March 2014) to analyze the taxonomic distribution from the filtered reads (supplementary fig. S3, Supplementary Material online). Results were visualized using Megan v5.3.5 (Huson et al. 2011). Reads were then simultaneously mapped against the five marsupial reference genomes used in the baits design using BWA v.0.5.9 (Li and Durbin 2009). As detailed in supplementary note S4, Supplementary Material online, we explored alternative parameter values affecting the mapping stringency, but ultimately retained the most stringent combination at the risk of rejecting some real endogenous sequences. We further refined the mapping analysis by using BLASTN to compare all reads against the NCBI nr database (March 2014) and subsequently discarding all reads that did not align to a known marsupial sequence in order to remove all potential contaminating DNA sequences.

We also performed an iterative mapping using TMAP and the *Dorcopsulus vanheurni* mtDNA sequence as initial reference, following the method described in Mitchell, Llamas, et al. (2014) and Mitchell, Wood, et al. (2014) (see supplementary note S5, Supplementary Material online). Iterative mapping did not incorporate more reads after six iterations for either extinct marsupial. Misincorporation patterns were assessed using MapDamage v0.3.6 (Ginolhac et al. 2011) (supplementary figs. S4–S7, Supplementary Material online). Reads from the initial and final iterations were aligned against the NCBI nr database (March 2014) using BLASTN to evaluate the presence of contaminant reads (fig. 2 and supplementary figs. S9 and S10, Supplementary Material online).

Molecular Data

Consensus sequences were generated for both *Simosthenurus* and *Protemnodon* using the mpileup command, the bcftools utilities, and the vcfutils.pl script from SAMtools v0.1.18 (Li et al. 2009). Nucleotides were called at each position covered by at least one read. Sites that received no coverage or insufficient coverage to confidently call a base were coded with IUPAC ambiguity symbols as appropriate. An alignment of 23 macropodoid and 2 phalangerid mitochondrial genomes (used as outgroup in the phylogenetic analyses; supplementary table S2, Supplementary Material online) was created

using Seaview v.4.2.12 (Gouy et al. 2010). Our consensus sequences were then included manually in the alignment, and we removed three independent single-nucleotide insertions in the ancient *Protemnodon* sequences (most probably PGM sequencing errors) that would disrupt the coding frame of protein-coding genes.

Morphological Data

We augmented our molecular data set with a morphological matrix of 83 skeletal characters taken from several recent studies (Prideaux and Warburton 2010; Prideaux and Tedford 2012). Eight species for which we included genetic data were scored as unknown in the morphological data set (*Trichosurus vulpecula*, *Phalanger interpositus*, *Ptrogale xanthopus*, *Aepyprymnus rufescens*, *Bettongia lesueur*, *Macropus robustus*, *M. rufogriseus*, and *Potorous longipes*), whereas two species were represented by congeners: *Dendrolagus bennettianus* in place of *De. lumholtzi* (McGreevy et al. 2012) and *Dorcopsis veterum* in place of *Do. hageni* (Groves and Flannery 1989). In an additional Bayesian analysis (see below), we added morphological data for four of the most complete kangaroo fossil taxa *Wanburoo*, *Dorcopsoides*, *Hadronomas*, and *Ngamaroo* (Prideaux and Warburton 2010).

Phylogenetic Analyses

The molecular data sets did not include the D-loop and consisted of eight partitions: Codon positions for the protein-coding genes (with ND6 codon positions in separate partitions), RNA stems, and RNA loops. All morphological characters were treated as unordered in all analyses, as per the original study (Prideaux and Warburton 2010). Bayesian Inference (MrBayes 3.2 [Ronquist et al. 2012]), ML (RAxML v.7.2.8 [Stamatakis 2006]), and Maximum Parsimony (PAUP* [Swofford 2002]) analyses were performed on the combined molecular and morphological data (supplementary note S6, Supplementary Material online). For the Bayesian and ML analyses, molecular substitution models and partitioning schemes were selected using the Bayesian information criterion as implemented by PartitionFinder v.1.0.1 (Lanfear et al. 2012), which favored a six-partition scheme with the selected models shown in supplementary table S4, Supplementary Material online. The morphological data formed the seventh partition. To ascertain the impact of the new aDNA data, combined analyses were performed with all morphological and molecular data, and then without aDNA (i.e., the extinct sthenurine and *Protemnodon* scored only for morphological traits, whereas all other taxa scored for molecular and—where available—morphological traits). Analyses were also performed with the molecular data alone (including aDNA).

We attempted to improve topological support by performing two additional Bayesian analyses using the combined molecular and morphological data:

i) A clock-based analysis in MrBayes using the IGR model and all morphological and molecular data (supplementary fig. S12, Supplementary Material online). Root age was modeled as a uniform distribution with a lower bound at approximately 25 Ma (the age of the oldest macropodoids) and an upper bound at 54.6 Ma (the age of the Murgon deposits from which no macropodoids have been described). This necessarily wide prior distribution means that estimated node ages are similarly uninformative.

ii) A dated Bayesian analysis (as above) with the addition of morphological characters from the most complete kangaroo fossil taxa *Wanburoo*, *Dorcopsoides*, *Hadronomas*, and *Ngamaroo* (Prideaux and Warburton 2010) (supplementary fig. S13, Supplementary Material online).

Supplementary Material

Supplementary notes S1–S6, references, figures S1–S13, and tables S1–S9 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

The authors are grateful to R. Beck and an anonymous reviewer for their helpful comments. They thank C. Reid from the Queen Victoria Museum and Art Gallery and M. Binnie from the South Australian Museum for granting access to paleontological collections; P. Darby and L. Gray for guiding us through the Mt Cripps karst; T. Worthy, J. Wood, N. Rawlence, F. Salt, H. Geraldene, and I. Household for assistance in the field; G. Prideaux for assistance in identifying specimens; J. Korlach and T. Clark for their help with sequencing samples at Pacific Biosciences; e-research SA for high-performance computing resources. Funding for field work at Mt Cripps and 14C dating at Oxford was provided by ARC Discovery Project DP0664562 to A.C. B.L. was a postdoctoral fellow from the ARC Discovery grant DP1095782.

References

Allentoft ME, Collins M, Harker D, Haile J, Oskam CL, Hale ML, Campos PF, Samaniego JA, Gilbert MT, Willerslev E, et al. 2012. The half-life of DNA in bone: measuring decay kinetics in 158 dated fossils. *Proc R Soc Lond B Biol Sci.* 279:4724–4733.

Archer M, Arena R, Bassarova M, Black K, Brammall J, Cooke BN, Crosby K, Godthelp H, Gott M, Hand SJ, et al. 1999. The evolutionary history and diversity of Australia's mammals. *Aust Mammal.* 21:1–45.

Avila-Arcos MC, Cappellini E, Romero-Navarro JA, Wales N, Moreno-Mayar JV, Rasmussen M, Fordyce SL, Montiel R, Vielle-Calzada J-P, Willerslev E, et al. 2011. Application and comparison of large-scale solution-based DNA capture-enrichment methods on ancient DNA. *Sci Rep.* 1:74.

Bragg LM, Stone G, Butler MK, Hugenholtz P, Tyson GW. 2013. Shining a light on dark sequencing: characterising errors in Ion Torrent PGM data. *PLoS Comput Biol.* 9:e1003031.

Briggs AW, Good JM, Green RE, Krause J, Maricic T, Stenzel U, Lalueza-Fox C, Rudan P, Brajkovic D, Kucan Z, et al. 2009. Targeted retrieval and analysis of five Neandertal mtDNA genomes. *Science* 325: 318–321.

Briggs AW, Stenzel U, Johnson PLF, Green RE, Kelso J, Prufer K, Meyer M, Krause J, Ronan MT, Lachmann M, et al. 2007. Patterns of damage in genomic DNA sequences from a Neandertal. *Proc Natl Acad Sci U S A.* 104:14616–14621.

Brotherton P, Haak W, Templeton J, Brandt G, Soubrier J, Jane Adler C, Richards SM, Sarkissian CD, Ganslmeier R, Friederich S, et al. 2013. Neolithic mitochondrial haplogroup H genomes and the genetic origins of Europeans. *Nat Commun.* 4:1764.

Bunce M, Worthy TH, Phillips MJ, Holdaway RN, Willerslev E, Haile J, Shapiro B, Scofield RP, Drummond A, Kamp PJ, et al. 2009. The evolutionary history of the extinct ratite moa and New Zealand Neogene paleogeography. *Proc Natl Acad Sci U S A.* 106: 20646–20651.

Burbano HA, Hodges E, Green RE, Briggs AW, Krause J, Meyer M, Good JM, Maricic T, Johnson PLF, Xuan Z, et al. 2010. Targeted investigation of the Neandertal genome by array-based sequence capture. *Science* 328:723–725.

Der Sarkissian C, Brotherton P, Balanovsky O, Templeton JEL, Llamas B, Soubrier J, Moiseyev V, Khartanovich V, Cooper A, Haak W, et al. 2014. Mitochondrial genome sequencing in Mesolithic North East Europe unearths a new sub-clade within the broadly distributed human haplogroup C1. *PLoS One* 9:e87612.

Flannery TF. 1983. Revision in the macropodid subfamily Sthenurinae (Marsupialia: Macropodoidea) and the relationships of the species of *Troposodon* and *Lagostrophus*. *Aust Mammal.* 6:15–28.

Flannery TF. 1989. Phylogeny of the Macropodidea: a study in convergence. In: Grigg G, Jarman P, Hume I, editors. Kangaroos, wallabies and rat kangaroos. Sydney (NSW): Surrey Beatty and Sons. p. 1–46.

Fu Q, Meyer M, Gao X, Stenzel U, Burbano HA, Kelso J, Paabo S. 2013. DNA analysis of an early modern human from Tianyuan Cave, China. *Proc Natl Acad Sci U S A.* 110:2223–2227.

Fu Q, Mittnik A, Johnson PL, Bos K, Lari M, Bollongino R, Sun C, Giemsch L, Schmitz R, Burger J, et al. 2013. A revised timescale for human evolution based on ancient mitochondrial genomes. *Curr Biol.* 23: 553–559.

Gillespie R, Camens AB, Worthy TH, Rawlence NJ, Reid C, Bertuch F, Levchenko V, Cooper A. 2012. Man and megafauna in Tasmania: closing the gap. *Quat Sci Rev.* 37:38–47.

Ginolhac A, Rasmussen M, Gilbert MT, Willerslev E, Orlando L. 2011. mapDamage: testing for damage patterns in ancient DNA sequences. *Bioinformatics* 27:2153–2155.

Gouy M, Guindon S, Gascuel O. 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol.* 27:221–224.

Green RE, Krause J, Briggs AW, Maricic T, Stenzel U, Kircher M, Patterson N, Li H, Zhai W, Fritz MHY, et al. 2010. A draft sequence of the Neandertal genome. *Science* 328:710–722.

Green RE, Malaspinas AS, Krause J, Briggs AW, Johnson PL, Uhler C, Meyer M, Good JM, Maricic T, Stenzel U, et al. 2008. A complete Neandertal mitochondrial genome sequence determined by high-throughput sequencing. *Cell* 134:416–426.

Groves CP, Flannery TF. 1989. Revision of the genus *Dorcopsis* (Macropodidae: Marsupialia). In: Grigg G, Jarman P, Hume I, editors. Kangaroos, wallabies and rat-kangaroos. Chipping Norton (NSW): Surrey Beatty & Sons Pty Ltd. p. 117–128.

Guthrie RD. 2006. New carbon dates link climatic change with human colonization and Pleistocene extinctions. *Nature* 441: 207–209.

Hahn C, Bachmann L, Chevreux B. 2013. Reconstructing mitochondrial genomes directly from genomic next-generation sequencing reads—a baiting and iterative mapping approach. *Nucleic Acids Res.* 41:e129.

Huson DH, Mitra S, Ruscheweyh H-J, Weber N, Schuster SC. 2011. Integrative analysis of environmental sequences using MEGAN4. *Genome Res.* 21:1552–1560.

Janis CM, Buttrill K, Figueirido B. 2014. Locomotion in extinct giant kangaroos: were sthenurines hop-less monsters? *PLoS One* 9: e109888.

Kircher M. 2012. Analysis of high-throughput ancient DNA sequencing data. *Methods Mol Biol.* 840:197–228.

Krause J, Briggs AW, Kircher M, Maricic T, Zwyns N, Derevianko A, Pääbo S. 2010. A complete mtDNA genome of an early modern human from Kostenki, Russia. *Curr Biol.* 20:231–236.

Lanfear R, Calcott B, Ho SY, Guindon S. 2012. Partitionfinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Mol Biol Evol.* 29:1695–1701.

Lee MSY, Camens AB. 2009. Strong morphological support for the molecular evolutionary tree of placental mammals. *J Evol Biol.* 22: 2243–2257.

Li C, Hofreiter M, Straube N, Corrigan S, Naylor GJ. 2013. Capturing protein-coding genes across highly divergent species. *Biotechniques* 54:321–326.

Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25:1754–1760.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Proc 1000 Genome Project Data. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25: 2078–2079.

Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, Pallen MJ. 2012. Performance comparison of benchtop high-throughput sequencing platforms. *Nat Biotechnol.* 30: 434–439.

Maricic T, Whitten M, Paabo S. 2010. Multiplexed DNA sequence capture of mitochondrial genomes using PCR products. *PLoS One* 5: e14004.

Mason VC, Li G, Helgen KM, Murphy WJ. 2011. Efficient cross-species capture hybridization and next-generation sequencing of mitochondrial genomes from noninvasively sampled museum specimens. *Genome Res.* 21:1695–1704.

McGreevy TJ, Dabek L, Husband TP. 2012. Tree kangaroo molecular systematics based on partial cytochrome b sequences: are Matschie's tree kangaroo (*Dendrolagus matschiei*) and Goodfellow's tree kangaroo (*D. goodfellowi buergersi*) sister taxa? *Aust Mammal.* 34:18.

Meredith RW, Westerman M, Springer MS. 2009a. A phylogeny of Diprotodontia (Marsupialia) based on sequences for five nuclear genes. *Mol Phylogenet Evol.* 51:554–571.

Meredith RW, Westerman M, Springer MS. 2009b. A phylogeny and timescale for the living genera of kangaroos and kin (Macropodiformes:Marsupialia) based on nuclear DNA sequences. *Aust J Zool.* 56:395–410.

Meyer M, Fu Q, Aximu-Petri A, Glocke I, Nickel B, Arsuaga JL, Martinez I, Gracia A, de Castro JM, Carbonell E, et al. 2013. A mitochondrial genome sequence of a hominin from Sima de los Huesos. *Nature* 505:403–406.

Meyer M, Kircher M, Gansauge M-T, Li H, Racimo F, Mallick S, Schraiber JG, Jay F, Pruefer K, de Filippo C, et al. 2012. A high-coverage genome sequence from an archaic Denisovan individual. *Science* 338: 222–226.

Miller W, Schuster SC, Welch AJ, Ratan A, Bedoya-Reina OC, Zhao F, Kim HL, Burhans RC, Drautz DI, Wittekindt NE, et al. 2012. Polar and brown bear genomes reveal ancient admixture and demographic footprints of past climate change. *Proc Natl Acad Sci U S A.* 109: E2382–E2390.

Mitchell KJ, Llamas B, Soubrier J, Rawlence NJ, Worthy TH, Wood J, Lee MSY, Cooper A. 2014. Ancient DNA reveals elephant birds and kiwi are sister taxa and clarifies ratite bird evolution. *Science* 344: 898–900.

Mitchell KJ, Pratt RC, Watson LN, Gibb GC, Llamas B, Kasper M, Edson J, Hopwood B, Male D, Armstrong K, et al. 2014. Molecular phylogeny, biogeography, and habitat preference evolution of marsupials. *Mol Biol Evol.* 31:2322–2330.

Mitchell KJ, Wood JR, Scofield RP, Llamas B, Cooper A. 2014. Ancient mitochondrial genome reveals unsuspected taxonomic affinity of the extinct Chatham duck (*Pachyanas chathamica*) and resolves divergence times for New Zealand and sub-Antarctic brown teals. *Mol Phylogenet Evol.* 70:420–428.

Murray DC, Haile J, Dortch J, White NE, Haouchar D, Bellgard MI, Allcock RJ, Prideaux GJ, Bunce M. 2013. Scrapheap challenge: a novel bulk-bone metabarcoding method to investigate ancient DNA in faunal assemblages. *Sci Rep.* 3:3371.

Murray DC, Pearson SG, Fullagar R, Chase BM, Houston J, Atchison J, White NE, Bellgard MI, Clarke E, Macphail M, et al. 2012. High-throughput sequencing of ancient plant and mammal DNA preserved in herbivore middens. *Quat Sci Rev.* 58:135–145.

Murray PF. 1995. The postcranial skeleton of the Miocene kangaroo, *Hadronomas puckridgi* Woodburne (Marsupialia, Macropodidae). *Alcheringa* 19:119–170.

Orlando L, Ginolhac A, Raghavan M, Vilstrup J, Rasmussen M, Magnussen K, Steinmann K, Kapranov P, Thompson JF, Zazula G, et al. 2011. True single-molecule DNA sequencing of a Pleistocene horse bone. *Genome Res.* 21:1705–1719.

Oskam CL, Haile J, Mclay E, Rigby P, Allentoft ME, Olsen ME, Bengtsson C, Miller GH, Schwenninger JL, Jacomb C, et al. 2010. Fossil avian eggshell preserves ancient DNA. *Proc R Soc Lond B Biol Sci.* 277: 1991–2000.

Phillips MJ, Haouchar D, Pratt RC, Gibb GC, Bunce M. 2013. Inferring kangaroo phylogeny from incongruent nuclear and mitochondrial genes. *PLoS One* 8:e57745.

Price GJ, Webb GE, Zhao J, Feng Y, Murray AS, Cooke BN, Hocknull SA, Sobbe IH. 2011. Dating megafaunal extinction on the Pleistocene Darling Downs, eastern Australia: the promise and pitfalls of dating as a test of extinction hypotheses. *Quat Sci Rev.* 30:899–914.

Prideaux GJ. 2004. Systematics and evolution of the sthenurine kangaroos. Berkeley (CA): University of California Press.

Prideaux GJ, Tedford RH. 2012. *Tjukuru wellsi*, gen. et sp. nov., a lagostrophine kangaroo (Diprotodontia, Macropodidae) from the Pliocene (Tirarian) of northern South Australia. *J Vertebr Paleontol.* 32:717–721.

Prideaux GJ, Warburton NM. 2010. An osteology-based appraisal of the phylogeny and evolution of kangaroos and wallabies (Macropodidae: Marsupialia). *Zool J Linn Soc.* 159:954–987.

Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, Bertoni A, Swerdlow HP, Gu Y. 2012. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* 13:341.

Reich D, Green RE, Kircher M, Krause J, Patterson N, Durand EY, Viola B, Briggs AW, Stenzel U, Johnson PLF, et al. 2010. Genetic history of an archaic hominin group from Denisova Cave in Siberia. *Nature* 468: 1053–1060.

Roberts RG, Flannery TF, Ayliffe LK, Yoshida H, Olley JM, Prideaux GJ, Laslett GM, Baynes A, Smith MA, Jones R, et al. 2001. New ages for the last Australian megafauna: Continent-wide extinction about 46,000 years ago. *Science* 292:1888–1892.

Rohland N, Reich D, Mallick S, Meyer M, Green RE, Georgiadis NJ, Roca AL, Hofreiter M. 2010. Genomic DNA sequences from mastodon and woolly mammoth reveal deep speciation of forest and savanna elephants. *PLoS Biol.* 8:e1000564.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol.* 61:539–542.

Sanchez-Quinto F, Schroeder H, Ramirez O, Avila-Arcos MC, Pybus M, Olalde I, Velazquez AM, Marcos ME, Encinas JM, Bertranpetit J, et al. 2012. Genomic affinities of two 7,000-year-old Iberian hunter-gatherers. *Curr Biol.* 22:1494–1499.

Sawyer S, Krause J, Guschanski K, Savolainen V, Paabo S. 2012. Temporal patterns of nucleotide misincorporations and DNA fragmentation in ancient DNA. *PLoS One* 7:e34131.

Schubert M, Ginolhac A, Lindgreen S, Thompson JF, Al-Rasheid KA, Willerslev E, Krogh A, Orlando L. 2012. Improving ancient DNA read mapping against modern reference genomes. *BMC Genomics* 13:178.

Smith CI, Chamberlain AT, Riley MS, Stringer C, Collins MJ. 2003. The thermal history of human fossils and the likelihood of successful DNA amplification. *J Hum Evol.* 45:203–217.

Springer MS, Burk-Herrick A, Meredith R, Eizirik E, Teeling E, O'Brien SJ, Murphy WJ. 2007. The adequacy of morphology for reconstructing the early history of placental mammals. *Syst Biol.* 56: 673–684.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22:2688–2690.

Swofford DL. 2002. PAUP*: phylogenetic analysis using parsimony (and other methods) 4.0 beta. Sunderland (MA): Sinauer Associates.

Westerman M, Burk A, Amrine-Madsen HM, Prideaux GJ, Case JA, Springer MS. 2002. Molecular evidence for the last survivor of an ancient kangaroo lineage. *J Mamm Evol.* 9:209–223.