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Thorough assessment of DNA preservation from fossil bone and sediments excavated from a late Pleistocene–Holocene cave deposit on Kangaroo Island, South Australia.

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Abstract

Fossils and sediments preserved in caves are an excellent source of information for investigating impacts of past environmental changes on biodiversity. Until recently

studies have relied on morphology-based palaeontological approaches, but recent advances in molecular analytical methods offer excellent potential for extracting a greater array of biological information from these sites. This study presents a thorough assessment of DNA preservation from late Pleistocene–Holocene vertebrate fossils and sediments from Kelly Hill Cave Kangaroo Island, South Australia. Using a combination of extraction techniques and sequencing technologies, ancient DNA was characterised from over 70 bones and 20 sediment samples from 15 stratigraphic layers ranging in age from > 20 ka to ~6.8 ka. A combination of primers targeting marsupial and placental mammals, reptiles and two universal plant primers were used to reveal genetic biodiversity for comparison with the mainland and with the morphological fossil record for Kelly Hill Cave. We demonstrate that Kelly Hill Cave has excellent long-term DNA preservation, back to at least 20 ka. This contrasts with the majority of Australian cave sites thus far explored for ancient DNA preservation, and highlights the great promise Kangaroo Island caves hold for yielding the hitherto-elusive DNA of extinct Australian Pleistocene species.

1. Introduction

Islands have long provided a natural laboratory for the study of evolutionary processes because evolutionary changes on them are often magnified, simplified and therefore more readily interpretable (e.g., Darwin and Wallace, 1858; Losos and Ricklefs, 2010; MacArthur and Wilson, 1967). The study of genetic variation on islands also has a long history (Lomolino et al., 1989; Van der Geer et al., 2010). However, ancient DNA (aDNA) analyses applied to stratified, dated faunal successions can add a temporal context, allowing the ebb and flow of genes, species

and communities to be assessed, particularly in combination with more traditional analyses of vertebrate and plant macrofossils and pollen. A necessary prerequisite for aDNA research is adequate biomolecular preservation. Cave systems represent an ideal environment for palaeontological investigations as they often contain relatively complete and undisturbed stratigraphic deposits that harbour several environmental proxies (Butzer, 2008; White, 2007) that have been subjected to minimal temperature and humidity fluctuations; conditions that favour DNA persistence (Stone, 2000). Such caves represent archives of well-preserved Quaternary vertebrate assemblages (Prideaux et al., 2010; Prideaux et al., 2007), with the ability to preserve invaluable repositories of past biodiversity. All samples (bones and sediments) analysed in this study were obtained directly from Kelly Hill Cave (KHC), Kangaroo Island (KI) with the aim of conducting a thorough assessment of DNA preservation in KHC to determine whether genetic data could enhance temporal information about faunal change on KI. Moreover, as part of this study the preservation of plant DNA obtained directly from sediments was assessed (Haile et al., 2007; Willerslev et al., 2007) with the aim to provide palaeovegetation data to complement fossil data.

In this study a combination of techniques such as a relatively new bulk-bone method (Murray et al., 2013) and High-Throughput Sequencing (HTS) technology was used in order to capture aDNA from a variety of samples collected from KHC. Also we show how the addition of a palaeontological molecular perspective may complement existing morphology based studies allowing identification of osteologically absent and cryptic species, and the investigation of genetic change over time. These results are overlayed upon the palaeogeographic history of KI, which provides a model context for studying mainland–island interactions. This is pertinent to KHC, because

it contains an excellent Late Quaternary vertebrate fossil assemblage (Pledge, 1979) that records the response of Australian native fauna to both the last glacial maximum (LGM) and isolation of KI caused by rising sea levels at 8.9 ka (McDowell, 2013).

2. Kangaroo Island

Kangaroo Island lies at the entrance to Gulf St Vincent in South Australia (Fig. 1), and is the third-largest land-bridge island in Australia (4,405 km^2), with a length of 145 km and width of 55 km at its widest point (Abbott, 1974; Hope et al., 1977; Lampert, 1981; Twidale and Bourne, 2002) (Fig. 1). It is geologically continuous with the adjacent Fleurieu Peninsula, but was isolated by glacial erosion during the Late Carboniferous and Early Permian (Belperio and Flint, 1999). Today it is separated from Yorke Peninsula by Investigator Strait, a 50-km stretch of 30–35 m deep water (Fig. 1).

During the late Pleistocene, sea levels were 120 m lower than at present (Chappell and Shackleton, 1986; Yokoyama et al., 2001) and KI was connected to the mainland. Global warming during the early Holocene caused a rapid rise in sea-level, isolating KI from the mainland at 8.9 ka (Belperio and Flint, 1999; Bradley, 1999; Cutler et al., 2003). Prior to its isolation, gene flow was presumably continuous between the mainland KI which supported a species-rich fauna (Abbott, 1974; McDowell, 2013). Once isolated, the newly marooned organisms would have become more susceptible to genetic drift; island flora and fauna tend to be diverse after initial isolation but subsequently suffer elevated selection pressures, loss of genetic diversity and elevated rates of extinction (Diamond, 1972; Foufopoulos and Ives, 1999; Stiller et al., 2010).

Despite the loss of genetic diversity caused by island living, many species that have become extinct or endangered on the mainland find refuge on islands due to relaxed competition and reduced predation pressures (Lister, 2004). KI retains the largest proportion of uncleared native vegetation of any southern Australian agricultural district. In addition KI remains free of rabbits and foxes that have had such a catastrophic impact on mainland biota (Robinson and Armstrong, 1999) enhancing its conservation importance. Biodiversity management can be complimented by aDNA analyses and assessments of the fossil record, providing parameters such as population sizes, levels of gene flow and population relatedness (De Bruyn et al., 2011; Leonard, 2008; Ramakrishnan et al., 2005).

2.1 Study site

The KHC complex on KI (35.83° S, 137.33° E) is ideally suited to explore biomolecule preservation as it has an already well-studied and well-dated palaeontological record that spans the terminal late Pleistocene to the middle Holocene (McDowell, 2013). In addition numerous surveys of the island's modern flora and fauna have also been made (Robinson and Armstrong, 1999).

KHC is the focus of an ongoing palaeontological project that investigates how climate change and isolation due to sea level rise has affected the fauna of KI (McDowell et al., 2013). To date, a fauna rich in mammals, birds, reptiles, frogs and land-snails has emerged from an excavation 4 m^2 x 1.5 m deep, and includes the remains of several species not previously recorded on KI. This site was selected based on the presence of fossil bones on the cave floor surface, depth of sediment, likelihood of stratigraphic integrity and likelihood of encountering speleothems that can be U/Th dated (McDowell, 2013; McDowell et al., 2013).

3. Materials & Methods

We used 2nd generation DNA sequencing technologies (Roche GS-Junior and Ion Torrent, Personal Genome Platforms (PGM) to target chloroplast (plastid) DNA (cpDNA) and mitochondrial DNA (mtDNA) from sediment samples and assessed aDNA preservation of bones collected from several sedimentary layers ranging in age from >20 to 6 ka (McDowell et al., 2013). This study uses a combination of techniques to recover aDNA from multiple samples including a novel bone-grind technique, hitherto applied only to two cave sites in Western Australia (Murray et al., 2013) To test the veracity or to refine morphology-based identifications, aDNA was extracted from 70 complete macropodid and reptile postcranial bones. Some specimens had been identified morphologically to species level, while others were only identified to family level (McDowell, 2013).

The layers span the terminal Late Pleistocene to the middle Holocene (>20 ka to 6 ka). Bones from layers 3 to 11 contained adequate collagen to be AMS radiocarbon dated (McDowell et al., 2013). Skeletal remains from layers 12 to 15 lacked viable collagen and what little remained was too degraded to be radiocarbon dated (McDowell et al., 2013). Standard aDNA protocols specifies that pre-PCR procedures are conducted in a dedicated aDNA clean room, with subsequent post-PCR work carried out in a separate laboratory in order to minimise contamination (Cooper and Poinar, 2000; Fulton, 2012; Pääbo, 1989). DNA extractions and amplifications were conducted at Murdoch University, whilst Sanger sequencing was performed at a commercial facility (South Korea) and HTS was carried out at Murdoch University (Roche, 454 GS-Junior) and the Lotterywest State Biomedical Facility Genomics

Node at Royal Perth Hospital (Ion Torrent, PGM). A more detailed version of the molecular methods (sections 3.2 to 3.4) can be found in the Supplementary data.

3.1 Background to sediments and sample collection

Numerous sediment core samples were collected from all exposed layers of the KHC excavation using sterile equipment and protective clothing to preserve their genetic integrity. Prior to sediment collection approximately 5 cm of surface soil was removed from the wall prior to coring to minimise possible contamination. A 50 mL falcon tube was pushed into the pit wall to core out the sediment of each distinctive layer. Each tube was sealed, labelled appropriately and stored at 4ºC to be used in subsequent aDNA work. Previous sedimentary analyses of all 15 layers has been conducted to assess grain composition, size, colour, petrography and geochemistry (McDowell et al., 2013). Subsamples from a total of 20 sediment cores were then subjected to aDNA analysis, targeting plant cpDNA and vertebrate mtDNA.

3.2 DNA extraction methods

All samples were extracted using methodologies designed for optimal aDNA recovery. Therefore, different extraction protocols were applied to bones and sediments and are described independently.

3.2.1 DNA extraction of Bone

Fossil remains were ground to powder using a Dremel tool (part no. 114: Germany) set to high rotational speeds using DNA extraction method described by (Haouchar et al., 2013). Briefly, ~0.2g of bone powder was collected in a 1.5 mL Eppendorf tube, weighed and stored at 4° C for later digestion. A number of bones

were individually extracted (i.e. one bone per extraction digest), although where bones were morphologically uninformative and unidentified, a bulk-bone method was applied. This entailed grinding up to six discrete bones together and extracting DNA from the resulting powder for molecular analysis (Murray et al., 2013).

Silica-based DNA extractions were performed (Haouchar et al., 2013) and all extracts quantitatively screened using primer sets targeting marsupials 12S and 16S rRNA markers (see Table 1 for primer combinations). Quantitative PCR assays were carried out using SYBR-green qPCR (Bunce et al., 2012) as described in the Supplementary data.

3.2.2 DNA extraction from sediment

All sediments were processed using the Sergey Bulat extraction method optimised for small amounts of material including controls (Haile, 2012). Briefly, ~2g of sediment were processed in the Bulat buffer and purified over silica columns (see Haile et al 2012). Like the bone, DNA extractions were screened using qPCR that employed two generic primer sets for plants; *trn*Lg/h and *rbc*L. The *trn*Lg/h assay amplifies short sections of the *trn*L intron (Taberlet et al., 2007; Taberlet et al., 1991) and the *rbc*L primers target a coding segment of the plastid *rbc*L gene (Table 1) (Chiang et al., 1998; Gielly and Taberlet, 1994; Kress and Erickson, 2007). Concentrations for the DNA digest, qPCR set up and cycling conditions are further described in the Supplementary data.

3.3 Sequencing of DNA from bone and sediments

All bone extracts were screened for DNA amplification. Any extracts, which successfully yielded amplicons of the target size were purified (see Supplementary

data for procedure) and sequenced. For single-source bone samples Sanger Sequencing was employed (using ABI, BigDye chemistry at Macrogen), for mixed samples and sediments a next generation HTS approach was used.

DNA extracts chosen for HTS were assigned a unique six base pair (bp) DNA tag (specifically a Multiplex Identifier-tag, MID-tag (Roche, 2009)) and built into primers as fusion tags. All fusion-tagged PCRs were carried out in 25 µl reactions (see Supplementary data for master mix) and imaged by qPCR (Bunce et al., 2012). The general cycling conditions and amplicon purification can be found in Supplementary data. After pooling the amplicons, the library was then quantified with calibrated standards using qPCR (Bunce et al., 2012) to determine appropriate templating ratios for HTS. Emulsion PCR and GS Junior 454 Sequencing were performed as per Roche GS Junior protocols for amplicon sequencing (http://www.454.com).

Sediment DNA was prepared for GS junior 454 and Ion Torrent PGM sequencing using both *trn*Lg/h and *rbc*L primer sets (Table 1). Extracts were quantified in the same manner as the 454. All qPCRs were generated in triplicate and pooled accordingly. Amplicon pools were cleaned using Agencourt AMPure XP PCR Purification Kit (Beckman Coulter Genomics, NSW, Aus) and separated by gel electrophoresis. GS Junior 454 Sequencing and emulsion PCR were performed as per Roche GS Junior protocols. Ion Torrent emulsion PCR was performed on One Touch 2 system and sequencing was performed on an Ion Torrent (PGM).

3.4 Data analysis and sequence identification

All sequence reads generated from the two platforms (454 GS Junior and Ion Torrent PGM) for both bone and sediment extracts were filtered in a similar manner. First, sequences were sorted into sample batches based on the unique MID-tags using the program Geneious v5.6.5 (Drummond et al., 2011). Tags and primers were trimmed from the sequences allowing for no mismatch in length or base composition. All sequence results that were seemingly less than the expected amplicon size depending on the primer set used, were removed from the analysis. Once sequences were searched to contain all relevant information, i.e., they all retained the unique MID-tag, forward and reverse primer and adaptor primer, they were trimmed and searched against the NCBI GenBank nucleotide database (Benson et al., 2006) using BLASTn version 2.2.23 (Altschul et al., 1990) to identify reads. BLASTn datafiles were analysed in the program MEtaGenome Analyzer v4 (MEGAN) (Huson et al., 2007) (see Supplementary data for a detailed description of the analyses).

4. Results and Discussion

4.1 Overview of sequence data of bones from KHC

Approximately 350,000 sequence reads were generated from multiple 454 (GS Junior) and Ion Torrent (PGM) runs. DNA from a range of taxa including *Macropus*, *Onychogalea*, *Potorous*, *Bettongia*, *Dasyurus*, *Rattus* and *Notechis* was amplified using an array of primers suitable to target the gene and organisms of interest (Table 1). The following results sections summarise the DNA analyses from KHC bones (Fig. 2). Initially, a number of bones that had previously been identified morphologically at family level were screened to investigate DNA preservation at the

site, and confirm the taxonomic identity. Approximately 70% of the 19 bones that were randomly selected (dispersed evenly throughout the 15 layers) from the site yielded DNA, including those from the deeper layers 14 and 15 enabling amplification of between 100–250bp sequences at a time. After this initial screening, another 45 bone fragments were subject to aDNA methods including an inventory of fragments from macropodid and murid limbs and vertebrae, and snake vertebrae.

4.2 Red kangaroo

Two postcranial bones identified as 'Macropodinae genus and species indeterminate' (sp. indet.) from layers 14 and 15 which are >20 ka (Fig. 2), yielded two sequences, one being 98% and the other 99% similar to red kangaroo (*Macropus rufus*), respectively. Morphologically diagnostic specimens of this species were not detected by McDowell (2013), but the species has previously been recorded on KI on the basis of three tooth fragments retrieved from Seton Rockshelter, nearby archaeological assemblage (Hope et al., 1977; Fig. 1). The presence of *M. rufus* in layers 14 and 15 likely represents a time when KI was connected to the mainland and probably preceded the LGM. Red kangaroos are currently widely distributed through the drier regions of mainland Australia but typically occur in arid and semi-arid regions (Jackson and Vernes, 2010; Van Dyck and Strahan, 2008). This finding is significant as it demonstrates that KHC is capable of long-term DNA preservation and provides additional impetus for further excavations to be carried out at this site. *M. rufus* probably became extirpated on KI as more wooded vegetation returned to KI.

4.3 Bridled Nail-tail Wallaby

Bridled nail-tail wallaby (*Onychogalea fraenata*) sequences were obtained from a combination of single-bone analysis and from bulk-bone sampling methods. A

total of four haplotypes were observed with genetic differences distinct from the mainland individuals (71% to *O. unguifera* and 98% to *O. fraenata*). GenBank lacks reference sequences of the Crescent Nail-tail Wallaby (*O. lunata*) and although the geographic ranges of *O. fraenata* and *O. lunata* overlapped on the mainland until the late 19th century, only *O. fraenata* has been identified from KI based on morphological evidence (McDowell, 2013). *O. fraenata* is now highly endangered (Gordon and Lawrie, 1980; McKnight, 2008) and persists only in managed populations (Kingsley et al., 2012; Van Dyck and Strahan, 2008). *O. lunata* is extinct and *O. unguifera* (Northern Nail-tail wallaby) is widespread across northern Australia. Prior to European settlement *O. fraenata* and *O. lunata* were common throughout southern and eastern Australia (Van Dyck and Strahan, 2008). The critical status of *O. fraenata,* and its susceptibility to foxes, stock grazing and habitat destruction has resulted in a massive decline over the last 100 years. Pending further research, KI might be considered as a potential reintroduction site for *O. fraenata.*

4.4 Potoroo

DNA sequences with a potoroo affinity were obtained using both a single-bone extraction and bulk-bone methods. Overall, three haplotypes were observed which varied considerably from the reference mainland potoroo sequences acquired from GenBank. Two bones identified as Macropodinae sp. Indet. were individually extracted and analysed. A number of bones (5 in particular) identified as *Potorous platyops* from various layers collected (including surface scatter, layers 5 and 7) were subjected to the bulk bone method. An unresolved polytomy (Fig. 3) is revealed from the three samples (two single bone extractions and one bulk sample) with low posterior support values for the four haplotypes observed on KI, making species

identification problematic. The cluster does however show that the species belongs in *Potorous*, since the percentage similarities between the mainland and island sequences show little discrepancy. Potoroo bone 1 (Fig. 3) and potoroo bone 2 share a similar match of 92.8% and 91.9% to *P. tridactylus* whilst haplotype 1 and 2 have a 98.3% and 98.5% similar match to *P. gilbertii*. As the species of *Potorous* is not clearly identified using this dataset, more bone fragments from KHC should be sequenced to identify whether the variability in this sequence cluster represents elevated genetic drift due to island isolation or potential for a new *Potorous* species, endemic to KI.

Prior to European settlement, potoroos were widely distributed across the continent. However, the combined pressure of habitat loss and introduced predators and competitors (Frankham et al., 2012) resulted in dramatic range reductions for most potoroos. *P. platyops* is extinct, *P. tridactyulus* is listed as vulnerable, *P. longipes* is endangered and *P. gilbertii* is critically endangered. Since the time of European settlement *P. platyops* was only recorded as living in the south-west of Western Australia and was likely already extinct on KI at that time (Robinson and Armstrong, 1999). Fossil remains of *P. tridactylus* have been recovered from KHC (McDowell, 2013), but it appears to have been extirpated well before European settlement.

Another bone recovered from KHC analysed using the single bone extraction method, showed a genetic similarity of 99.3% to sequence from mainland specimens of the Brushtail Bettong or Woylie (*Bettongia penicillata*) (Fig. 3; Bettongia bone 1). Bettongs have also suffered dramatic range reductions due to anthropogenic effects

and introduced predators throughout Australia (Claridge et al., 2007). The now extinct subspecies *Bettongia penicillata penicillata* was once plentiful on the South Australian mainland, whilst the Burrowing Bettong or Boodie (*B. lesueur*) and *B. penicillata* have been identified from KI fossil bones (Hope et al., 1977; McDowell, 2013; Robinson and Armstrong, 1999). Both have been now extirpated from the island. Attempts to reintroduce the burrowing bettong (*B. lesueur*) back onto KI have met with limited success (Robinson and Armstrong, 1999; Short et al., 1992).

4.5 Dasyuridae

Sequences of quoll species (*Dasyurus*) were obtained using a single-bone extraction method. The results show that one yielded a DNA sequence 99% similar to Tiger Quoll, *D. maculatus* (Fig. 3; *Dasyurus* bone 1). Whilst DNA extracted from a small right femur provisionally identified by one of us (MCM) as cf. *Phascogale*, was found to be 99.5% similar genetically to mainland Eastern Quoll, *D. viverrinus* (Fig. 3; *Dasyurus* bone 2). These findings support physical fossil evidence that both species were present on KI. *D. viverrinus* appears to have been lost from KI during the mid-Holocene (McDowell, 2013) but *D. maculatus* persisted until it was extirpated by Europeans. During the 19th century, quolls were reported as being extant on KI until ca 1886 (Robinson and Armstrong, 1999), although no specimens are known to have been lodged in a museum. The presence of *D. maculatus* in a 200-year-old European fur-trapper accumulation at Bales Bay (Walshe, In press) strongly points to this as the persistent species. Rehabilitation efforts and applications of genetic management to conserve quolls on the mainland (Jones et al., 2003) have taken place to reintroduce certain species back to their former ranges (Firestone et al., 1999; Firestone et al.,

2000). Direct evidence of fossils (McDowell, 2013), and now *Dasyurus* mtDNA haplotypes from KI suggests this top predator may be a candidate for reintroduction.

4.6 Muridae

A number of rodents have been collected from KI e.g., *Pseudomys shortridgei* (Heath rat), *Rattus lutreolus* (Swamp rat) and *R. fuscipes* (Bush rat). In this study crania morphologically identified as *R. fuscipes,* were genetically assessed using the bulk-bone method to confirm their species identity. The results show that all three groups were 98–99% similar to *R. fuscipes,* confirming the morphological identifications. Genetic variations within single nucleotide polymorphisms (SNPs) occur between samples and layers and probably between the different individuals sampled. However to prove this, further analyses of all bones sampled should be reanalysed individually using a single-bone extraction method in order to compare the SNPs throughout the layers and further compare this to the genetic population on the mainland.

R. fuscipes is common on KI and the mainland as well as 13 other continental islands off the coast of South Australia (Hinten et al., 2003). As a result of elevated sea levels following the LGM (Barrows et al., 2002; Petherick et al., 2008), genetically isolated populations developed on several of the newly formed islands (Hinten et al., 2003; Schmitt, 1978; Seddon and Baverstock, 2002). In distinct contrast to continental islands off Australia, a combination of mtDNA and microsatellite analyses (Hinten et al., 2003) indicate that the KI population of *R. fuscipes* has a greater level of genetic diversity than the adjacent mainland.

R. fuscipes has experienced population bottlenecking on the mainland (Hinten et al., 2003). Combined pressures imposed by feral cats, foxes and habitat fragmentation may be further contributing to these dwindling populations (Hinten et al., 2003). Given that KI has remained free from foxes, it serves as an ideal place in which to preserve the already diverse and self-maintaining *R.fuscipes* population and to host insurance populations.

4.7 Elapidae

The Pygmy Copperhead (*Austrelaps labialis*), Little Whip Snake (*Suta flagellum*) and Eastern Black Tiger Snake (*Notechis ater*, although now believed to be synonymised with *Notechis scutatus*) were common on KI (Houston and Tyler, 2002; Robinson and Armstrong, 1999). Individuals of these species have 200–400 vertebrae that vary morphologically along the vertebral column making them difficult to identify to species when found as fossils. However, because each snake has so many vertebrae they are common in Australian palaeontological excavations. Elapid vertebrae consist largely of thick cortical bone and typically preserve high-quality DNA that can be specifically identified using molecular methods (Polly and Head, 2004).

The bulk-bone method (Murray et al., 2013) allowed rapid initial identification of the snake species present, and then single bone extractions verified the layers in which the species occurred (see Fig. 2). Elapid DNA was amplified from strata that have been radiometrically dated to >20 ka (layer 11: McDowell et al., 2013) and yielded 100% and 99.5% similar matches to the *Notechis ater* and *Austrelaps labialis* respectively (the only two species recognised from excavated crania). Little genetic difference was

observed within species over time, nor when compared to the modern mainland reference sequences. The Pygmy Copperhead, which is widespread and common on KI, has a depauperate mainland range and is found primarily restricted to the southern Mount Lofty ranges and Fleurieu Peninsula. Habitat destruction is probably the main force driving the decline of this species (Houston and Tyler, 2002; Robinson and Armstrong, 1999).

4.8 Overview of plant data from sediments

A total of eight plant families were detected (Table 2) from an initial assessment of ancient DNA preservation in sediment (*sed*aDNA). DNA was screened for 12 sediment sub-samples ranging from >20 ka to approximately 9 ka. Sediment DNA was also tested for mtDNA although no animal DNA was detected. A total of six sediment samples (Fig. 2 and Table 2) successfully yielded DNA using a two-locus approach targeting the chloroplast *trn*L and *rbc*L genes resulting in approximately 100,000 sequences from several HTS runs. Sequences were identified using BLAST (Altschul et al., 1990), and results interpreted with MEGAN (Huson et al., 2007) (Table 2). As with previous sedimentary ancient plant DNA literature using these chloroplast loci (Jorgensen et al., 2011; Parducci et al., 2005), taxonomic resolution is reliant upon comparative database coverage, and within certain families is constrained by the degree of interspecific variation. Nevertheless, the results provide some interesting insights. There are nearly 900 native plant species recorded on KI which currently include 40 endemic species (Holiday et al., 2003). Families which are well represented on KI are those characteristic of the higher rainfall areas of South Australia in general such as Myrtaceae (*e.g.*, eucalyptus), Cyperaceae (*e.g.*,

sedges), Liliaceae (*e.g.,* lilies), Ericaceae (formerly - Epacridaceae e.g., heath shrubs and herbs) and Apiaceae (formerly - Umbelliferae *e.g*., heath shrubs).

The majority of species present in the layers were found on KI and/or the adjacent mainland. However, some of the deeper layers yielded taxa not found on KI or mainland South Australia. For example layer 12 (dated at >20 ka) yielded *Piper* sp. indet. (Piperaceae – pepper family) (Table 2), from *sed*aDNA, but has never been recorded on the island before. Closely related species appear to be common in the eastern states of Australia, particularly all along the coast of Queensland and New South Wales (Atlas of living Australia; http://www.ala.org.au/). Species of the Myrtaceae family, which are considerably common throughout KI, was found in four sedimentary layers, and included a number of taxa that could be identified to genus (Table 2). *Eucalyptus sp., Leptospermum sp.* and *Syzygium sp.*, were present in the deeper excavation layers and with the exception of *Syzigium sp.,* which remains common on the mainland, all can be found on KI today.

The study of past plant species distribution (palynology), which principally relies on pollen, has a long and venerable history. However, until the discovery of aDNA persistence in sediments (Willerslev et al., 2003), the absence of macro-fossil remains (seeds, buds or vegetative tissue) and micro-fossils (pollen) was a serious limitation (Jorgensen et al., 2011). Ancient sediments, in particular permafrost deposits, have proven to be an excellent archive for the long-term preservation of environmental ancient DNA (*sed*aDNA) (Willerslev et al., 2003), making them useful for palaeo-reconstructions. However, *sed*aDNA preservation in less favorable climates like that of Australia can be somewhat challenging and so far have only been reproducible in a minority of plant aDNA studies. To date no record has been made on the preservation of plant macro- and micro-remains in the sediments of KHC,

therefore any information that can be gathered of past floral assemblages will provide a significant contribution to our understanding of paleovegetation and climate changes.

5. Conclusion

This is the first aDNA assessment of vertebrate fossils and plant DNA from KI in South Australia. Outcomes presented here add significant value to the late Pleistocene–mid-Holocene paleontological record of KI. We demonstrate the utility of aDNA techniques when applied to KI cave deposits and build upon results of morphological studies by confirming existing identifications and revealing additional species not preserved as diagnostic fossil specimens. The excellent preservation of both animal and plant DNA extracted from KHC bones and sediments are fundamental in providing such valuable information about the past biodiversity of KI. This study provides a detailed molecular record of animal and plant species that once lived in the KHC region, including some species that have been extirpated from KI. We reveal an array of taxa ranging from marsupials and reptiles to shrubs and trees, some of which were deposited > 20 ka. Our results also highlight the potential of the ongoing excavation that will delve even deeper (> 15 layers) into the past and may provide insights into the extinction of megafaunal species previously recorded on KI (Hope et al., 1977) and at the KHC site (McDowell, 2013).

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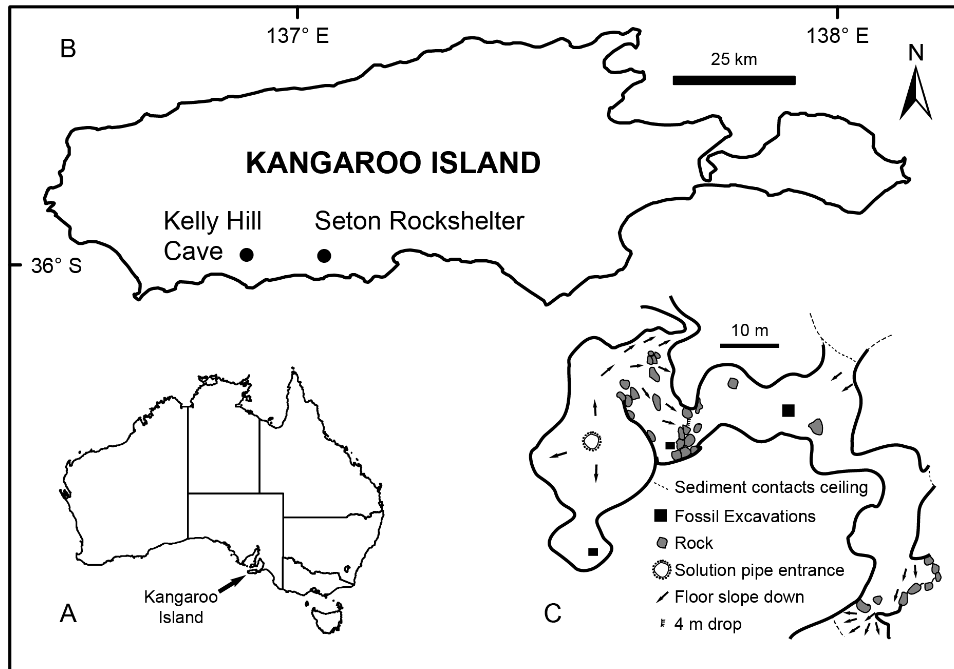

Fig. 1. A. Location of Kangaroo Island relative to the Australian mainland. B. Location of Kelly Hill Cave and Seton Rock Shelter, Kangaroo Island. C. Relevant map section of Kelly Hill Cave showing the location of modern solution pipe entrances, fossil excavation and the blocked palaeo-entrance through which excavated sediments and bones entered the cave (McDowell, 2013)

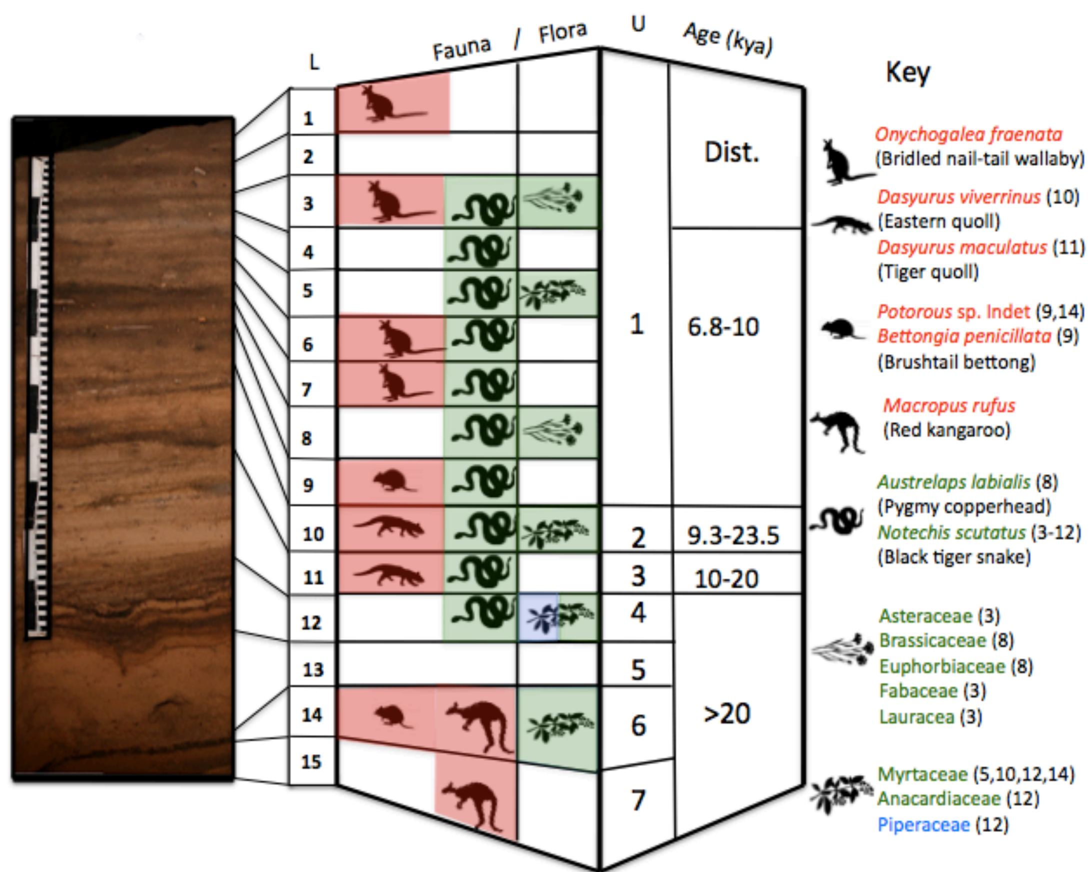

Fig. 2. A snapshot of the fauna and flora recovered throughout the layers of the excavation site in KHC. Units are indicated (U: 1-7) as well as Layers (L: 1-15). Age (ka) indicates the time over which each accumulated based on both U-Th and radiocarbon ages. Dating results suggest that a depositional hiatus occurred between accumulation of Units 3 and 2 and that Unit 2 has been reworked. The first three layers represent a disturbance (Dist.) followed by twelve undisturbed layers (4-15). Scale bar is 1.0 meter in length (McDowell et al., 2013). Red shading indicates fauna and flora extirpated from the island; green shading indicates species still present on KI today; blue shading shows species not previously found on KI. Numbers in the key represent the layers each species was found, when multiple species are found in one layer.

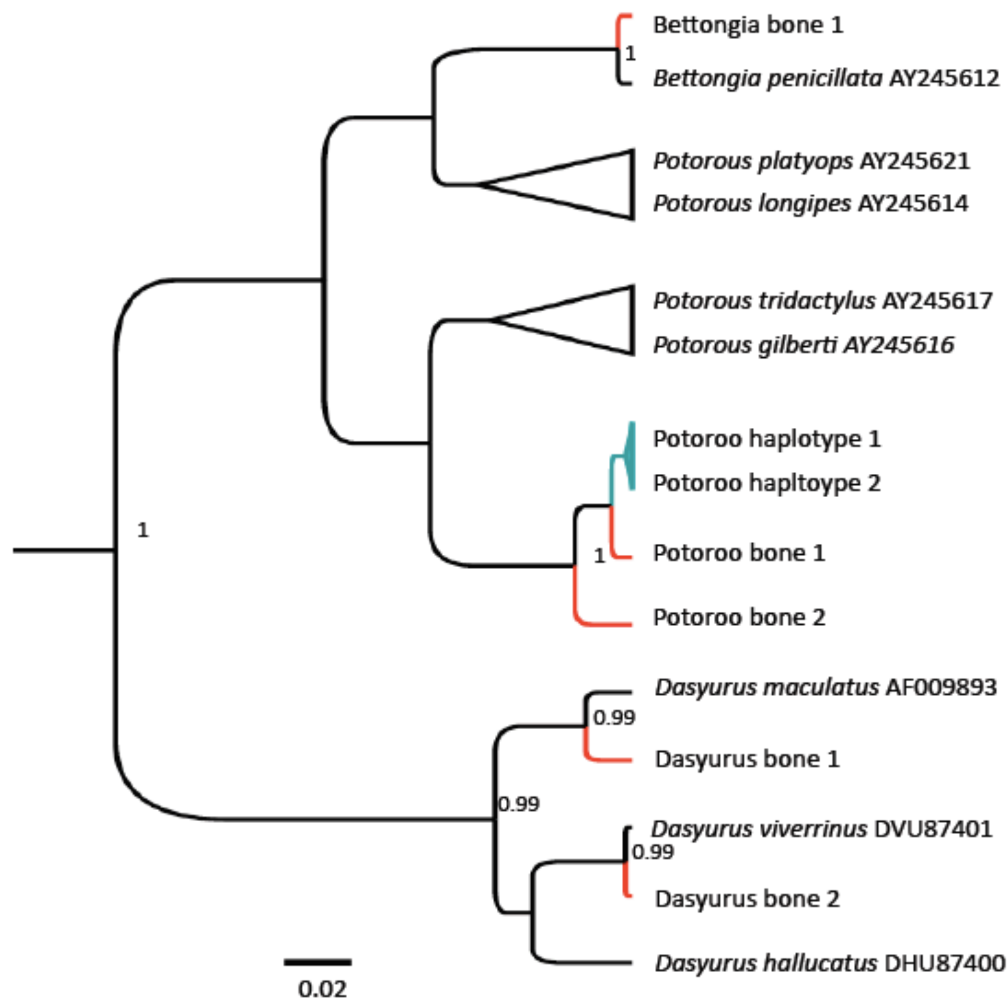

Fig. 3. Bayesian 12s rRNA phylogeny from 180bp alignment showing the closest genetic match of the fossils from KHC to the mainland reference sequences retrieved from GenBank (accession numbers shown). Sequence data illustrates the relationships between *Bettongia*, *Potoroo* and *Dasyurus*. Blue coloured nodes are the result from bulk bone sampling; whilst red coloured nodes are single bones, single extraction samples. The tree was built using a HKY model and Yule tree prior with invariant gamma sites and imposed with a relaxed molecular clock. Values on node show >90% posterior probabilities. The scale represents the number of nucleotide substitutions per site.

Table 1. Mitochondrial 12s rRNA/16s rRNA and Cytochrome *b* primer sequences and conditions used in this study to genetically characterise marsupial/mammal fossils and plants from sediments from KHC

Primer name	Primer sequence 5' → 3'	Annealing temperature (°C)	Amplicon size (bp)	Primer info
Primers for mammal/marsupial fossils				
12s_Macro_40F	GAYCTACACATGCAAGTTTCCGC	53	175	This study
12s_Macro_240R	CGGTGGCTGGCACGAGATTTAC			
12s_Macro_725F	GGAAAGYAATGGGCTACATTTTCTAA	60	115	This study
12s_Macro_843R	GCCTATTTCAATTAAGCTCTCTATTCT			
12s_Mars_520F	GGTCATAGCATTAACCCAAATTAACAG	55	170	This study
12s_Mars_690R	CTAATCCCAGTTTGTCTCTTAGCT			
16s_Mam1_F	CGGTTGGGGTGACCTCGGA	54	150	(Taylor, 1996)
16s_Mam1_R	GCTGTTATCCCTAGGGTAACT			
Cytb_Macropod_250F	CACGCTAACGGAGCATCCATATTC	56	160	This study
Cytb_Macropod_450R	GCCGATGTAGGGGATAGCGG			
Cytb_Macropod_400F	TACCGTGAGGACAAATATCATTCTGA	56	160	This study
Cytb_Macropod_600R	GAGCCTGTTTCGTGTAGGAATAG			
Primers for plants from sediment				
*trn*Lg	GGGCAATCCTGAGCCAA	54	90	(Taberlet et al., 2007)
*trn*Lh	TTGAGTCTCTGCACCTATC			
*rbc*L_F	GGCAGCATTCCGAGTAACTCCTC	53	100	(Chiang et al., 1998)
*rbc*L_*R*	CGTCCTTTGTAACGATCAAG			
Primers for snake fossils				
Cytb_Snake_55F1	CTCCACCTGATGAAACTTCGG	54	145	This study
Cytb_Snake_220R1	ATATGGATGCGCCGATTGCG			

Table 2. Summary of plants achieved using High-Throughput DNA sequencing and number of sequences from sediment cores taken from six layers in KHC, KI. Eight families were detected using a two-locus approach targeting the chloroplast *trn*L and *rbc*L genes, resulting in over 100,000 sequences. Families indicated in bold and genus is italicized.

Plant taxa identification	Sedimentary layer and sampling depth from surface (KHC, KI)					
	Layer 3 (-10cm):	Layer 5 (-18cm):	Layer 8 (-59cm):	Layer 10 (-80cm):	Layer 12 (-98cm)	Layer 14 (-130cm)
Anacardiaceae					n=100	
Anacardium sp.					n=36	
Asteraceae	n=200#§					
Brassicaceae			n=11400#§			
Brassica sp.			n=10§			
Euphorbiaceae			n=260#§			
Hevea sp.			n=160			
Fabaceae	n=4000#§					
Lauraceae	n=350 #§					
Cinnamomum sp.	n=7§					
Myrtaceae		n=26500#§		n=10500#§	n=20700 #§	n=14200#§
Myrcia sp.		n=7			n=24	n=5
Eucalyptus sp				n=13#§	n=22#§	n=6#§
Leptospremum sp.		n=765#§		n=250#§	n=875#§	n=375#§
Syzygium sp.						n=8§
Piperaceae					n=1000	
Piper sp.					n=263	

Key: # - Presence documented on Kangaroo Island; § - presence documented on South Australia mainland.

n = number of sequences in each family/genera detected using HTS.