Identifying remains of extinct kangaroos in Late Pleistocene deposits using collagen fingerprinting

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ABSTRACT: Our knowledge of past animal populations, including the geographical ranges of extinct species, has largely been derived from morphological analyses of skeletal fossil remains. However, a major barrier to the identification of the remains of extinct megafaunal species in archaeological and palaeontological sites is the highly fragmented nature of the material, which often precludes confident taxonomic identifications based on morphology. Biomolecular techniques are able to go beyond these limitations and are increasingly being used to make such identifications. Protein analysis offers a promising alternative to DNA techniques because they can be much cheaper, more amenable to high-throughput processing and work on much older specimens. Here we demonstrate the potential of collagen fingerprinting in an Australian context by extracting collagen from 50-ka kangaroo fossils from two caves in Tasmania, and identify several species including the extinct short-faced kangaroo Simosthenurus occidentalis. Importantly, of the five fossil bones sampled that had hitherto been ascribed morphology-based identifications below the family level, three had been incorrectly identified during an initial assessment of photographs taken in the field. Our results highlight the utility of using protein-based methods for making genus-level identification of marsupial bone, especially those that may form a basis for broader arguments such as that of late-surviving megafaunal species.

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KEYWORDS: bone fragments; collagen fingerprinting; marsupial species identification; short-faced kangaroo; Sthenurine.

Introduction

Many larger terrestrial species became extinct during the Late Pleistocene, but at different times on different continents (Barnosky et al., 2004). The relative roles of humans and climate change as drivers have been heavily debated, nowhere more so than in Australia, which appears to have lost around 90% of larger species (‘megafauna’) before the Last Glacial Maximum (LGM) (Cosgrove et al., 2010; Prideaux et al., 2010; Rule et al., 2012; Field et al., 2013; Wroe et al., 2013; Saltik et al., 2016). Archaeological dates confirm the widespread presence of people in Australia by at least 47 000 cal a BP (Allen and O’Connell, 2014; O’Connell and Allen, 2015; Hamm et al., 2016) and potentially as early as 55 ka (Clarkson et al., 2015), but direct associations of megafaunal remains and archaeological evidence have only been documented in three sites on the continent: Cuddie Springs, and Nombi and Warraty rock shelters (Field et al., 2008; Fillios et al., 2010; Hamm et al., 2016).

The earliest evidence for human occupation in Tasmania is ca. 40 000 cal a BP (Cosgrove et al., 2010), after people crossed the exposed Bassian land bridge linking Tasmania and the mainland (Lambeck and Chappell, 2001). Evidence for extended Late Pleistocene occupation of limestone cave sites comes from the south-west region, and dates obtained from basal cultural deposits from two sites (see Fig. 1 for locations), Warreen Cave (39°906 ± 879 cal a BP) and Parmerpar Meethaner shelter (39°310 ± 1151 cal a BP), indicate early settlement (Cosgrove et al., 2010). These caves were abandoned at around 13 000 cal a BP probably due to climate change following the LGM (Pike-Tay et al., 2008; Cosgrove et al., 2014). Throughout this occupation the Aboriginal terrestrial economy was based on the hunting of two medium-sized prey animals, the red-necked or Bennett’s wallaby (Macropus rufogriseus) and common wombat (Vombatus ursinus). Of a total of ca. 95 000 bones analysed from very small to medium-sized animals, ca. 15% were attributable to M. rufogriseus which are thought to form up to 75–80% of the human prey (Cosgrove et al., 1990; Cosgrove and Allen, 2001). It is noteworthy that Cosgrove and Allen (2001, p. 424) were unable to attribute any of the ~95 000 fragmented bones to large, now-extinct kangaroos known from Late Pleistocene palaeontological sites in Tasmania, including the short-faced kangaroo Simosthenurus occidentalis and the giant kangaroos Macropus giganteus titan and Proteusmodon anak. Further work in the Florentine River Valley similarly found that, despite apparent regional chronological overlap between humans and both M. giganteus titan and P. anak, no bones of these animals were identified in any archaeological site (Cosgrove et al., 2010). Direct dating of megafaunal remains from three caves in the Florentine River Valley (Cosgrove et al., 2010) to between 53 000 ± 4000 and 44 700 ± 3300 cal a BP has been used to support the view that species including Thylacoleo carnifex (marsupial lion), Sarcophilus laniarius (giant Tasmanian devil) and Simosthenurus occidentalis were extinct before people arrived in the region. However, absence of morphologically identifiable evidence for later-surviving megafaunal species and exploitation by humans does not equate to evidence of absence from the archaeological record. Although none of the ~95 000 bones were attributed to megafaunal species, the overwhelming majority are fragmentary and not morphologically identifiable to species (Garvey, 2006). Most of these fragments are within the size range expected for M. rufogriseus and V. ursinus, but two bones...
were a closer match for Macropus giganteus in size (Garvey, 2006). Biomolecular methods hold great potential for identifying nondescript bone fragments to species, as has been demonstrated recently in Australia using ancient DNA (aDNA)-based methods (Murray et al., 2013; Grealy et al., 2015; Llamas et al., 2015). Nevertheless, archaeological and palaeontological sites with good aDNA preservation are rare in non-frozen landscapes (Poinar and Stankiewicz, 1999). Bone protein, however, may be retained in abundance for much longer periods, in some cases for millions of years (Rybczynski et al., 2013). Here we explore the potential of a recently developed collagen fingerprinting method, also known as Zooarchaeology by Mass Spectrometry (ZooMS; Buckley et al., 2008, 2009, 2014), in studying Australian vertebrate palaeobiodiversity that includes extinct taxa from over 50,000 years ago.

Methods

Modern collagen samples were obtained from bones of the short-beaked echidna (Tachyglossus aculeatus), Tasmanian devil (Sarcophilus harrisii), eastern quoll (Dasyurus viverrinus), southern brown bandicoot (Isodon obesulus), common wombat (Vombatus ursinus), common brushtail possum (Trichosurus vulpecula), common ringtail possum (Pseudocheirus peregrinus), red kangaroo (Macropus rufus), eastern grey kangaroo (M. giganteus) and Bennett’s wallaby (Macropus rufogriseus). Tissue samples were obtained from road-killed specimens collected during fieldwork in Tasmania under permits from the Australian Government (permit numbers FA13095, FA14370 and FA15013) and other reference material held in the Department of Archaeology and History, La Trobe University. No permits were required for this aspect of the described study, which complied with all relevant regulations. The modern specimens collected as roadkill are also now housed in the Department of Archaeology and History, La Trobe University, under the appropriate permits described above.

Protein extraction and collagen fingerprinting

Initial identifications were made by visual morphological comparison with reference specimens. Some were made only from photographs, but this proved to be problematic due to the inability to assess all distinguishing morphological features and to compare with other positively identified fossil specimens. Samples were then drilled and collagen was extracted following previously published methods (Buckley et al., 2009). In summary, ~50 mg of bone powder was demineralized with 1 mL 0.6 M hydrochloric acid overnight. Following centrifugation, the acid-insoluble pellet was then gelatinized with 300 μL 50 mM ammonium bicarbonate for 3 h. The solution was then digested with 0.4 μg sequencing grade trypsin at 37°C for 18 h. The resultant peptide solution was then purified using C18 ziptips and dried by centrifugal evaporation. The samples were resuspended with 10 μL 0.1% trifluoroacetic acid and 1 μL co-crystallized onto a stainless steel matrix assisted laser desorption ionization (MALDI) target plate with a further 1 μL of a-cyano hydroxycinnamic acid matrix. MALDI Time of Flight (ToF) mass spectrometric analysis was carried out using a Bruker Ultraflex II mass spectrometer with up to 2000 laser acquisitions; tandem mass spectrometry (MS) analysis was attempted using the MALDI instrument with up to 10,000 laser acquisitions (e.g. Supplementary Information Fig. S1). Further sequencing was carried...
out using an UltiMate™ 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA, USA) coupled to an Orbitrap Elite (Thermo, Fisher Scientific, Waltham, MA, USA) mass spectrometer (120k resolution, full scan, positive mode, normal mass range 350–1500) following extraction and analysis methods described by Wadsworth and Buckley (2014).

Peptides were separated on a 75 mm × 250 μm ethylene bridged hybrid (BEH) C18 analytical column (Waters, UK) using a gradient from 92% A (0.1% formic acid in water) and 8% B (0.1% formic acid in acetonitrile) to 33% B in 44 min at a flow rate of 300 nL min⁻¹ and automatically selected for fragmentation by data-dependent analysis; six MS/MS scans (Velos ion trap, product ion scans, rapid scan rate, Centroid data; scan event: 500 count minimum signal threshold, top 6) were acquired per cycle employing dynamic exclusion with one repeat scan (i.e. two MS/MS scans in total) acquired over 30 s with that precursor being excluded for the subsequent 30 s (activation: collision-induced dissociation (CID), 2⁻ default charge state, 2 m/z isolation width, 35 eV normalized collision energy, 0.25 activation Q, 10.0 ms activation time). Peptide homology was assisted through Error Tolerant searches using Mascot (e.g. Figs S2–S7) against the SwissProt database (Buckley et al., 2015).

Results and discussion

Taxonomic resolution

We evaluated the extent of (collagen) peptide mass fingerprint variability in all 10 extant species (e.g. Fig. 3). Although a wide range of peaks across entire spectra could potentially be used to make identifications (explored in various means by Buckley et al., 2009; Buckley, 2016), the minimum number of robust markers are typically identified to overcome variations in fingerprint quality, with fewer peaks observed in more degraded specimens (e.g. Buckley and Collins, 2011). Here we observed several peptide markers that are specific to particular groups (e.g. m/z 2177.1 for both dasyurid taxa), but there are also many distinctive markers between all genera included in this study, with the homologous markers to those published previously defined in Table 1.

Fossil bone identifications

Proteins were extracted from the six ca. 50-ka macropod fossils from several cave sites in Tasmania. One, a cranium (Fig. 2) embedded within the cave wall in Emu Cave (JF154), preserves the distinctive morphological attributes of Simosthenurus occidentalis, including a broad neurocranium and finely crenulated molar enamel (Prideaux, 2004). Protein extracts were then digested (into peptides) and peptide mass fingerprints (PMFs) were evaluated using MALDI-ToF-MS (e.g. Fig. 4) with further sequencing of selected peptides carried out using high-resolution LC-Orbitrap-MS/MS. Before in-depth LC-MS/MS analyses, the Simosthenurus collagen PMF (Fig. 4) was compared with those of other mammalian groups and, as expected, a greater similarity between Simosthenurus and macropod in terms of number of shared peaks could be observed than for other taxa (e.g. wombat; Fig. 3; see also Fig. S8).

The taphonomy of the south-west Tasmanian limestone caves has been dealt with in detail previously and will not be reiterated here (Cosgrove and Allen, 2001; Cosgrove et al., 2010; Garvey, 2010). However, it is worth pointing out that these caves have exceptional bone preservation, where even delicate mouse scapula are preserved in the pre-35 000 BP layers. Micromorphological analysis of sediments in a number of caves suggests that only small amounts of bone have been lost to diagenesis (Cosgrove, 1995). The natural pitfall sites that we have sampled for this project also appear to exhibit high-quality preservation of bone, unsurprising given that the bones of these very large macropods come from protected locations within the caves. It is likewise not surprising that collagen fingerprints were obtained in all fossil remains analysed, allowing for taxonomic determination (Table 2). Due to the observation of several conflicts with initial morphological interpretation, the morphology was revised in light of the PMF-based identifications under closer scrutiny (Table 2).
It is significant that one of the ‘macropod’ fossils (JF155-3-talus) could be confidently referred to a sthenurine (probably Simosthenurus occidentalis in this context) following the species markers described in this study (Table 1), reflecting the first identification of an extinct taxon using collagen fingerprinting (Table 2). They also included a suspected S. occidentalis innominate (JF155-2/JF155_146), due to size/robustness, which most closely matched the fingerprint typical for the macropodines.

The femur (JF155-1/JF155_144) originated from the roof deposit in cave JF155 (Cosgrove et al., 2010) and was much larger and more robust than the largest extant macropod in Tasmania, Macropus giganteus. Following Helgen et al. (2006), measurements were taken of the circumference of the femur and were found to closely match the metrics of Simosthenurus (Fig. 5); however, these were clearly shown here to be unreliable as an indicator of taxonomic identity as the fingerprint did not match the peptide markers for S. occidentalis retrieved from the cranial specimen JF154-1/JF154_13. Upon closer inspection, the shape of the greater trochanter, which ascends smoothly from the femoral shaft, supports the indication that it does not belong to a sthenurine.

Table 1. List of collagen tryptic peptide markers for the eight extant taxa and extinct short-faced kangaroo; peptide labels follow Buckley (2016); values estimated to those expected from sequence information.

| Taxonomy      | Genus      | 2t43   | 2t76   | 2t69   | 1t55/56 | 2t3   | 2t67   |
|---------------|------------|--------|--------|--------|---------|-------|--------|
| Macropodidae  | *Macropus* | 1453.7 | 1652.8 | 2145.1 | 2897.4  | 2975.4| 2943.4 |
| Macropodidae  | *Simosthenurus* | 1453.7 | 1652.8 | 2145.1 | 2897.4  | 2975.4| 2943.4 |
| Phalangeridae | *Trichosurus* | 1439.7 | 1624.8 | 2161.1 | 2897.4  | 2975.4| 2959.4 |
| Phalangeridae | *Pseudocheirus* | 1439.7 | 1624.8 | 2161.1 | 2897.4  | 2975.4| 2929.4 |
| Vombatidae     | *Vombatus* | 1453.7 | 1624.8 | 2161.1 | 2897.4  | 2975.4| 2929.4 |
| Peramelidae    | *Isodon*   | 1453.7 | 1624.8 | 2161.1 | 2897.4  | 2975.4| 2929.4 |
| Dasyuridae     | *Sarcophilus* | 1453.7 | 1652.8 | 2177.1 | 2897.4  | 2975.4| 2929.4 |
| Dasyuridae     | *Dasyurus* | 1453.7 | 1652.8 | 2177.1 | 2897.4  | 2975.4| 2929.4 |
| Tachyglossidae | *Tachyglossus* | 1453.7 | 1606.8 | 2121.1 | 2873.4  | 3009.4| 3015.4 |

Figure 3. MALDI-ToF-MS spectra of collagen digests from various reference species showing the collagen fingerprint variability across various marsupial and monotreme taxa; labels identify markers from Table 1 (fingerprints of the remaining reference species are shown in Fig. S8).
The overall shape of bone ‘JF155_M_fulig?’ indicates that it is an innominate of a macropodid. The ilium and ischium are aligned in the same plane, which indicates that it does not belong to a sthenurine (confirmed by the collagen fingerprint analysis). The ilium itself, although incomplete, was clearly broad and, although initially considered a potential *Macropus* fragment, closer inspection identified it as being significantly broader than is characteristic of species of *Macropus*. This isolates *Protemnodon anak* as the most likely candidate species.

The overall shape of bone ‘JF155_Protem_sp’ indicated that it is a partial tibia of a macropod, but the distal half of the specimen is missing, along with most of the anterior crest. Macropodine and sthenurine tibiae may be most easily distinguished by the shapes of the anterior crest and the distal epiphysis (missing here); in macropodines the anterior crest is

![Figure 4. MALDI-ToF-MS spectra of collagen digests from some of the fossil samples in this study including the known *Simosthenurus occidentalis*; inset: one of the most abundant peptide biomarkers that separate the sthenurines from the macropodines (see Table 2 for specimen details).](image)

Table 2. Previous known context and morphological species identifications presented alongside the results from the protein analysis.

| Lab. number | Field ID       | Age* (BP) | Skeletal element | Initial morphological ID (from photographs) | PMF-based ID | Revised morphological ID |
|-------------|----------------|-----------|------------------|--------------------------------------------|--------------|--------------------------|
| n/a         | JF154-3-42     | No date   | Ulna             | Macropod                                   | Macropodine  | Large macropod           |
| n/a         | JF155-4-talus  | No date   | Femur            | Macropod                                   | Macropodine  | *                        |
| n/a         | JF155-3-talus  | No date   | Tibia            | Macropod                                   | Sthenurine   | *                        |
| Wk-39988    | JF155-2/JF155_146 | No date | Pelvis           | *Simosthenurus! (Fig. S9)                  | Macropodine  | Large macropod           |
| ACRF-2073   | JF155-1/JF155_144 | >46 200 | Femur            | Simosthenurus (Fig. 5 & S10)               | Macropodine  | *Protemnodon sp. indet, most likely *P. anak* |
| Wk-39989    | JF155_M_fulig? | 46        | Right pelvis     | Large *Macropus* (Fig. S11)               | Macropodine  | *Protemnodon sp. indet, most likely *P. anak* |
| Wk-39987    | JF155_Protem_sp | 44       | Left tibia       | *Protemnodon* (Fig. S12)                  | Sthenurine   | *                        |
| ACRF-2074   | JF154-1/JF154_13 | >45 400 | Zygomatic (crania) | *Simosthenurus occidentalis (Fig. 2)       | Sthenurine   | *                        |

*No change between initial and revised morphological identifications.*

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distinctly stepped, whereas in sthenurines it curves smoothly from the main tibial shaft, but the position of the break on this specimen dictates significant uncertainty in the subfamilial identification based on morphology alone.

Overall, three initial morphology-based identifications of post-cranial elements were found to be incorrect in light of the collagen data, with the JF155-1/JF155_144 femur and JF155-2/JF155_146 innominate described above corrected as macropodines and the JF155_Protem_sp tibia corrected as sthenurine, which clearly highlights the utility of this method for taxonomically identifying highly fragmented remains. Of the three undiagnostic macropod bone fragments, one was found to be sthenurine (Simosthenurus) highlighting the potential of collagen fingerprinting at discovering the remains of extinct taxa from an otherwise underutilised resource.

Although this research is based on a relatively small number of ancient specimens, the likelihood for errors in species determination for these macropods of variable size but similar skeletal morphology is clearly notable. Given the propensity of mammals to increase in size as they approach colder latitudes (Bergmann’s Rule), this method is particularly suited to Late Pleistocene Tasmanian archaeological sites where the size of the bones can lie outside the expected range of the extant human prey animals which may otherwise give rise to the false attribution of bones to either specific large extant or extinct megafauna species where none exist. Additionally, it has been previously shown that Ice Age Tasmanian Aboriginal people systematically butchered their prey, resulting in low representations of some skeletal elements making it back to the cave sites (Allen et al., 2016). It has also been noted that one explanation for the lack of megafauna in these archaeological sites is that they were cut up away from caves and transported as meat, rather than their bones brought back to sites (Cosgrove et al., 2010). However, some could consider it surprising that no megafaunal marrow bones are present in the archaeological sites given a human record of over 25,000 years of occupation (Cosgrove and Allen, 2001, p. 424). Several detailed explanations have been proposed elsewhere to account for this pattern (Cosgrove et al., 2010). For example, it has been recently shown that the marrow of large macropods like M. giganteus does not contain the same high-value unsaturated fatty acids as smaller ones (i.e. Bennett’s wallaby; Garvey, 2010) and may be one reason why these bones are not found in archaeological sites (Allen et al., 2016). Irrespective of this, one cannot discount the human utilization of large macropods as prey animals during the Late Pleistocene through which the marrow-harvested bones would be more likely to be fragmented beyond species identification using morphology alone, increasing the need for utilizing a biomolecular approach such as the collagen fingerprinting technique proposed here.

### Practical applications to archaeology

Although the taxonomic resolution is not as great as that of DNA-based methods, collagen fingerprinting for species identification does have two main advantages: (i) results can confidently be obtained from much older specimens with
collagen typically surviving much longer (see Nielsen-Marsh, 2002) with fingerprints obtained from >3.5 Ma specimens (e.g. Rybczynski et al., 2013); and (ii) identifications can be obtained with much greater high-throughput, with previous investigations studying >12,000 specimens from a single cave site (Buckley et al., 2016, 2017; >1000 specimens being fingerprinted weekly in some instances, even by manual approaches). Additionally, the method can easily be applied to a wide range of vertebrate taxa worldwide and although more useful in identifying fragmentary bone that cannot be identified morphologically, in cases with morphologically similar taxa such as this, or with sheep/goat (Buckley et al., 2010; Buckley and Kansa, 2011), the approach could be much more cost-effective and, at times, more reliable than hiring a specialist morphologist; depending on sample size, these analyses can also leave the remains morphologically intact so that future investigations can be carried out on the fragmentary assemblage. The method is also highly complementary with other archaeological science methods that are based on collagen extraction, such as radiocarbon dating (Harvey et al., 2016) and stable-isotope analysis (van der Sluis et al., 2014). Investigators could utilize the combined approaches to better understand the palaeoecology of particular regions with improved chronologies of particular taxa.

Conclusions

Here we present a new tool for species identification that could be routinely used to screen fragmentary Australian faunal remains for clear indications of both extant and extinct species from the period crucial for answering the debate on human- or climate-caused extinction. However, although none of the ~950,000 bones from the seven excavated limestone archaeological sites discussed within approached the robustness of the bones identified at Emu Cave JF154 and Un-named Cave JF155 (Cosgrove et al., 2010). If megaunal species did lay among the unidentified fragmentary bones, then applying collagen fingerprinting would be an excellent method to identify them. Further development and use of collagen fingerprinting to not only identify currently undetectable bone fragments but also potentially assist the screening of specimens for dating (Harvey et al., 2016) will allow a clearer pattern of chronology and species composition to emerge regarding the roles played by people, climate and habitat modification on the extinction of Australia’s megafauna.

Supplementary information

Fig. S1. MALDI-ToF tandem mass spectra of m/z 2881.4 and 2897.4.

Figs S1-S7. LC-MS/MS spectra of novel peptide markers.

Fig. S8. MALDI-ToF mass spectra of collagen tryptic digests from red and grey kangaroo, brown bandicoot, ring-tail possum and the eastern quoll.

Fig. S9. Photograph of JF155_146.

Fig. S10. Photograph of JF155-1/JF155_144.

Fig. S11. Photograph of JF155_M_fulig.

Fig. S12. Photograph of JF155_Protem_sp.

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Abbreviations. aDNA, ancient DNA; CID, collision-induced dissociation; LGM, Last Glacial Maximum; MALDI, matrix assisted laser desorption/ionization; PMF, peptide mass fingerprint.

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