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A critical evaluation of how ancient DNA bulk bone metabarcoding complements traditional morphological analysis of fossil assemblages

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ABSTRACT

When pooled for extraction as a bulk sample, the DNA within morphologically unidentifiable fossil bones can, using next-generation sequencing, yield valuable taxonomic data. This method has been proposed as a means to rapidly and cost-effectively assess general ancient DNA preservation at a site, and to investigate temporal and spatial changes in biodiversity; however, several caveats have yet to be considered. We critically evaluated the bulk bone metabarcoding (BBM) method in terms of its: (i) repeatability, by quantifying sampling and technical variance through a nested experimental design containing subsamples and replicates at several stages; (ii) accuracy, by comparing morphological and molecular family-level identifications; and (iii) overall utility, by applying the approach to two independent Holocene fossil deposits, Bat Cave (Kangaroo Island, Australia) and Finsch's Folly (Canterbury, New Zealand). For both sites, bone and bone powder sub-sampling were found to contribute significantly to variance in molecularly identified family assemblage, while the contribution of library preparation and sequencing was almost negligible. Nevertheless, total variance was small. Sampling over 80% fewer bones than was required to morphologically identify the taxonomic assemblages, we found that the families identified molecularly are a subset of the families identified morphologically and, for the most part, represent the most abundant families in the fossil record. In addition, we detected a range of extinct, extant and endangered taxa, including some that are rare in the fossil record. Given the relatively low sampling effort of the BBM approach compared with morphological approaches, these results suggest that BBM is largely consistent, accurate, sensitive, and therefore widely applicable. Furthermore, we assessed the overall benefits and caveats of the method, and suggest a workflow for palaeontologists, archaeologists, and geneticists that will help mitigate these caveats. Our results show that DNA analysis of bulk bone samples can be a universally useful tool for studying past biodiversity, when integrated with existing morphology-based approaches. Despite several limitations that remain, the BBM method offers a cost-effective and efficient way of studying fossil assemblages, offering complementary insights into evolution, extinction, and conservation.

1. Introduction

For over a century, the study of fossils has played a major role in understanding prehistoric life and evolutionary processes. In particular, morphological analyses of fossils can reveal species that existed in the past, help elucidate the evolutionary relationships of extinct and extant species (e.g., Donoghue et al., 1989; Deméré et al., 2005; Manos et al., 2007), and assist the development of palaeoenvironment reconstructions that provide insights into the evolutionary and ecological impacts of environmental changes (e.g., Rodríguez-Aranda and Calvo, 1998; Zhang et al., 2008). However, such traditional methods have limitations. For instance, taxonomic assignments of fossils have been necessarily reliant on morphological distinctions, making the identification of

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fragmented or taxonomically-mixed fossil material challenging, if not impossible. This limitation can be partially overcome in some Late Quaternary contexts with the application of ancient DNA (aDNA) techniques. Over the past two decades, aDNA has proved to be a useful complement to the morphological study of fossils, and is rapidly growing in popularity, accessibility, and applicability. In combination with next-generation sequencing (NGS), aDNA has been used to test phylogenetic relationships, and timing of speciation and extinction trajectories (e.g., Krause et al., 2010), resolve taxonomy (e.g., Rohland et al., 2010), reconstruct palaeoenvironments (e.g., Willerslev et al., 2003), and measure historic genetic diversity (e.g., Larson et al., 2002; Allentoft et al., 2014).

Despite the utility of aDNA analysis, unidentifiable bone fragments that are retrieved from palaeontological and archaeological excavations are often too numerous and small to justify the expense of aDNA analysis. However, if such bones are pooled for aDNA extraction as one bulk sample, the pool may be sequenced cost-effectively to yield valuable systematic data useful for assessing past biodiversity over time and space (Murray et al., 2013). In addition, bulk bone samples may be useful for evaluating general aDNA preservation at a site, without requiring the destruction of complete or precious fossil specimens (Murray et al., 2013). The bulk bone method employs a metabarcoding approach (Taberlet et al., 2012), which involves: (1) simultaneous extraction of aDNA from multiple unidentifiable fragments of bone; (2) amplification of short, 'diagnostic' regions of mitochondrial genes by polymerase chain reaction (PCR); and (3) sequencing (via NGS) of these amplicons to identify the species present by comparison with a genetic database of known species (e.g., GenBank; Altschul et al., 1990; Benson et al., 2006). Metabarcoding has been used to evaluate both present and past biodiversity (Epp et al., 2012) through the analysis of environmental samples such as sediments (e.g., Jørgensen et al., 2011; Andersen et al., 2012; Pedersen et al., 2013; Epp et al., 2015; Pansu et al., 2015), seawater (e.g., Minamoto et al., 2012; Thomsen et al., 2012), coprolites (e.g., Hofreiter et al., 2003), and middens (e.g., Murray et al., 2012), and has even been able to detect taxa that were considered extinct based on the macrofossil record (e.g., Haile et al., 2009; Haouchar et al., 2014). Using a metabarcoding approach to generate biodiversity data has the potential to significantly reduce workload and costs compared with a morphological approach that can be labour intensive, or require large amounts of taxonomic expertise and time investment (Ji et al., 2013). When combined with the use of indexing (Binladen et al., 2007; Meyer et al., 2007; Kircher et al., 2012) (where DNA from each bulk sample is 'tagged' with a few unique bases), multiple DNA samples can be combined with equimolarity and sequenced in parallel (i.e., 'sample multiplexing') on an NGS platform, increasing throughput and further reducing cost and time.

Although the bulk bone metabarcoding (BBM) method has been implemented in several recent studies (e.g., Murray et al., 2013; Haouchar et al., 2014), several caveats and biases of this method have yet to be addressed. Firstly, the amount of variance attributable to experimental error in the BBM method, as well as other environmental metabarcoding methods (Andersen et al., 2012; Pedersen et al., 2013; Porter et al., 2013), has not been measured. As such, it is unknown at what step, and to what extent, efforts need to be concentrated to minimise experimental error (Earp et al., 2011), and an optimal experimental protocol has not been developed. This is important if we wish to confidently compare how biodiversity has changed across time or space, in order to reliably determine what has driven those changes (Wooley et al., 2010). The "ability of the researcher to obtain a statistically significant result" (Kitchen et al., 2010) is influenced by the treatment effect, and repeatability (or precision), which is affected by biological variability and technical noise. For the BBM method, the treatment effect is the variance in biodiversity *between* samples that arises from differences between palaeontological collection sites (space) or stratigraphic layers (time); biological variability refers to the differences in biodiversity *within* samples resulting from sub-sampling effort and differential DNA preservation in the fossils; and technical noise is the variability in biodiversity introduced by the experimental protocol itself (including sub-sampling bone powder for DNA extraction, human error, random contamination, stochastic variations in quantitative PCR and amplification biases, aDNA damage, PCR and sequencing errors, and amplicon pooling during the creation of NGS libraries). In order to isolate the treatment effect from the background (Kitchen et al., 2010), we can quantify the contribution that each of these factors makes to the total variance in the data (the 'experimental error') through a careful experimental design containing multiple sub-samples, biological and technical replicates, stringent laboratory protocols, and the use of multiple blank controls at each step (Kuehl, 2000; Macgregor, 2007; Kitchen et al., 2010).

Secondly, most metabarcoding studies of environmental samples have found discrepancies between estimates of biodiversity obtained from DNA metabarcoding methods and those obtained from traditional biodiversity sampling methods (Ji et al., 2013) because some species identified morphologically were not identified via DNA methods, and vice versa (Hajibabaei et al., 2011; Murray et al., 2013). These discrepancies arise from differences in the biomass and behaviour of animals (Andersen et al., 2012), as well as sampling effort, differential preservation of both fossils and the aDNA within them, technical 'noise' (such as amplification bias, PCR and sequencing error; Fonseca et al., 2012), and deficiencies in reference genetic databases, such as GenBank (Pedersen et al., 2014). It is likely that BBM studies would be affected by similar biases (Murray et al., 2013); however, the extent to which bulk-bone taxonomic identifications reflect those arising from the fossil record has yet to be examined.

In this paper we critically evaluate the BBM approach in terms of its repeatability, accuracy and overall utility. Repeatability was assessed by estimating the contribution to variance made by experimental error using a nested experimental design containing pooled bone sub-samples (biological variability), bone powder (extraction) sub-samples, and library preparation and sequencing run replicates (technical variability)—this allows us to determine where sampling effort and replication need to be concentrated in order to reduce variance in the detection of families and operational taxonomic units (OTUs). Accuracy was assessed by comparing the family assemblages derived from morphological identification of fossil collections with those derived from a subset subjected to BBM analysis. Finally, overall utility was assessed by applying the approach to two independent Holocene fossil deposits, Bat Cave (BC; Kangaroo Island, Australia) and Finsch's Folly (FF; Canterbury, New Zealand). These methods enabled us to gauge the strengths, limitations, and biases of the BBM approach in order to assess how it complements traditional palaeontological methods.

2. Materials and methods

2.1. Study systems and sample collection

2.1.1. Bat Cave

Located in the Kelly Hill Caves Conservation Park, south-west Kangaroo Island, South Australia (SI Fig. Ia), Bat Cave (BC; 35° 59′ S, 136° 54′ E; Cave Exploration Group of South Australia no. 5K65) consists of a single chamber with a rock-pile entrance (SI I). The taphonomic characteristics of the BC assemblage (maximum species body mass, presence of invertebrate remains, degree of