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Molecular and morphological analyses of avian eggshell excavated from a late thirteenth century earth oven.

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Abstract

Using ancient DNA (aDNA) extracted from eggshell of the extinct moa (Aves: Dinornithiformes) we determined the species composition and number of eggs found in a late thirteenth century earth oven feature at Wairau Bar (South Island, New Zealand) – one of New Zealand's most significant archaeological sites. Mitochondrial and nuclear DNA signatures confirmed this oven feature contained fragments of at least 31 moa eggs, representing three moa genera: *Emeus*; *Euryapteryx*; *Dinornis*. We demonstrated through the genetic identification of 127 moa eggshell fragments that thickness is an unreliable character for species assignment. We also present a protocol for assessing the preservation likelihood of DNA in burnt eggshell. This is useful because eggshell fragments found in archaeological contexts have often been thermally modified, and heat significantly increases DNA fragmentation. Eggshell is widely used in radiocarbon dating and stable isotope research, this study showcases how aDNA can also add to our knowledge of eggshell in both archaeological and palaeoecological contexts.

Keywords: ancient DNA, fossil eggshell, moa, archaeology, midden

1. Introduction

Avian eggshell is often found in abundance in archaeological and palaeontological sites. This can be ascribed to the bioceramic calcium carbonate matrix (calcite, ~97 percent of mass) that can withstand diagenic changes. Eggshell also contains an organic component (~3 percent of mass) within the matrix; protected by the matrix, biomolecules, including DNA (Oskam *et al.* 2010) and amino acids (Johnson *et al.* 1997; Clarke *et al.* 2006) can be very well preserved and are resistant to microbial attack and diffusional loss (Miller *et al.* 1992; 1997). The refractory nature of eggshell has favoured its extensive use in geochronological studies (Higham 1994; Magee *et al.* 2008), as well as research in palaeoecology and palaeodiets by stable isotopic profiles (Miller *et al.* 2005; Emslie & Patterson 2007).

The extensive use of birds by early humans is apparent in many archaeological deposits, notably by the presence of bone and eggshell. Intact eggs can have cultural importance and are, together with other possessions and elaborate jewellery, sometimes found alongside human skeletal remains (Duff 1950; Dell & Falla 1972; Houghton 1975). Eggs are also an excellent source of nutrition (Sales *et al.* 1996).

Those of large birds, such as ratites, can contain the equivalent of a dozen or more chicken eggs. Eggs found in archaeological features, such as middens, have often been thermally modified, and show characteristic sooting or charring. This may result from either cooking whole eggs on the fire or, after consumption of the contents, when waste fragments were disposed of in the fire or embers (Clarke *et al.* 2007). The presence or absence of burned eggshell can allow archaeological sites to be distinguished from nesting sites, although post-depositional heating by subsequent natural or anthropogenic fire(s) in a natural site can also affect eggshell fragments (Anderson *et al.* 2005).

Robust identification of eggshell fragments to species level is essential for accurate assessments of the historical use of birds and eggs, and for measuring faunal diversity in zooarchaeological assemblages. This is especially important if eggshell is to be used in archaeological or palaeontological studies analysing species-specific patterns, such as stable isotope signatures. Yet, because of the difficulties in obtaining a secure species identification, whole eggs or eggshell fragments collected from archaeological sites are most often generically labelled in museum collections as 'avian eggshell' (Scofield *et al.* 2003). Identification of eggshell fragments by rapid, economical methods, such as visual or microscopic examination or measurement, would be preferable but taphonomic processes, such as fading or disappearance of pigments (Keepax 1981), and morphological alteration of the shell induced by high temperatures (Clarke *et al.* 2007), often makes a visual identification of fossil eggshell impossible. Moreover, various factors, including dietary stress, age of the bird, environmental variables (Keepax 1981), uptake of calcium by the developing embryo (Reynolds 2010), taphonomy, and human interaction can also influence eggshell morphology and size, making it difficult to identify a fragment even to genus level.

The ability to assign a species to an egg or eggshell fragment is particularly relevant to our understanding of archaeological 'moa hunting' sites in New Zealand. New Zealand was one of the last major landmasses to become occupied by humans. Polynesians settled in New Zealand c.700 years ago (Anderson 1991) and exploited the megafauna of pinnipeds and giant birds as ready sources of protein and energy. Before humans arrived, the moa (Aves: Dinornithiformes) occupied habitats ranging from alpine fellfield to coastal shrublands (Worthy & Holdaway 2002) but within about a century, all taxa of moa were extinct as a result of human hunting and habitat

destruction (Holdaway & Jacomb 2000) (Fig. 1). Archaeological evidence indicates that the New Zealand Polynesians cooked moa in large earth ovens.

The potential of archaeological sites to contain eggshell of any of the six, often sympatric, genera of moa (Bunce *et al.* 2009) found together with the remains of other large birds (kiwi, rails and seabirds) makes the species identification of eggshell even more difficult in New Zealand than elsewhere. It has previously been suggested that some eggshell morphological characteristics are diagnostic of moa genera (Gill 2000; Gill 2007; Gill 2010). Using recently developed ancient DNA techniques (Oskam *et al.* 2010), morphological identifications, primarily eggshell thickness, can now be directly tested for fidelity.

There are three aims of our research: (I) to investigate DNA preservation and morphological changes in thermally-modified eggshell obtained from an archaeological oven feature, (II) to determine whether or not eggshell morphology is a reliable indicator of species identity in moa as opposed to more expensive and time-consuming molecular techniques, and (III) to illustrate how eggshell palaeogenetics can be used to interpret zooarchaeological assemblages. Our research focuses on a single site, Wairau Bar, one of New Zealand's oldest and most scientifically and culturally significant archaeological sites, dated to the late thirteenth century (Higham *et al.* 1999).

2. Materials and methods

2.1 Site information and eggshell sampling

Wairau Bar (174.06218°E 41.50715°S) is the site of a village established by some of New Zealand's earliest settlers at the end of the thirteenth century (Higham *et al.* 1999). These settlers consumed a variety of marine and terrestrial food resources, including some cultivated plants and several taxa of moa (Brooks *et al.* 2009). Archaeological excavations in early 2009 (directed by co-authors CJ and RW) identified a group of six large (5-6 m diameter) earth ovens (Fig. 1) that were used for cooking large vertebrates, including moa. One of these ovens (Area 4-5) was found to have been used as a midden dump after having been used for cooking. The midden exhibited some amorphous banding but no clear stratigraphy, and was therefore excavated in arbitrary 100 mm spits. The eggshell was distributed more or less evenly throughout the deposit, with a concentration near the centre, which had been

subjected to extreme heating evidenced by charring and distortion of the eggshell fragments. More than 1135 fragments (356 g) of eggshell were recovered from the oven feature. Seventy-six of these fragments were selected for species identification using molecular methods. Fragments were selected based on their distribution (within four quadrants of each spit of a metre square column sample in order to provide a representative sample both horizontally and vertically), and the fragment size to allow sufficient material for DNA analysis, stable isotopic profiling and radiocarbon dating.

Seventy-one eggshell fragments were also selected from collections made at Hukanui Pool (39.2472°S 176.5344°E) and Hukanui #7a (39.2502°S 176.5337°E) sites (Holdaway & Beavan 1999). Both are natural sites in the east-to-central North Island, which were nesting shelters used by a different suite of moa taxa than those that were present near Wairau Bar. The Hukanui sites, because they are natural nesting sites and anthropogenic environmental stress post-dated their deposition, were chosen as further tests of the applicability of eggshell thickness as a feature for moa species identification.

2.2 Thickness and morphological analyses

Eggshell thickness was measured using digital Vernier callipers at various points across each eggshell fragment, as outlined previously (Gill 2007), to obtain a thickness profile for each fragment. Unlike bone, for which thermal modification in archaeological deposits have been studied extensively, both molecularly and morphologically (Shipman *et al.* 1984; Asmussen 2009; Ottoni *et al.* 2009), there has only been one preliminary investigation into thermal modifications of archaeological eggshells (Texier *et al.* 2010). Based on colour, appearance, and texture, we propose four categories of preservation/alteration of eggshell. We present eggshell images together with aDNA success rates as a way of visually assess the likelihood of aDNA survival in fossil eggshell from archaeological deposits.

2.3 Molecular analyses of eggshell fragments

Eggshell fragments were sampled, digested and extracted as described in Oskam *et al.* (2010, fig. S1), with the modifications outlined in the Supplementary Information. Following aDNA guidelines, all samples were prepared and DNA was extracted in a dedicated aDNA facility at Murdoch University, Perth, Western Australia. The sampling area and tools were thoroughly decontaminated between

processing each sample to prevent cross-sample contamination (Cooper & Poinar 2000; Allentoft *et al.* 2009). PCRs were conducted with multiple extraction controls and non-template controls throughout the study.

To assist in discriminating between eggshell fragments from different moa individuals, two approaches were used. Firstly, mitochondrial DNA (mtDNA) control region sequences were obtained using a quantitative PCR (qPCR) assay (Primers CR262F/441R) described in Oskam *et al.* (2010). Species assignment was conducted by comparing eggshell mtDNA sequences with a large number of reference moa sequences (derived from fossil bone) available on GenBank. Sequences were aligned and analysed using Geneious 5.3.6 (Biomatters, New Zealand) and deposited on GenBank (accession numbers JF927651-JF927706). Haplotype assignment was based upon differences observed in the mtDNA sequence. Secondly, a single moa-specific microsatellite marker (Moa_MS2) was amplified and genotyped according to methods presented in Allentoft *et al.* (2009). The excellent preservation of DNA in many of the eggshell allows nuclear DNA to be amplified as well – more specifically polymorphic microsatellite markers (Allentoft *et al.* 2009; Allentoft *et al.* 2011). The minimum number of individual eggs (MNIE) was therefore determined by assessing, in combination, the mitochondrial haplotype and Moa_MS2 microsatellite genotype for each eggshell fragment. Both DNA damage and allelic dropout were taken into consideration when determining MNIE (Allentoft *et al.* 2011). Eggshell fragments with different mtDNA sequences cannot be from eggs laid by the same individual (cognisant of DNA damage effects). However, when identical mtDNA haplotypes were obtained from different samples, the allelic combination in a moa-specific microsatellite marker (Moa_MS2) could be used to further discriminate between different individuals. Only when two individuals showed different mtDNA profiles and/or different Moa_MS2 profiles were they accepted as remains from different individuals, relevant to estimate MNIE.

3. Results and discussion

3.1 Assessing site distribution and quantity of eggshell

Before this study, no comprehensive assessment of moa eggshell abundance in archaeological sites across New Zealand had been conducted. Figure 1 illustrates all palaeontological and archaeological sites containing moa material in New Zealand, known up to 2001. There is a clear tendency for sites of the moa-hunting period to be

coastal, but this does not reflect the actual distribution of the moa (Worthy & Holdaway 2002; Bunce *et al.* 2009). Most of New Zealand's Archaic period sites are within a few kilometres of the coast, where a range of non-moa resources, such as fish and shellfish, were available. The quantity of eggshell in New Zealand archaeological deposits has never been systematically recorded, but an indication of its abundance is evidenced by the fact that c. 3500 g of eggshell has been recovered from the Wairau Bar site alone.

3.2 Morphology and ancient DNA preservation of archaeological eggshell.

Some of the fossil eggshell fragments collected from the oven feature at Wairau Bar (Fig. 1) had clearly been altered by heat. Analysis of the thermal modification of eggshells may provide insights into how these large eggs were cooked and also provide a simple visual method to assess the likelihood of DNA recovery. We developed a four-point category based on morphological features, including decolouration and texture (Table 1).

Thermally-modified eggshell fragments from Wairau Bar exhibited a continuum of discolouration that resulted from limited exposure to heat (minimal colour change, neutral white/pale yellow) to those exposed to much higher temperatures (up to 900°C see Shipman *et al.* 1984) which were more heavily discoloured (light brown to reddish/dark brown) and dark gray/black fragments of calcined eggshell (Table 1). The observed colour differences in eggshell were most likely the result of diagenesis of the organic constituents, accelerated by heating, as previously described in bone (Shipman *et al.* 1984; Asmussen 2009).

Our assessment revealed that eggshell fragments that have presumably had limited exposure to heat showed a shiny, marbled appearance and the mammillary and palisade layers were distinct (Table 1). In fragments exposed to progressively greater heat, the layers were less distinct, culminating in the coalescence of the crystals into a homogeneous structure. Eggshell fragments assigned to categories I and II retained their curvature, but some of those assigned to categories III and IV were flattened or warped by heat. These highly modified eggshells gave off an earthy, burnt ash odour during sample grinding.

Of 1135 eggshell fragments excavated from the feature, 23 percent showed clear signs of thermal damage or charring. This fraction of burnt eggshell corresponded to the recorded failure rate (26 percent) (Table 1: SI T1) for DNA

amplification. Mitochondrial DNA amplification (~200 bp in length) was achieved only from eggshell that showed evidence of limited or no thermal modifications, i.e. categories I and II and a decrease in the Moa_MS2 microsatellite amplification was also observed between these two categories. Ancient nuclear DNA was not amplified from eggshell fragments that were assigned to categories III and IV (Table 1), although relatively few were attempted because we determined at an early stage that these calcined eggshell fragments contained poorly preserved biomolecules. In contrast, samples of moa eggshell from the natural fossil sites yielded a 100 percent success rate (SI T2).

A previous investigation of the preservation of aDNA in thermally modified bones demonstrated that authentic aDNA could be amplified successfully from bones exposed to temperatures of >140°C (Ottoni *et al.* 2009). However, theoretical depurination kinetics suggest that DNA does not survive in bones at temperatures heated to >170°C (Ottoni *et al.* 2009). Temperatures in hearth fires and open oven pits can exceed 400-900°C (Shipman *et al.* 1984), which is far beyond the limits of DNA preservation. We do not know for how long and at what temperatures the moa eggshell fragments from Wairau Bar were exposed. Although the eggshell thermal modification categories proposed here do not correlate exactly with the levels of DNA preservation estimated by the rates of successful recovery, they are useful indicators of the state of DNA preservation in eggshell and may also be in other archaeological contexts. Our results are consistent with findings by Ottoni *et al.* (2009) and Miller *et al.* (1999) in suggesting that heating is detrimental to preservation of biomolecules.

Very little is known about how, or indeed if, moa eggs were cooked. As New Zealand Polynesians lacked pottery, eggs may have been eaten raw, cooked in their shell or cooked outside their shell in combination with other ingredients. Analogies may be drawn from ethnographic records of the cooking of the eggs of other ratites, such as *Struthio* (ostrich), *Rhea* (rhea) and *Dromaius* (emu). Some Australian aboriginal tribes cooked emu eggs in hot ashes, placing them in a hole dug in the ground for the purpose (Bourne 1953). In Patagonia, rhea (*Rhea* spp.) eggs were cooked by puncturing one end, removing a little of the white, and setting the egg vertically on a slow fire (Moreno 1879).

Given the level of DNA preservation in the eggshell in the Wairau Bar oven, coupled with our ability to identify moa species genetically, the next step was to

determine if a morphological feature, thickness of the eggshell, was a reliable identification character as has been proposed (Gill 2000; Gill 2007; Gill 2010).

3.3 Species identification of avian eggshell fragments from an archaeological oven: morphological and genetic approaches

We tested the accuracy of species assignments based on eggshell thickness by amplifying the mtDNA control region from eggshell fragments and compared the sequences to those available on GenBank. Fifty-six eggshell fragments from the Wairau Bar archaeological site and 71 fragments from the natural sites at Hukanui (Holdaway & Beavan 1999) were measured. This dataset contained a total of 127 genetically identified moa eggshell encompassing 5 of the 6 moa genera. We considered it was important to include eggshell from both archaeological and natural contexts when assessing thickness because anthropogenic environment change, (e.g. forest burning), could have altered moa diet and consequently egg morphology.

Our results showed that eggshell thickness varied significantly within, as well as between, genera, between and within species, and also within the same egg (Fig. 2). The natural Hukanui sites demonstrated that eggshell fragments from two moa genera, known to be represented there, could not be separated based on eggshell thickness. Although 50% of the eggshell thicknesses varied by 0.10-0.28 mm, thickness could vary by up to 0.80 mm within a genus, as seen in *Pachyornis* (Fig. 2). Our results mirror to some extent the observations of Huynen *et al.* (2010) with respect to eggshell thickness variation within and between species (Fig. S1). However, Huynen *et al.* (2010) propose that differences in the thickness of *Euryapteryx* eggshell likely represents new species - a claim that our data do not support. Moreover, re-examination of the Huynen *et al.* (2010) data shows overlap between the proposed *Euryapteryx* 'classes' (*Euryapteryx* Class I, 0.98-1.60 mm thick, cf. Class II taxon eggshell 0.74-1.11 mm thick where the 1.11 mm Class II measurement was omitted (Huynen *et al.* 2010). As an example of more profound thickness-differences, Tennyson *et al.* (2010) observed three classes of eggshell thickness associated with possible Archaic bird or moa from the Miocene; one 'thin' class <0.5 mm; and two 'thick' classes: 0.5-1.19 mm and >1.19mm. Our results however, illustrated that there is a continuum of thicknesses within each of the moa species, causing considerable inter-specific overlap. This variation in eggshell thickness could be a reflection of age differences among the female birds, the

environment in which the birds lived, stress-levels (Keepax 1981) and variation in a range of taphonomic variables.

Taken together, our data illustrate that eggshell thickness is not a reliable character on which to identify moa. Our data agree with Huynen *et al.*'s (2010) suggestion that *D. robustus* and *E. curtus* laid the thickest-shelled eggs of all South Island moa. However, a *Euryapteryx* eggshell fragment in the lower quartile range could equally be assigned to another genus on that criterion (Fig. 2). Importantly, we demonstrate that, because of the wide variation in thickness, meaningful insights into moa hunting practices require (see below) a robust method of species identifications, and mtDNA signatures are currently the only reliable approach.

3.4 Genetic profiling of eggshell from Wairau Bar; a New Zealand moa-hunting site.

Of the 76 eggshell fragments subjected to aDNA analysis from this site, only 56 (74 percent) yielded amplifiable DNA. The samples that failed to yield DNA had been too degraded by heating (see Table 1). The mtDNA control region sequence data clearly shows that the eggs of three moa species (*E. curtus*; *E. crassus*; *D. robustus*) were represented within the single large oven excavated in 2009 at Wairau Bar (Fig. 3). Differences in mtDNA sequences, combined with microsatellite profiles, enabled us to identify the remains of at least 31 individual eggs in the Wairau Bar oven.

The presence of at least 31 moa eggs in a single oven shows that they represented a significant part of the diet of the people at Wairau Bar. It indicates a significant exploitation of breeding moa (Scofield *et al.* 2003), which would have exacerbated the more damaging effects of predation on the adults (Holdaway & Jacomb 2000). Although the three species identified in this paper have been observed from earlier excavations at the site (Scofield *et al.* 2003), *Dinornis* has been observed more rarely. Its presence, however, is not surprising as this species inhabited the edges of the drier forests characteristic of the eastern South Island, as well as wet forests such as those immediately to the north, on the other side of the Wairau River from the site.

Eggshell fragments excavated from archaeological middens may often represent more than one egg and more than one species. However, without genetic analysis, the fragments cannot be reliably assigned to species, nor can the minimum number of individual eggs be quantified. This, in turn, influences the level of inference we can draw about human hunting pressure on the moa breeding population

and about foraging practices of prehistoric people. Genetic analysis of the fragments allows a more detailed reconstruction of their diet and hunting practices, and a better assessment of the impact they had on the area they occupied.

4. Concluding remarks

Here we have demonstrated the utility of aDNA for analysing collections of eggshell fragments from archaeological sites. Such eggshell have often been cooked or subjected to heat, so we developed a four-point category by which eggshell specimens with the potential for good aDNA preservation can be identified. The cooking, and particularly charring, of eggs dramatically affected the preservation of both mitochondrial and nuclear DNA in the eggshell fragments.

Eggshell thickness is a poor discriminator for species assignment of shell fragments in moa; intra-specific variation is high, and we observed overlap in thickness of eggshell in five of the six recognised genera that were identified. Therefore the molecular techniques applied in this study can be used to significantly increase the level of information from zooarchaeological assemblages and collections of avian eggshell held in museum collections worldwide. Eggshell fragments can be assigned to individual eggs by the use of mtDNA and microsatellite profiling techniques. In addition to representing a valuable tool for quantification, such genetic profiling of eggshell fragments can be used as a screening tool to eliminate the risk of duplicating analyses on fragments of the same egg when selecting fragments for radiocarbon dating or stable isotopic analyses.

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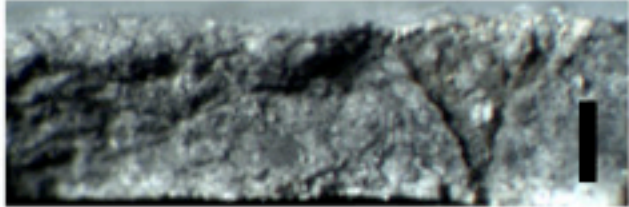
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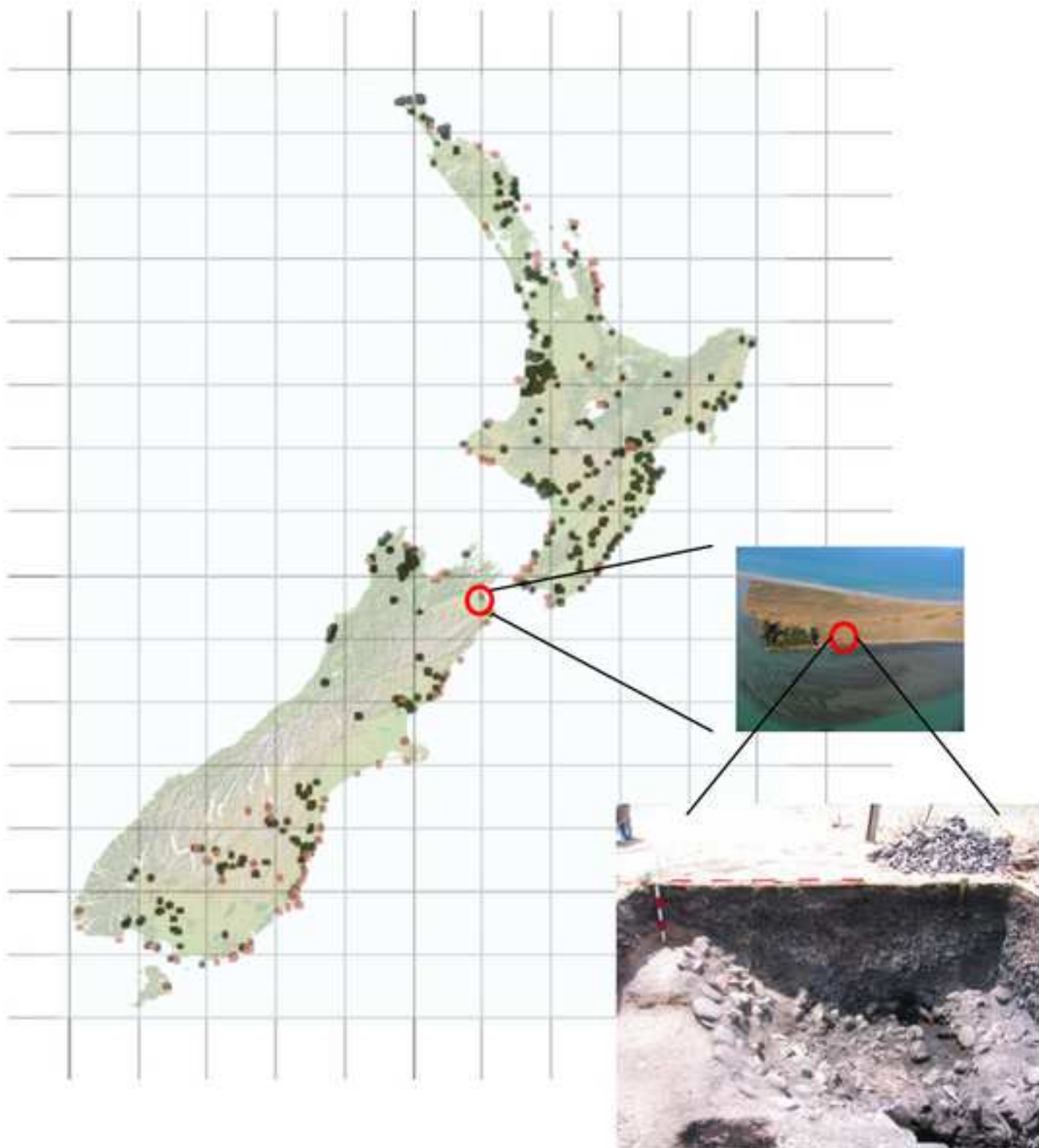
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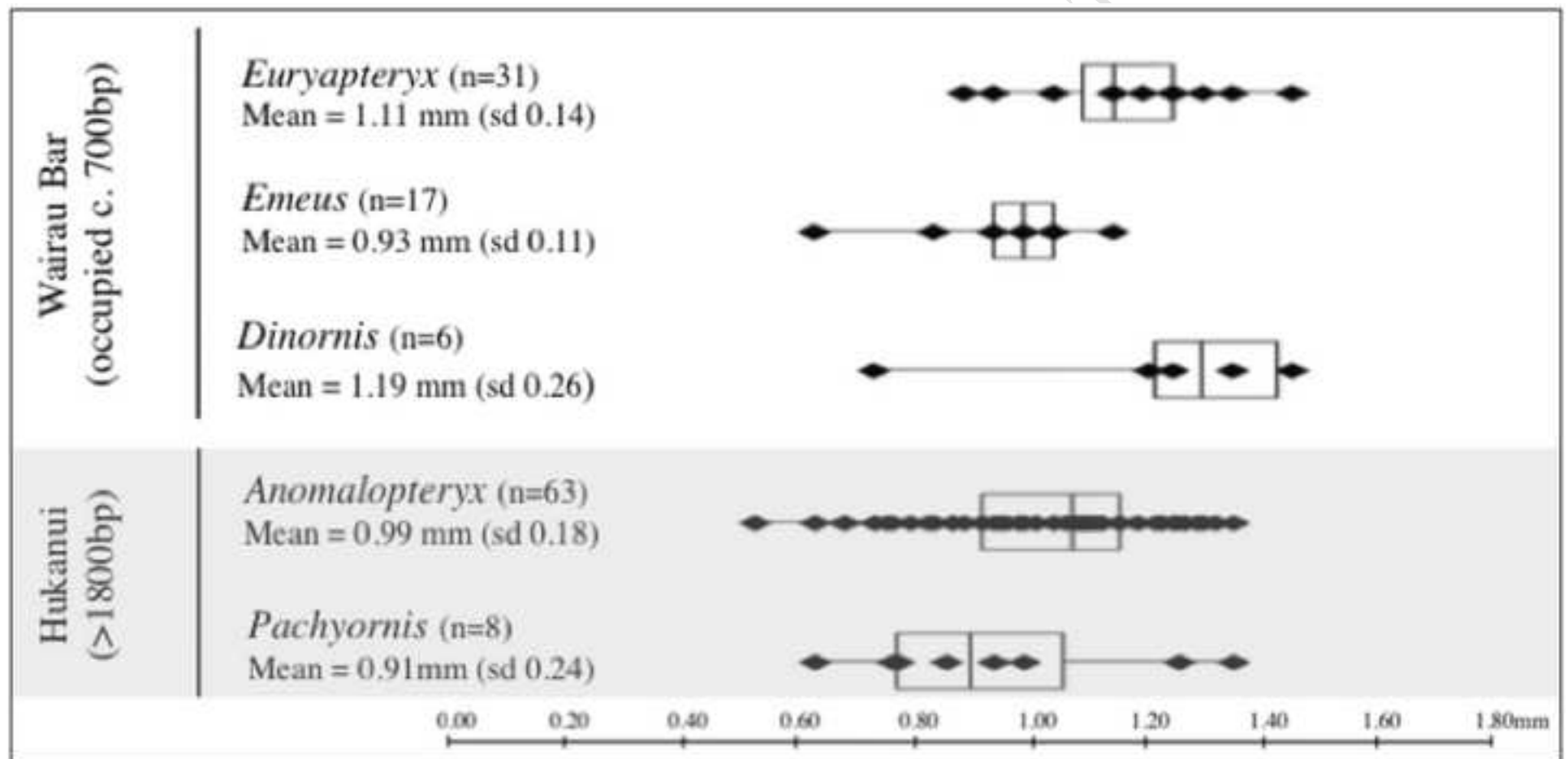
Table 1

Archaeological eggshell characteristics and mitochondrial and nuclear DNA amplification success rates from moa (Aves: Dinornithiformes) eggshells collected from an archaeological site in New Zealand, Wairau Bar.

Archaeological eggshell characteristics				N	mtDNA	nuDNA
Category	Image	Colour/Appearance	Texture			
I		Neutral white-yellow palisade, with distinct white-grey mammillary	Marbled	51	96% (49)	60% (29)
II		Light brown palisade, with distinct light brown mammillary	Powdery crystals	19	37% (7)	14% (1)
III		Mid/dark reddish-brown, mammillary layer nearly coalesced with palisade layer	Chalky crystals	5	0%	na
IV		Dark grey/black, no distinct layers, crystals completely coalesced	Calcined	2*	0%	na
Total				76	74% (56)	39% (30)

Scale bar on image – 0.5mm *Because of the gradual decline in success rate from category II to III eggshell, only 1 eggshell from category IV was analysed for the presence of DNA. Values in parenthesis are the total number of successful samples. Lower edge = mammillary layer. Images were taken on a dissecting microscope 4X objective lens using *Moticam 1000* and images modified in *Motic Images Plus 2.0.10*.



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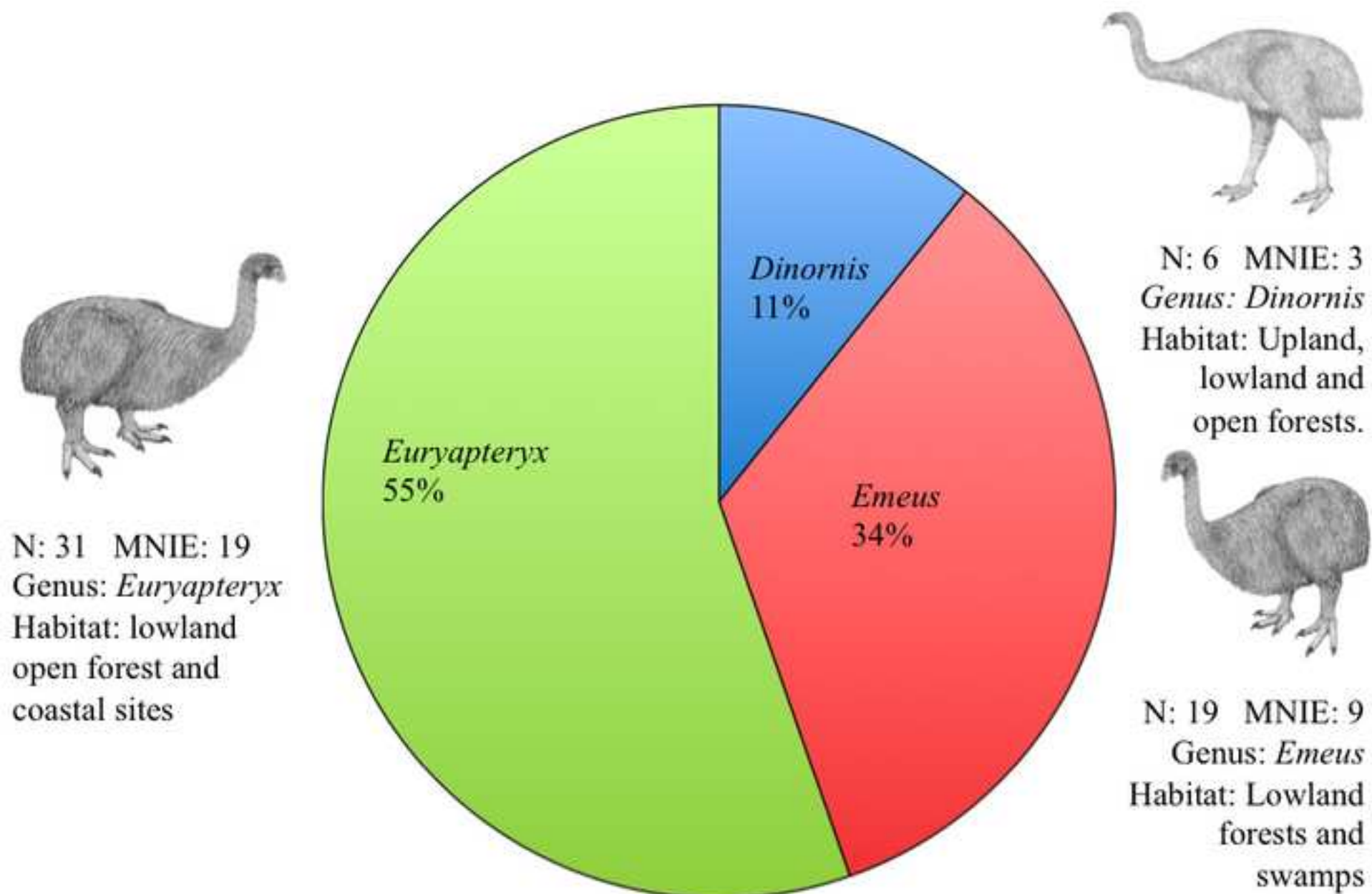


Fig. 1. Left. Archaeological (red) and Paleontological (black) moa deposits identified on New Zealand's North and South Islands. Upper right. Aerial view of Wairau Bar. Lower Right. Large oven feature (Area 4-5) at Wairau Bar from which 1135 eggshells were collected (356 g) and 56 were genetically identified. The coloured divisions on the scale bar represent 100 mm.

Fig. 2. Intraspecific variation in moa eggshell thicknesses in five of the six moa genera. Box plot represents upper and lower quartiles, minimum, maximum and the median eggshell thicknesses in mm. Eggshells were collected from two dated sites; Hukanui - a palaeontological moa nesting site (North Island) and the Area 4-5 oven feature at the archaeological Wairau Bar deposit. Species identification of the eggshell was determined by comparing the mitochondrial control region DNA with a large number of moa sequences (of known species identity) present on GenBank (see Bunce *et al.* 2009).

Fig. 3. Moa composition within the Area 4-5 oven feature at Wairau Bar. Percentage represents the proportion of moa genera within the 56 eggshells that were genetically identified. N, number of fragments assigned to genus. MNIE, minimum number of individual eggs within each genus that had unique mtDNA (control region) and/or microsatellite (Moa_MS2) DNA signatures.