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ORIGINAL ARTICLE

Ancient DNA microsatellite analyses of the extinct New Zealand giant moa (*Dinornis robustus*) identify relatives within a single fossil site

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By analysing ancient DNA (aDNA) from 74 ^{14}C-dated individuals of the extinct South Island giant moa (*Dinornis robustus*) of New Zealand, we identified four dyads of closely related adult females. Although our total sample included bones from four fossil deposits located within a 10 km radius, these eight individuals had all been excavated from the same locality. Indications of kinship were based on high pairwise genetic relatedness (r_{XY}) in six microsatellite markers genotyped from aDNA, coupled with overlapping radiocarbon ages. The observed r_{XY} values in the four dyads exceeded a conservative cutoff value for potential relatives obtained from simulated data. In three of the four dyads, the kinship was further supported by observing shared and rare mitochondrial haplotypes. Simulations demonstrated that the proportion of observed dyads above the cutoff value was at least 20 times higher than expected in a randomly mating population with temporal sampling, also when introducing population structure in the simulations. We conclude that the results must reflect social structure in the moa population and we discuss the implications for future aDNA research.
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INTRODUCTION

Because of the challenges in characterising nuclear DNA from degraded substrates, microsatellite-based analyses such as that of genetic structuring (Pritchard *et al.*, 2000; Hardy and Vekemans, 2002), migration rates (Beerli and Felsenstein, 1999; Wilson and Rannala, 2003) and kinship and relatedness (Queller *et al.*, 1993; Kalinowski *et al.*, 2006) have been largely absent from ancient DNA (aDNA) research. However, with new methods available for identifying microsatellites markers from aDNA (Allentoft *et al.*, 2009) and the development of strict protocols for the subsequent data generation (Taberlet *et al.*, 1996; Allentoft *et al.*, 2011), microsatellite analyses of extinct taxa are no longer beyond reach (Ishida *et al*, 2012; Nyström *et al*, 2012; Allentoft *et al*, 2014). Here we employ microsatellite DNA profiling on a large assemblage of radiocarbon-dated fossils of the extinct New Zealand moa (Aves: Dinornithiformes) in an attempt to gain new insights into the palaeobiology of this extinct megafauna.

Nine species of moa are currently recognised (Bunce *et al*, 2009), all of which were large browsing ratites endemic to New Zealand. They became extinct as a result of overhunting and habitat destruction shortly after Polynesians settled in the thirteenth century (Anderson, 1989; Holdaway and Jacomb, 2000; Allentoft *et al.*, 2014; Holdaway *et al.*, 2014). As is the case for most extinct taxa, little is known about their biology except some insights into their feeding habits, gained from their gut contents and genetic and morphological analyses of coprolites (e.g., Burrows *et al.*, 1981; Burrows, 1989; Wood *et al.*, 2008, 2013). It has also been demonstrated that moa displayed reverse sexual dimorphism with the females being up to 280% heavier than the males (Bunce *et al.*, 2003; Huynen *et al.*, 2003). Moreover, Allentoft *et al.* (2010) demonstrated by a molecular sexing technique that there was an overall 5:1 excess of females among 267 individuals sampled in North Canterbury. In particular, moa fossils from the shallow lake at Pyramid Valley, excavated periodically since 1939, displayed an extreme excess of 49 females to 2 males of South Island giant moa (*Dinornis robustus*). Hypotheses involving female territoriality and seasonal egg incubation by males (both observed among extant ratites) have been invoked to explain the absence of males from some deposits. From the observation of an even sex ratio among juvenile moa of the taxon *Euryapteryx curtus* (Allentoft *et al.*, 2010), but not among the adults, a higher mortality among males has also been suggested. Incubation by males has been suggested by Huynen *et al.* (2010) who identified putative moa male DNA on the surface of their eggshell.

Although these are just fragmentary insights into moa biology, they still place moa among the best studied extinct animals (summarised in Worthy and Holdaway, 2002 and Allentoft and Rawlence, 2012), and promote them as promising 'model organisms' for research into the social structure and behaviour of extinct species. In an attempt to obtain a deeper understanding of moa biology, and explore the potential in using nuclear genetic data from fossils, we re-analysed a recently published aDNA data set (Allentoft *et al.*, 2014). This data

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set included both mitochondrial DNA (mtDNA) sequences and six genotyped microsatellite loci in 74 radiocarbon-dated individuals of *D. robustus*, excavated from four sites within a 10 km radius in North Canterbury, South Island (Figure 1), and covering ~4200 years of the mid-to-late Holocene. *D. robustus* was the largest of the moa species with females weighing up to 250 kg (Worthy and Holdaway, 2002; Bunce *et al.*, 2003; Brassey *et al.*, 2013). We employed a range of methods to explore the genetic relatedness among the 74 individuals. The application of microsatellite profiling to fossil assemblages is interesting from both biological and methodological perspectives. If such 'DNA fingerprinting' technology can be successfully applied to extinct species, it could result in significant insights into their biology and behaviour, taking population palaeogenetics to a new level.

MATERIALS AND METHODS

Data generation

The microsatellite, mtDNA, and radiocarbon age data analysed in this study were first presented in Allentoft *et al.* (2014), where details of sampling, DNA extraction, genotyping validation and radiocarbon dating are given. The *D. robustus* data set includes mtDNA control region sequences from 87 radiocarbon dated individuals and multilocus microsatellite profiles from 74 of these. The 74 samples are from four fossil deposits (Pyramid Valley; Bell Hill Vineyard; Glencrieff; Glenmark) within a 10 km radius in North Canterbury, South Island, New Zealand (Figure 1).

Briefly, left tibiotarsi of moa were sampled by 'coring' at mid-shaft: 200 mg of bone material was powdered and incubated overnight in a buffer (EDTA, proteinase K, dithiothreitol and Triton-X). The DNA was then isolated and purified using Qiagen (Valencia, CA, USA) silica spin columns. A mtDNA control region sequence of 340 bp (excluding primers) was amplified from 87 individuals using two sets of overlapping primers (Bunce *et al.*, 2003). The associated GenBank accession numbers for the individual mtDNA sequences are presented in Allentoft *et al.* (2014). Six moa microsatellite markers were amplified successfully in 74 of the 87 individuals using rigorous methods for generating high-quality microsatellite data from ancient DNA as outlined previously (Allentoft *et al.*, 2011).

ML-RELATE

Two methods were used to identify relatives in the data: (i) a maximum likelihood-based approach, as implemented in the software ML-RELATE (Kalinowski *et al.*, 2006) and (ii) a more qualitative *ad hoc* assessment of observed and simulated relatedness indices (r_{XY}), combined with radiocarbon dates and mtDNA control region haplotypes.

ML-RELATE applies a maximum likelihood method based on relatedness values to estimate if each pair of individuals (dyads) have a higher probability of being unrelated, full siblings, half siblings or parent–offspring (Kalinowski *et al.*, 2006). The confidence intervals for all pairwise relationships among the 74 individuals were assessed according to a 95% significance level and 10 000 permutations.

Simulating relatives

In the second method, pairwise r_{XY} values (Queller and Goodnight (1989), adapted by Oliehoek *et al.* (2006)) were calculated between all dyads using the R package DEMERELATE (Kraemer and Gerlach, 2013). Closely related moa individuals should show high allele sharing and similar geological ages (calibrated ^{14}C ages), because each passing generation of random mating reduces the relatedness of individuals on the same genealogical branch by half. To examine this relationship we plotted the r_{XY} value for each dyad against the number of years separating the two individuals.

To assess the significance of the r_{XY} values in our observed data, we simulated 10 000 random offspring dyads of each of the relatedness classes 'non-related', 'half siblings', and 'full siblings' and calculated the r_{XY} distribution in each class (Supplementary Figure S1). The 95% quantile for non-related individuals was 0.49, meaning that 95% of the dyads with no close family

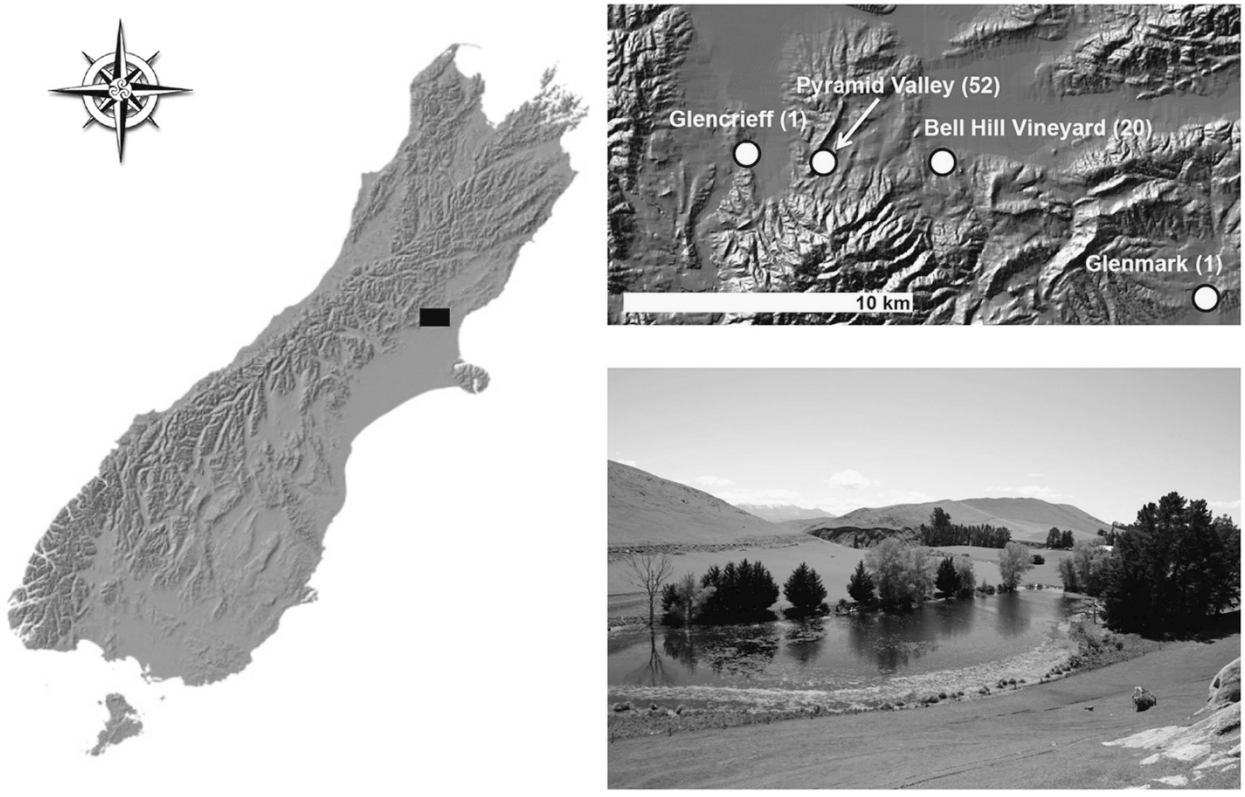

Figure 1 Sampling localities. Map of New Zealand's South Island with the sampling area in North Canterbury indicated. This study includes genetic data from four proximate fossil sites but all four identified dyads of related *Dinornis robustus* females were from Pyramid Valley (lower image), which has been excavated periodically since the 1930s. The exact locations at Pyramid Valley are shown in Supplementary Figure S4.

relations displayed r_{XY} values below 0.49. The corresponding 95% values for simulated dyads of half siblings and full siblings were 0.68 and 0.90, respectively (Supplementary Figure S1). We therefore conservatively selected an r_{XY} of 0.68 as a cutoff value for considering dyads in our empirical data set as potential relatives.

Temporal sampling simulations

As it is not known how the temporal structure of the sampling could influence the distribution of relatedness, we performed an additional series of simulations. These were conducted using FASTSIMCOAL2 (Excoffier *et al*, 2013) to emulate the empirical distribution of sampling times (that is, median-calibrated radiocarbon ages) from the moa population. We simulated data from six microsatellite loci under a random mating population with a constant effective population size (N_e) of 2000 individuals, which yielded an expected heterozygosity (H_E) and number of alleles very similar to the observed ones. This N_e was chosen based on opportunistic trial and error, as there is to our knowledge no way of analytically deriving the equilibrium theta under a generalised microsatellite mutation model (Fu and Chakraborty, 1998) such as that implemented in FASTSIMCOAL2. Note that these simulations were done using a constant-sized population at mutation-drift equilibrium, hence theta deviates slightly from that inferred in Allentoft *et al.* (2014) where a flexible demographic model was used. We performed 100 replicates of this simulation and calculated r_{XY} values as above. To compare the empirical r_{XY} values with this temporal null distribution of simulated relatedness, we calculated how many of the pseudo-observed dyads (99×2701 dyads in total) that showed an $r_{XY} > 0.68$. Moreover, we calculated how often this simulation would produce dyads with $r_{XY} > 0.68$ that were separated by 50 years or less ($dyads_{50}$ in the following), which could be argued as the realistic span of temporal separation for parent–offspring or siblings. Hence we could assess the significance of observing such dyads in our empirical data. We also plotted the distribution of r_{XY} values among the $dyads_{50}$ only.

Spatiotemporal sampling simulations

The presence of population subdivision (that is, spatial structure) in *D. robustus* could lead to elevated r_{XY} values in our spatially restricted sampling because we would tend to sample relatives more often than in a random mating, unstructured population of the same effective size. To assess the combined impact of population subdivision and temporal sampling, we performed another series of simulations in FASTSIMCOAL2 where we introduced an island-model type of structure in addition to the temporal sampling described above. We assumed ten demes each of $N_e = 200$, connected by three different levels of migration: high ($m = 0.05$), medium ($m = 0.005$) and low ($m = 0.0005$). We sampled just one of the ten demes to mimic spatially restricted sampling in a structured population. For each scenario we performed 100 replicate simulations and calculated the pairwise r_{XY} among all dyads in each replication.

mtDNA

We assessed the mtDNA similarity of the proposed relatives. Different mtDNA profiles would exclude a number of possible family relationships such as full siblings, half siblings related through the mother and mother–offspring. Conversely, identical haplotypes support kinship, but the strength of this as evidence depends on the frequency of the given haplotype in the population. On the basis of the 87 available mtDNA profiles (Allentoft *et al.*, 2014), we could estimate the haplotype frequencies in the *D. robustus* population and use this information in our evaluation. To visualise the genetic structure and diversity of the mtDNA sequences we generated a median-joining haplotype network with NETWORK v.4.5 (Fluxus-engineering.com; Bandelt *et al*, 1999).

RESULTS

Detailed descriptions of the genetic diversity and the radiocarbon ages have been presented previously (Allentoft *et al.*, 2012a, 2012b, 2014) and will be mentioned only briefly here. The total data set encompassed 74 individuals genotyped in six microsatellite markers, except for four individuals that could only be genotyped in five of the markers despite several attempts. The median calibrated radiocarbon ages of the 74 individuals profiled for microsatellites ranged from 4837 to 602 years before present (Allentoft *et al*, 2014).

Deviation from Hardy–Weinberg proportions ($\alpha = 0.05$) was observed in one marker but not after Bonferroni correction and no tests for linkage disequilibrium among loci were significant (Allentoft *et al.*, 2014). Moreover, we did not find evidence of scoring bias from null alleles, allelic drop-out, or stuttering in the data (Allentoft *et al.*, 2014). The Holocene *D. robustus* population of North Canterbury displayed an expected microsatellite heterozygosity (H_E) of 0.72 (see allele frequencies in Supplementary Table S1) and had 29 haplotypes in the targeted mtDNA control region (Allentoft *et al.*, 2014).

ML-RELATE identified a surprisingly high number of potential relatives in the sample. Of 2701 pairwise relationships tested among the 74 individuals, ML-RELATE estimated 551 family relations (71 full siblings, 344 half siblings and 136 parent–offspring relationships). The number seems unrealistically high, given the >4000 year timeframe covered by the data and the fact that many of these putative relatives were separated by hundreds of years. The average age separation between identified full siblings in ML-RELATE was 988 years, whereas the same numbers were 1019 years and 1021 years for parent–offspring and unrelated individuals, respectively. Although the numbers indicated a weak tendency towards a smaller average age separation in related individuals, it was not significant (*t*-test, $P = 0.50$). We conclude that ML-RELATE overestimated the number of family relations in the data, which is likely the result of the limited resolution power of the six microsatellite markers. When excluding all pairwise relations where the confidence sets included a status of 'unrelated', 113 dyads remained.

The simulation cutoff approach was more conservative in inferring possible relatives. Of the 2701 pairwise combinations (dyads) among the 74 individuals in our observed data set, only 13 had r_{XY} values exceeding the 0.68 cutoff for relatives obtained through simulations (Figure 2). Again, some of these putative relatives were separated by centuries or millennia (Figure 2), and assuming that the radiocarbon dates have normal levels of accuracy, these could not have been related and therefore constitue false positives. Four of the dyads (0.15%), however, clustered out with both high r_{XY} values (0.78–0.88) and similar calibrated radiocarbon ages (Figure 2, Table 1). Among the 124 dyads in which the two individuals were separated by <50 years of sampling time ($dyads_{50}$; Figure 2), these four dyads with high r_{XY} constitute 3.2%, which is ~22 times higher frequency than found across all dyads. The eight individuals involved were all adult females excavated from the same area of the Pyramid Valley site (Figure 1, Table 1, Supplementary Figure S4).

In the temporally simulated data, emulating a randomly mating population sampled through time, we observed 554 (0.2%) of 270 100 dyads across the 100 simulation runs that had r_{XY} values exceeding the 0.68 cutoff. However, only 21 (0.008%) of these were also $dyads_{50}$ and hence qualifying as potential relatives according to our criteria. This is ~19 times less than observed in the empirical data (0.15%). Equivalently, given the frequency of putative relatives in the simulated null distribution (0.008%), the binomial probability of observing four or more dyads of relatives among the 2701 observed dyads is just 0.008%. Hence our observed data are highly unlikely to have been drawn from a null distribution representing random mating. When focusing on the r_{XY} distribution among the 12 400 simulated $dyads_{50}$ there were only 21 (0.16%) with $r_{XY} > 0.68$, which is 20 times less than observed among our empirical $dyads_{50}$ (3.2%) (Supplementary Figure S2). Moreover, none of the 100 replicate simulation runs had more than two $dyads_{50}$ with $r_{XY} > 0.68$, and >80% of the simulations

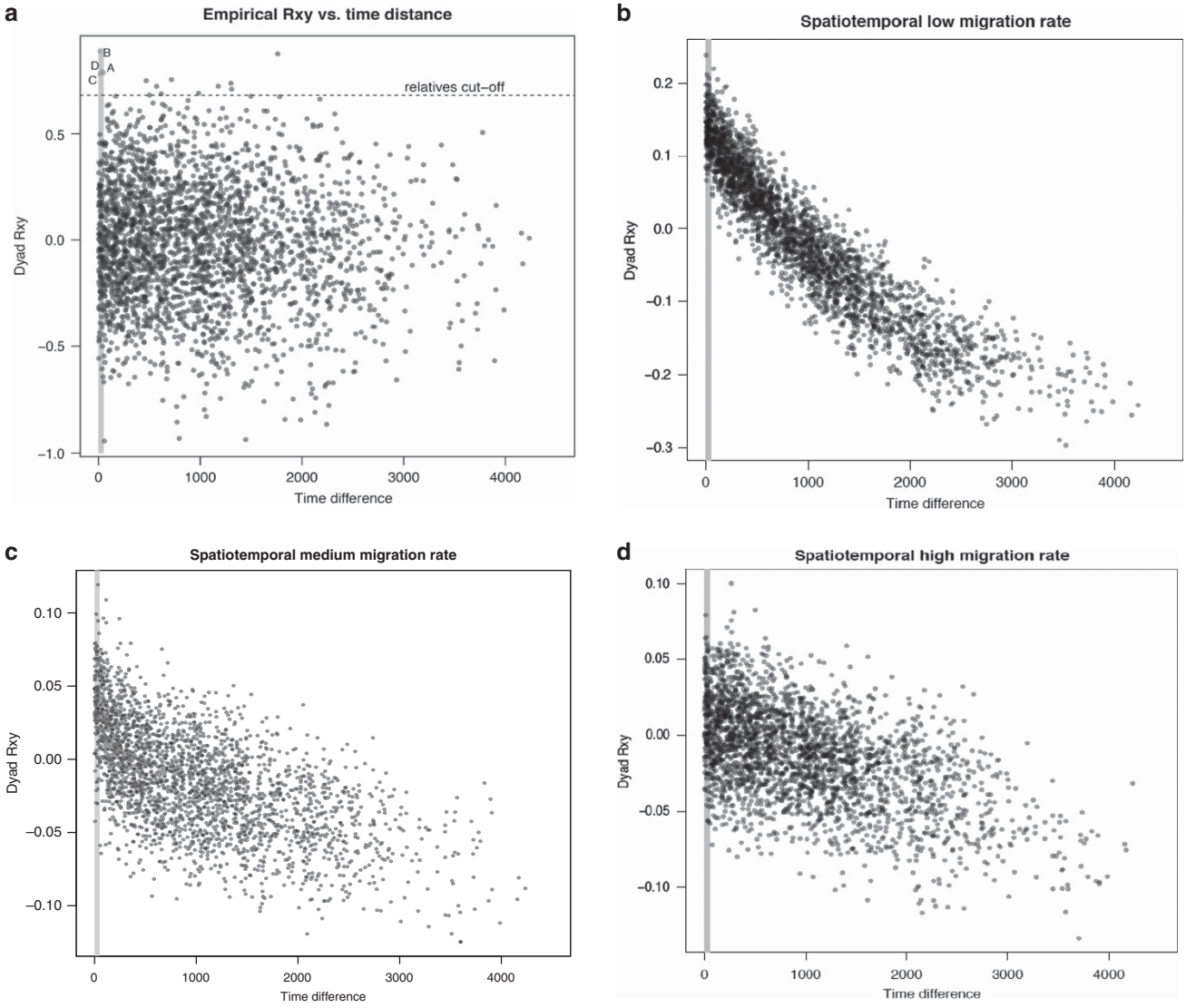

Figure 2 Relatedness. (**a**) Relatedness (r_{XY}) values between all 74 individuals (2701 dyads) plotted against their difference in radiocarbon age. The 95% upper boundary for simulated half siblings ($r_{XY}=0.68$) was used as cutoff for potential relatives. An age difference of 0–50 years is highlighted as it represents the realistic temporal gap between related individuals (parent–offspring or siblings). Four dyads containing putative relatives were identified with an r_{XY} >0.68 and within the 50-year-separation window. (**b**–**d**) r_{XY} Distributions for simulated spatio-temporal data sets based on three different migration rates.

Table 1 The four dyads of putative relatives

Catalogue #	r_{XY}	*Haplotype*	*Age*	*Dyads* r_{XY}> n	*Dyads*$_{50}$ r_{XY}> n	*Site*	*Sex*	*Maturity*
CM AV 8471	0.78	A: 4.6%	2406 BP	0.05%	0.03%	PV	F	VI
CM AV 8493			2357 BP			PV	F	VI
CM AV 8468	0.88	B: 4.7%	2331 BP	0.00%	0.02%	PV	F	VI
CM AV 8447			2318 BP			PV	F	VI
AMNH 7301	0.78	C: 2.7%	1326 BP	0.05%	0.03%	PV	F	VI
CM AV 8475			1313 BP			PV	F	VI
CM AV 8466	0.79	D: not shared	1766 BP	0.04%	0.02%	PV	F	V–VI
CM AV 8486			1801 BP			PV	F	VI

All eight individuals were adult females of maturity stage V or VI (Turvey and Holdaway, 2005) from the Pyramid Valley (PV) site. Museum catalogue numbers (CM, Canterbury Museum); r_{XY}, relatedness; mtDNA haplotypes (labelled as on Figure 3) shown with the frequency in the population; the median calibrated radiocarbon age (Allentoft *et al*, 2014); proportion of dyads in the temporal simulations displaying a higher r_{XY} than the observed putative relatives. This proportion is indicated for both the total simulated dataset and within the dyads$_{50}$ only.

had zero (Supplementary Figure S3). Again, this is in contrast to the four such dyads observed in the empirical data (Figure 2).

The spatiotemporal simulations did not produce more relatives according to the threshold criteria (Figure 2). In addition, the overall distribution of pairwise r_{XY} values differed considerably from our observed distribution (Figure 2). When structure was introduced in the simulations there was a clear negative correlation between r_{XY} and the temporal separation between two individuals in the sampled subpopulation. This is not surprising as allele sharing at any point in time is higher within than among demes in structured populations. This genetic similarity will, however, decrease with temporal separation because of genetic drift and because incoming migrants are likely to contribute new alleles from demes with different allelic frequencies.

mtDNA

In three of the four putatively related dyads, the two individuals shared the same mitochondrial haplotype. The frequencies of these haplotypes in the North Canterbury population of *D. robustus* were 0.046 (A), 0.047 (B) and 0.027 (C) (Figure 3, Table 1). The probability of randomly picking the same haplotype in two individuals of a panmictic population is equal to the haplotype frequency. Even allowing for sampling uncertainty in the estimate of the haplotype frequencies, the low frequencies of the shared mtDNA haplotypes in dyads A, B and C strongly support their family relationships. The two individuals in dyad D had different haplotypes that could be explained by the two individuals being related only through the father.

DISCUSSION

By combining three different sources of information represented by microsatellites, mtDNA and radiocarbon ages, we were able to identify related individuals among fossil moa bones that are thousands of years old. However, the resolution power of six microsatellite markers can be limited when determining relatedness. This is evident from the wide distribution of r_{XY} values in each category of simulated relatives (Supplementary Figure S1), and therefore we did not attempt to determine the exact degree of kinship for the putative relatives. Regardless, our temporal simulations demonstrated that related females were in excess in our empirical data set compared with a population with random mating, and we were able to reject population structure and the nature of our sampling as a potential driver for this observation. We therefore conclude that moa individuals of different social and relatedness classes were not randomly distributed in the landscape and that some behavioural aspect of moa biology must account for these results.

Other observations support this notion. For example, all four putative related dyads consisted of adult females that died in close proximity at Pyramid Valley (Table 1, Supplementary Figure S4). Although 60% of the samples were from females from Pyramid Valley, the naive probability of all four related dyads belonging to this class of individuals is only 1.5%. Also, Allentoft *et al.* (2010) showed a significant difference in the distribution of sexes and juveniles from the proximate fossil sites at Pyramid Valley and Bell Hill Vineyard (Figure 1). At Pyramid Valley, only two of 49 *D. robustus* individuals sampled were identified genetically as males and this extreme skew is likely a combination of higher mortality among males combined with behavioural differences or differential habitat preferences between the sexes (Allentoft *et al.*, 2010). The social behaviour in extant ratites varies greatly between taxa but in southern cassowaries (*Casuarius casuarius*), for example, males and females have separate territories (Moore, 2007), except for a short period when breeding. Ostriches (*Struthio camelus*) often form flocks with many females (related or not) and only one male. If adult female moa from each cohort were particularly attracted to the habitat around Pyramid Valley, whether foraging in groups of females or alone, it could explain the non-random relatedness distribution in our sample. It has previously been suggested that moa females maintained home ranges around the lakes, whereas males may have been more mobile (Allentoft *et al.*, 2010).

The social structure reflected in our data could not have been uncovered with mtDNA data alone. However, nuclear genetic data are challenging to obtain from ancient samples and for microsatellites there are fundamental issues with allelic dropout that need to be carefully assessed when working with degraded DNA (Navidi *et al.*, 1992; Taberlet *et al.*, 1996; Miller *et al.*, 2002; Roeder *et al.*, 2009; Allentoft *et al.*, 2011). In addition, most of the methods developed to

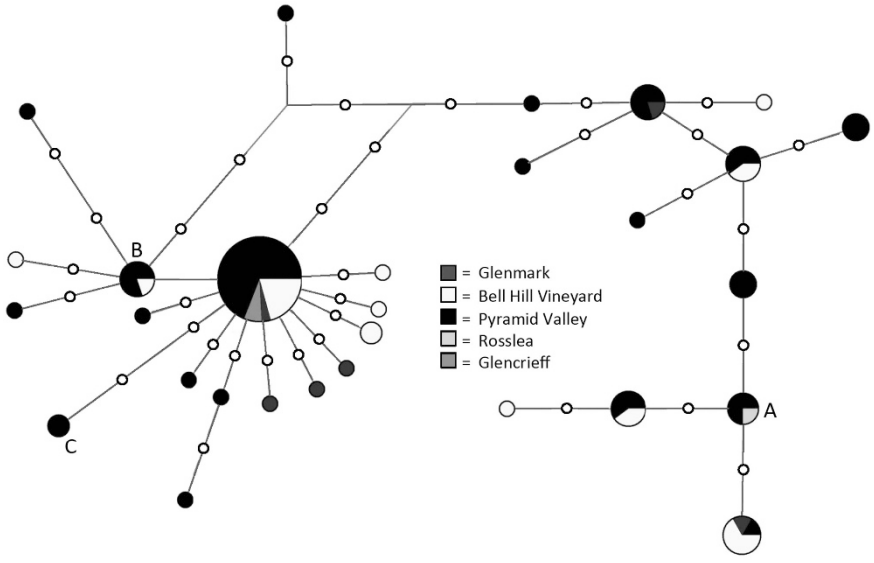

Figure 3 mtDNA haplotype network. Haplotype network based on ~340 bp of mtDNA control region from 87 *D. robustus* individuals. The colour composition is defined by the locality where each specimen was found. A, B and C mark the three rare haplotypes that are shared respectively within three of the four dyads with relatives (see Table 1 for details). Open circles reflect single mutations separating the haplotypes.

analyse microsatellite markers are not readily compatible with ancient data, which often represent individuals who lived at different points in time. Hence, our study has exploratory value in evaluating the kind of biological information that can be extracted from a highly heterochronous microsatellite data sets. Whereas the ML-RELATE method identified many false-positive relatives, the data simulation approach provided a more sensible null distribution of r_{XY} values for comparison with the observed distribution. This is likely because the simulation framework offers a possibility to mimic temporal sampling, which is crucial when the samples are fossils spanning several millennia. This also explains why data simulation approaches such as approximate Bayesian computation (ABC) are being increasingly applied in aDNA studies when past population dynamics are under scrutiny (Anderson *et al.*, 2005; Chan *et al.*, 2006; Bramanti *et al.*, 2009; Ramakrishnan and Hadly., 2009; Ghirotto *et al.*, 2010; Gamba *et al.*, 2012; Allentoft *et al.*, 2014).

We have here shown that data simulations can be used not only to recover large scale ancient population dynamics from aDNA data but also to investigate patterns of social structure and kinship in past populations. This implies in principle that palaeobiological research can explore an area of population genetics that has, until now, been restricted to extant species. However, it is clear that large assemblages of fossils with excellent biomolecule preservation are rare, and the comprehensive moa fossil record in New Zealand is arguably unique in terms of chronological precision (Holdaway *et al.*, 2014). But the methods we present are not only applicable to large assemblages of extinct megafauna with hundreds of associated radiocarbon dates. Microsatellites are often applied to measure changes in genetic diversity across centuries or decades in extant species, based on museum collections (Groombridge *et al.*, 2000; Whitehouse and Harley, 2001; Johnson *et al*, 2004; Harper *et al*, 2006; Petren *et al*, 2010; Bourke *et al*, 2010; White *et al*, 2014) and our simulation approach should be equally suitable to address question of kinship or social structure in such data sets. Our methodological framework could also be applied in association with aDNA recovered from human burials where the chronology is usually well established and where questions related to social structure and kinship are often of great interest to the archaeological community.

DATA ARCHIVING

As noted in the text, radiocarbon dates and Genbank accession numbers for all mtDNA sequences are provided elsewhere (Allentoft *et al.*, 2014). Microsatellite allele frequences are provided in the online Supplementary Table S1 and individual allele calls are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.hf876.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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