

ORIGINAL ARTICLE

# Influence of Holocene environmental change and anthropogenic impact on the diversity and distribution of roe deer

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Extant patterns of population structure and levels of diversity are a consequence of factors that vary in both space and time. Our objective in this study is to investigate a species that has responded to both natural and anthropogenic changes in ways that have shaped modern populations and provide insight into the key processes. The roe deer (*Capreolus capreolus*) is one of the two species of deer native to Britain. During the last glacial maximum (LGM), the British habitat was largely under ice and there was a land bridge to mainland Europe. As the Earth warmed during the early Holocene, the land bridge was lost. Subsequent hunting on the British mainland left the southern region extirpated of roe deer, whereas a refugial population remained in the north. Later reintroductions from Europe led to population expansion, especially in southern United Kingdom. Here, we combine data from ancient and modern DNA to track population dynamics and patterns of connectivity, and test hypotheses about the influence of natural and anthropogenic environmental change. We find that past expansion and divergence events coincided with a warming environment and the subsequent closure of the land bridge between Europe and the United Kingdom. We also find turnover in British roe deer haplotypes between the late-Holocene and modern day that have likely resulted from recent human disturbance activities such as habitat perturbation, overhunting and restocking. *Heredity* (2014) **112**, 607–615; doi:10.1038/hdy.2013.142; published online 22 January 2014

**Keywords:** *Capreolus capreolus*; ancient DNA; LGM; genetic diversity

## INTRODUCTION

Evolutionary process determines the level and structure of genetic diversity within and between natural populations. This is influenced by both spatial and temporal factors, and both adaptation and genetic drift. Understanding the relationship between specific pressures on populations and the consequences for evolutionary potential is a core objective of conservation genetics, as well as an essential aspect of understanding evolution. In this study we focus on how environmental factors (including those of anthropogenic origin) have contributed to the population dynamics and distribution of a widespread terrestrial mammal, and how these processes may have helped shape the level and pattern of genetic diversity in modern populations.

Our study species is a large mammal that is currently the most common and widespread cervid in temperate habitat across Europe, the European roe deer (*Capreolus capreolus*; see Andersen *et al.*, 1998). The European roe deer is well represented in the fossil record from the Middle to Late Pleistocene (Lister *et al.*, 1998; Sommer and Zachos, 2009), and have coexisted with many other large mammal species that perished around the Pleistocene/Holocene transition (for example, mammoth, cave bear, steppe bison, giant deer). During the last glacial maximum (LGM, 23 000–18 000 years before present (YBP); Kukla *et al.*, 2002), roe deer were likely forced into southern refugial populations along with other temperate species, later recolonising northern Europe following climatic warming and deglaciation.

Signatures of this past history are evident in the phylogeographic patterns of modern roe deer from across Europe (for review, see Sommer *et al.*, 2009). Genetic variation of the European roe deer divides into central, eastern and western lineages. The central lineage is widespread throughout Europe, whereas the eastern lineage is found mainly in Greece and Serbia and the western lineage is mainly in Spain and Portugal (see Lorenzini *et al.*, 2003; Randi *et al.*, 2004; Lorenzini and Lovari, 2006). In addition to these divisions, significant internal structuring has been detected in roe sampled from the Italian and Iberian peninsulas. This supports the existence of a subspecies in central–southern Italy (*C. c. italicus*) (Lorenzini *et al.*, 2002; Randi *et al.*, 2004) and an additional Celtic–Iberian group in central–southern Spain (Royo *et al.*, 2007). Taken together, these data likely reflect the existence of several glacial refugia (Sommer *et al.*, 2009). Following the end of the LGM, roe deer recolonised Britain via the expanse of land known as Doggerland that once provided a direct connection to continental Europe (Yalden, 1999).

Although climatic change has been a major force shaping the evolutionary history of the roe deer, in modern times the ever-growing impact of humans has also been significant (Baker and Hoelzel, 2013). Cervids across Europe have long been under the influence of humans through hunting, habitat modifications and restocking. In Britain, it was not until the last few centuries (fourteenth–eighteenth) that the impact of human activity through hunting and deforestation began to significantly affect populations of the British roe deer. These activities were believed to have caused

local extinction in most areas of southern United Kingdom (Ritson, 1933), and restricted roe populations to parts of Scotland and possibly some of the northern border English counties (Whitehead 1964). With the turn of the nineteenth century, large-scale replanting of woodland provided suitable habitat for remnant populations over much of the north to recolonise uninhabited areas (Taylor, 1948). In the south, reforestation helped facilitate the successful reintroduction of roe deer (using both native and nonnative stocks) across much of southern United Kingdom (Whitehead, 1964; Prior, 1995; Baker and Hoelzel, 2013). Reforestation and reintroductions have been so successful that roe deer populations are showing continual expansion and repopulation over much of their historic range (Whitehead, 1964; Ward, 2005). A recent study suggested that genetic structure and diversity of British roe deer populations has been strongly influenced by recent bottlenecks and restocking activity, as well as specific aspects of life history and behaviour (Baker and Hoelzel, 2013).

In this study we focus on the British population using both ancient and modern DNA, and integrate these data into published studies on roe deer genetic diversity across Europe. We test the hypothesis that historical founder and colonisation events (both natural and anthropogenic) were instrumental in defining modern patterns of diversity and population structure in this species. We also test the hypothesis that there was free movement across the land bridge at Doggerland until the land bridge was flooded, isolating two established populations either side of the British Channel. Our results provide inference about the role of historical demographic events on the modern genetic diversity of a widespread species, and reinforce earlier inference about the role of life history characteristics (Baker and Hoelzel, 2013).

## MATERIALS AND METHODS

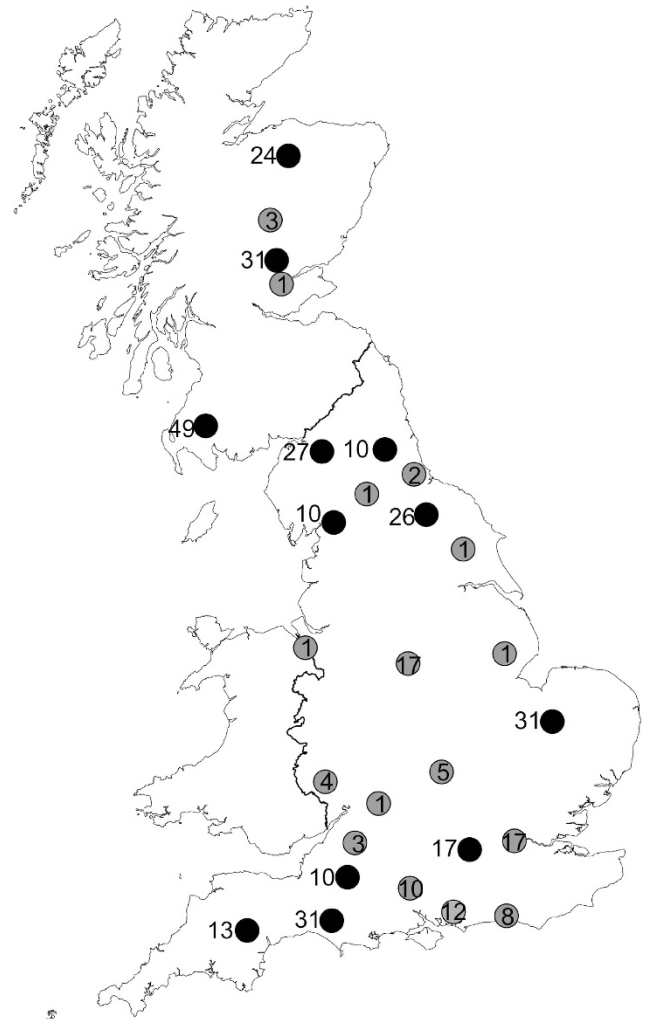
Ancient samples ( $N=140$ ) were collected from across the United Kingdom (Table 1 and Supplementary Table S1). Samples that showed amplification success are represented on a map of Britain ( $N=86$ ; Figure 1 and Supplementary Table S1). DNA was extracted from 0.05 g of bone powder using a QIAquick purification kit (Qiagen, Hilden, Germany) following the manufacturer's guidelines. Precautions to avoid contamination were taken during every stage of ancient DNA (aDNA) extraction and PCR set-up that took place in a separate laboratory dedicated to aDNA research free from contemporary DNA or PCR product. No laboratory materials or clothing were transferred from the postamplification rooms to the ancient laboratory. All work surfaces and equipment were thoroughly cleaned with 10% bleach (sodium hypochlorite) followed by 70% ethanol. Surfaces, equipment and solutions were also routinely exposed to ultraviolet light for at least 10 min. All extractions and PCR work were carried out in class II PCR hoods. Negative extraction and PCR controls (1 sample in every 5) were included to detect potential contamination in reagents and cross-contamination between samples. In addition, 50% of samples were replicated by extracting twice from independent samples of the same bone. In addition, all samples with unique aDNA haplotypes were repeated with an independent PCR amplification. DNA sequences (from both independent DNA extractions and PCRs) were considered authentic when independent replicates from the same individual yielded identical sequences. In rare cases when consistent differences between any replicates were detected, a third replicate was used to verify the actual nucleotide position.

Two overlapping fragments of the hypervariable section of the mitochondrial control region were designed to overlap with database modern sequences. The primer pairs were Roe\_1F: 5'-ATTATATGCCCCATGCTTAT-3' and Roe\_1R: 5'-CCTGAAGAAAGAACCAGATG-3'; Roe\_2F: 5'-AACCAAGAACTTACCAG-3' and Roe\_2R: 5'-GGGACATAATGTACTATG-3'. These primer pairs amplified fragments of 244 and 267 bp, respectively (including primers). PCR Reactions (25  $\mu$ l) contained 0.2  $\mu$ M  $\mu$ l<sup>-1</sup> each primer, 0.2 mM of each dNTP, Platinum 1X High Fidelity Buffer (60 mM Tris-SO<sub>4</sub>, pH 8.9/18 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Invitrogen)), 1.5 mM MgCl<sub>2</sub> and 1 unit of Taq High Fidelity DNA

**Table 1** Pairwise  $F_{ST}$ s (above diagonal) and  $\Phi_{ST}$  (below diagonal) for roe deer between European lineages, *C. c. Italicus*, the Celtic-Iberian group and ancient and modern UK populations for 388 bp of the mitochondrial DNA control region

|                           | 1           | 2           | 3           | 4           | 5           | 6           | 7           |
|---------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| 1. Central lineage        | 0           | <b>0.11</b> | <b>0.09</b> | <b>0.31</b> | <b>0.19</b> | <b>0.06</b> | <b>0.13</b> |
| 2. Western lineage        | <b>0.55</b> | 0           | <b>0.12</b> | <b>0.39</b> | <b>0.22</b> | <b>0.13</b> | <b>0.19</b> |
| 3. Eastern lineage        | <b>0.58</b> | <b>0.68</b> | 0           | <b>0.34</b> | <b>0.20</b> | <b>0.11</b> | <b>0.18</b> |
| 4. <i>C. c. Italicus</i>  | <b>0.39</b> | <b>0.75</b> | <b>0.65</b> | 0           | <b>0.50</b> | <b>0.39</b> | <b>0.40</b> |
| 5. Celtic-Iberian lineage | <b>0.51</b> | <b>0.73</b> | <b>0.52</b> | <b>0.83</b> | 0           | <b>0.22</b> | <b>0.28</b> |
| 6. Ancient UK             | <b>0.09</b> | <b>0.56</b> | <b>0.58</b> | <b>0.61</b> | <b>0.59</b> | 0           | <b>0.09</b> |
| 7. Modern UK              | <b>0.15</b> | <b>0.61</b> | <b>0.61</b> | <b>0.60</b> | <b>0.57</b> | <b>0.10</b> | 0           |

Values in bold indicate significance after Bonferroni correction.



**Figure 1** Map showing the locations from where modern (black circles) and ancient (grey circles) mtDNA sequences originated from. The sample numbers are shown either alongside or within each sample circle.

polymerase (Invitrogen). Then, 2  $\mu$ l of DNA template was added. Amplifications were performed with the following cycles: 95 °C for 5 min; 45 cycles at 94 °C for 45 s, 51 °C (roe\_1F, 1R) or 55 °C (roe\_2F, 2R) for 45 s and 68 °C for 45 s; 68 °C for 5 min. Amplicons were sequenced in both directions and PCR products were cleaned using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

### Phylogeography, diversity and expansion signals

Our aDNA sequence data were compared against three published modern data sets (Randi *et al.*, 2004; Royo *et al.*, 2007; Baker and Hoelzel, 2013). Details of the exact data used and corresponding accession numbers are provided in Supplementary Table S4. All sequences were aligned against each other using the programme CLUSTAL X (Larkin *et al.*, 2007). A 388-bp region of the consensus mitochondrial DNA (mtDNA) control region was used for subsequent analyses. The European data set was divided into the lineages eastern, western and central as well as the subspecies *C. c. italicus* (as previously described by Randi *et al.*, 2004) and the Celtic–Iberian group (as previously described by Royo *et al.*, 2007).

Summary statistics were calculated in DnaSP10.4.9 (Rozas *et al.*, 2003) for each of the lineages individually and combined, as well as for the ancient and modern UK samples individually and combined. The following statistics were computed: number of segregating (polymorphic) sites (*S*); number of unique haplotypes (*h*); haplotype diversity (*H*); average number of pairwise nucleotide differences (*k*); and nucleotide diversity ( $\pi$ ).

The relationship between European and UK populations (using both ancient and modern data) was investigated using a Median Joining Network constructed using the programme NETWORK 4.5 (<http://www.fluxus-engineering.com>), chosen to account for possible missing nodes and alternative connections (Bandelt *et al.*, 1999). The  $F_{ST}$  and  $\Phi_{ST}$  values were calculated using Arlequin v 3.0 (Excoffier *et al.*, 2005). Two neutrality tests were performed in DnaSP: Tajima's *D* (Tajima, 1989) and Fu's  $F_s$  (Fu, 1997). Mismatch distributions were also used to evaluate possible events of expansion and decline (Rogers and Harpending, 1992) using the sudden expansion model and goodness-of-fit tests (sum of squared deviation (SSD); Harpending's raggedness index *R*; Schneider and Excoffier, 1999). Tau ( $\tau$ ; calculated using ARLEQUIN 3.5) was used to estimate expansion time (*T*) using the equation:  $T = \tau/2\mu$  where  $\mu$  is the mutation rate in units of substitutions per locus per generation (Rogers and Harpending, 1992). The roe deer generation time was taken to be 3 years (after Randi *et al.*, 1998, 2004).

### Demographic analyses

Given that our UK ancient sample set reflected a substantial sample size, covered a time frame of over 5000 years and was less likely to be affected by continental introductions, all demographic analyses were based on just these samples. The programme BEAST v 1.4.8 (Drummond and Rambaut, 2007) was used to obtain substitution parameters (based on the ancient sample set) and explore past demographic change in roe deer. Substitution rates were estimated from temporally spaced sequence data (Drummond *et al.*, 2002) obtained by stratigraphic dating. For some samples, stratigraphic dates had wide date ranges and therefore an average date was used across all samples. All dates were provided as YBP. Input files were first generated with BEAUTi version 1.4.2. (Drummond and Rambaut, 2007).

Three independent Markov chain Monte Carlo runs of four chains each were run for 20 000 000 iterations, of which the first 10% were discarded as burn-in. Samples from two runs (that yielded similar results) were combined to estimate model parameters. Genealogies and model parameters were sampled every 2000 iterations. An explicit post-mortem damage model was incorporated into each run (Rambaut *et al.*, 2009) that takes into account the potential for sequence damage to influence the outcome of the aDNA analyses. Demographic inferences were essentially the same with or without the incorporation of the post-mortem damage model (details available from authors). A strict molecular clock model was applied. To determine the model of sequence evolution to use in this programme, a hierarchical likelihood test in Mr. MODELTEST 2.2 (Posada and Crandall, 1998) was performed. The substitution model chosen was Hasegawa, Kishino and Yano (HKY) (Hasegawa *et al.*, 1985). Independent runs were combined using Tracer 1.4 (Rambaut and Drummond, 2007) to generate credibility intervals that represent the coalescent model and phylogenetic uncertainty and to produce final estimates. For combined runs, effective sample sizes for each parameter exceeded 100, which indicated efficient mixing (that is, low autocorrelation in the Markov chain) and sufficient sampling of model parameters. The Bayesian skyline plot (BSP) demographic model was applied (Drummond *et al.*, 2005).

The programme isolation with migration (IM; Hey and Nielsen, 2004) was used to estimate divergence times between the ancient UK population and its

most closely related European populations. To minimise substructure within sample sets, the mainland European data set was restricted to sequences from central Europe (Germany and France), resulting in a comparison between 37 central European and 86 ancient UK sequences. The substitution rate estimated from BEAST was incorporated and the HKY model of evolution applied. Three runs were conducted using a two-step heating increment. Each Markov chain was run for 100 000 000 generations after discarding 10% burn-in. The first run was conducted to determine appropriate priors for subsequent runs; unrealistic upper bounds for priors were used in this preliminary run. Uninformative priors (that is, ranges that encompassed the entire posterior distributions) were then set for the final two runs. The final runs were conducted using identical conditions but different random number seeds to test whether multiple runs gave similar results. To ensure convergence, simulations were run until the smallest effective sample size estimates were at least 100 (Hey, 2005). Results from replicate runs did not differ, and hence data from the longer of the two runs are presented. The mode is reported along with the 95% highest posterior densities (HPDs; Hey and Nielsen, 2004).

### Direct comparison between ancient and modern populations

To compare phylogenies between modern and ancient haplotypes, median joining networks were created in NETWORK 4.5 (Bandelt *et al.*, 1999). Inferences about possible native and nonnative haplotypes were made by mapping haplotypes common to ancient and modern populations. To investigate relationships between ancient and modern populations, pairwise  $F_{ST}$  values (Weir and Cockerham, 1984) were calculated for mtDNA with 1000 permutations in Arlequin 3.0 (Excoffier *et al.*, 2005). For the calculation of  $F_{ST}$  values, the ancient population could only be divided as north and south because of restrictions of small sample sizes per area, whereas the modern populations were separated by area into six different populations. Bonferroni correction was applied to correct for type 1 error in multiple tests.

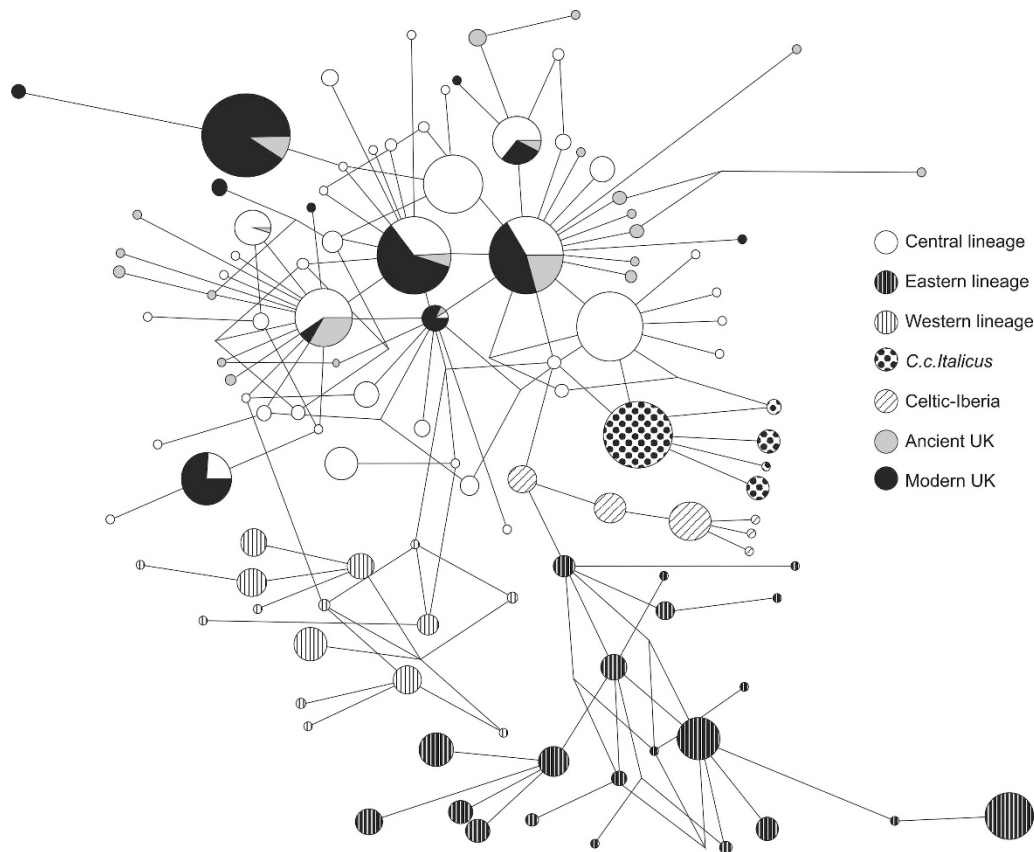
## RESULTS

### Phylogeography, genetic diversity and expansion signals

The data taken from Randi *et al.* (2004) and Royo *et al.* (2007) were divided into the following five separate groups (thought to reflect historical geographic refugial populations): an eastern, western, central, and Celtic–Iberian groups and the *C. c. italicus* subspecies. With the addition of UK samples, this structure was maintained (Figure 2). The inclusion of UK data increased the number of haplotypes found across Europe from 95 (see Royo *et al.*, 2007) to 115. Some of the haplotypes found in both ancient and modern UK populations were shared with the central European lineage, indicating a close association (Figure 2). The  $F_{ST}$  and  $\Phi_{ST}$  values were used to quantify the degree of differentiation among these lineages and in comparison with UK data (Table 1; Randi *et al.*, 2004).

Overall, levels of mtDNA diversity showed a wide range within Europe (see Table 2). The central European lineage had the highest levels of diversity and the subspecies *C. c. italicus* the lowest. Diversity in ancient UK populations was similar to the main European lineages, whereas modern UK populations were less diverse. Fu's  $F_s$  indicated a significant departure from neutrality, indicating expansion for the central lineage all European lineages combined with *C. c. italicus* and ancient UK and modern and ancient UK data combined (Table 2). The more conservative Tajima's *D* test indicated negative but nonsignificant deviations from neutrality for these same sample sets (Table 2).

The goodness-of-fit tests comparing an expansion model to the observed mismatch distributions revealed strong evidence for expansion for all European lineages combined with *C. c. italicus* (SSD = 0.003,  $P = 0.41$ ;  $R = 0.012$ ,  $P = 0.47$ ), the ancient UK population (SSD = 0.007,  $P = 0.68$ ;  $R = 0.036$ ,  $P = 0.34$ ), modern and ancient UK combined (SSD = 0.010,  $P = 0.28$ ;  $R = 0.035$ ,  $P = 0.46$ ) but not for the central lineage (SSD = 0.019,  $P = 0.00$ ;  $R = 0.089$ ,  $P = 0.00$ ).



**Figure 2** Median joining network (MJN) computed using 115 haplotypes from continental European data sets along with the ancient and modern UK data sets. Circle size is proportional to haplotype frequencies. The shaded circles represent the lineages, subspecies and groups formerly defined by Randi *et al.* (2004) and Royo *et al.* (2007). A full colour version of this figure is available at the *Heredity* journal online where the central lineage is yellow, eastern lineage is green, western lineage is blue, the subspecies *C. c. italicus* is white, and the Celtic-Iberian group is purple. For the UK populations from this study, ancient UK is grey and modern UK is black.

**Table 2** Population genetic summary and demographic statistics for European and UK populations

|   | <i>n</i> | <i>h</i> | <i>Hd</i> ± <i>s.d.</i> | $\pi$  | <i>k</i> | <i>F<sub>s</sub></i> | <i>P-value</i> | <i>D</i> | <i>P-value</i> |
|---|----------|----------|-------------------------|--------|----------|----------------------|----------------|----------|----------------|
| Central lineage                                 | 394      | 44       | 0.91 ± 0.0006           | 0.0075 | 2.98     | <b>-26.94</b>        | <b>0.000</b>   | -1.12    | >0.10          |
| Eastern Lineage                                 | 188      | 22       | 0.89 ± 0.0001           | 0.0080 | 3.20     | <b>-4.75</b>         | <b>0.05</b>    | -0.32    | >0.10          |
| Western lineage                                 | 90       | 15       | 0.87 ± 0.014            | 0.0068 | 2.63     | -3.07                | 0.06           | 0.13     | >0.10          |
| Celtic-Iberian lineage                          | 59       | 6        | 0.68 ± 0.035            | 0.0025 | 0.96     | -0.99                | 0.143          | -1.88    | >0.10          |
| <i>C. c. Italicus</i>                           | 105      | 5        | 0.36 ± 0.0570           | 0.0009 | 0.39     | -2.10                | 0.075          | -0.28    | >0.10          |
| All European lineages and <i>C. c. Italicus</i> | 836      | 92       | 0.962 ± 0.002           | 0.0130 | 4.86     | <b>-34.44</b>        | <b>0.000</b>   | -0.96    | >0.10          |
| Ancient UK                                      | 86       | 24       | 0.88 ± 0.0004           | 0.0069 | 2.76     | <b>-12.40</b>        | <b>0.000</b>   | -1.34    | >0.10          |
| Modern UK                                       | 279      | 12       | 0.76 ± 0.0003           | 0.0062 | 2.47     | 0.32                 | 0.131          | 0.43     | >0.10          |
| Ancient and Modern UK combined                  | 366      | 30       | 0.82 ± 0.0130           | 0.0067 | 2.64     | <b>-11.75</b>        | <b>0.000</b>   | -1.25    | >0.10          |

Abbreviations: *D*, Tajima's *D*; *F<sub>s</sub>*: Fu's *F<sub>s</sub>*; *h*, number of haplotypes; *Hd*, haplotype diversity (*s.d.*); *k*, average number of nucleotide differences; *n*, number of individuals;  $\pi$ , nucleotide diversity. Values in bold indicates significant values.

Mismatch analyses, from which  $\tau$  was calculated, are represented in Figures 3a–d. Possible expansion events occurred at  $\tau = 3.08$  (7014 YBP; 95% HPD 7919–3932YBP) for the central lineage and at  $\tau = 5.31$  (12 069 YBP; 95% HPD 19 479–4984) for all European lineages combined with *C. c. Italicus*. For ancient UK populations the data indicate a possible expansion event at  $\tau = 2.76$  (6274 YBP; 95% HPD 10 273–2072 YBP).

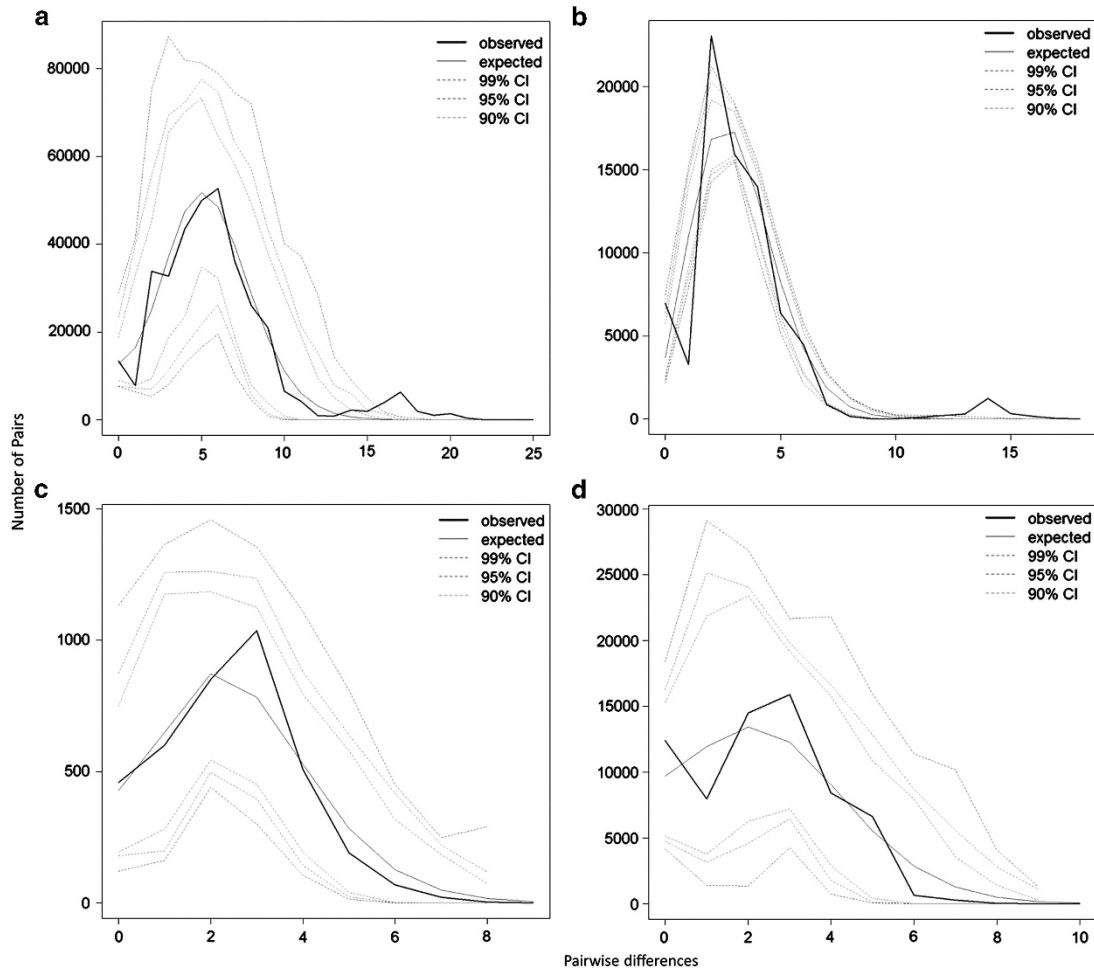
#### Demographic analyses

The substitution rate estimated by BSP in BEAST was  $3.69 \times 10^{-7}$  (95% HPD interval  $1.82 \times 10^{-7}$  to  $5.82 \times 10^{-7}$ ) substitutions per site

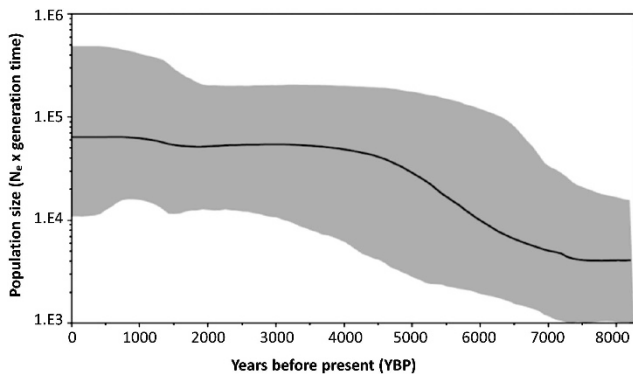
per year (Supplementary Figure S1). A coalescent reconstruction of past population dynamics (BSP) of British roe deer based on aDNA shows a rapid expansion in the effective number of roe deer between 5000 and 6000 YBP. After that time frame, roe deer numbers appear to remain relatively stable (Figure 4).

The divergence time for ancient UK and central Europe calculated in IMA is well resolved (Table 3 and Supplementary Figure S2), with a posterior distribution that has a distinct peak and bounds that fall within the prior distribution. Comparing ancient UK and the central European populations, the position of the peak indicates a population split at 5369 YBP (95% HPD 3317–12 901 YBP; Table 3).





**Figure 3** Mismatch distributions for mitochondrial DNA haplotypes sampled from the central lineage (a), all European lineages and *C. c. italicus* combined (b), ancient UK (c) and ancient and modern UK populations combined (d).



**Figure 4** A Bayesian skyline plot derived from ancient UK roe deer mtDNA D-loop sequences. The x axis is in units of years before present, and the y axis is equal to population size (the product of the effective population size and the generation length in years: 3). The black line is the median estimate, and the blue area shows the 95% highest posterior density intervals.

The effective population size for ancient United Kingdom is estimated to be largest, but central Europe and ancient United Kingdom are similar (with overlapping confidence interval ranges). The estimated rate of mtDNA gene flow into the United Kingdom

**Table 3** Maximum likelihood estimates and 95% highest posterior density (HPD) intervals (in parentheses) of isolation and migration model parameters and their respective demographic conversions for the UK population and central Europe

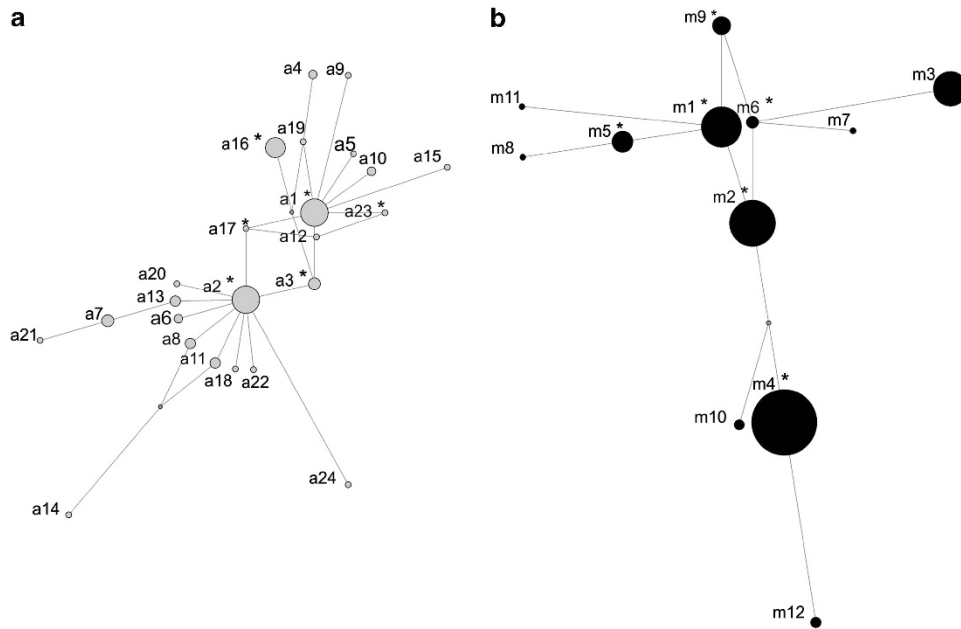
| Parameter                        | Ancient UK             | Central Europe       |
|----------------------------------|------------------------|----------------------|
| <i>t</i>                         | 0.84 (0.52–2.01)       |                      |
| <i>t</i>                         | 5368 (3317–12868)      |                      |
| <i>N<sub>e</sub></i>             | 32 676 (18 414–59 616) | 13 216 (4595–39 205) |
| <i>N<sub>e</sub></i> (ancestral) | 2313 (728–14 293)      |                      |
| <i>m</i>                         | 0.01 (0.01–1.07)       | 0.01 (0.03–4.05)     |
| <i>m</i>                         | 0.0047 (0.0047–0.500)  | 0.0047 (0.014–1.89)  |

The model parameters given in italics (*t* and *m*) are scaled by  $\mu$ . The demographic parameters (not italicised) are based on an estimate of  $\mu$  (see text) where  $N_e$  is effective population size, *t* is divergence time in years and *m* is average number of migrants per 1000 generations per gene copy.

and Europe is very small, suggesting that after separation the populations remained isolated.

#### Direct comparison between ancient and modern DNA

Between ancient and modern periods, haplotype number, haplotype diversity, nucleotide diversity and *k* decreased (Figure 5 and Table 2). We successfully sequenced 86 (61.4%) of the ancient UK samples



**Figure 5** A direct comparison of ancient (a) versus modern (b) median joining networks computed in NETWORK. In (a), grey circles indicate ancient haplotypes ( $n=24$ ; a1–24; see Supplementary Table S2). In (b), black circles indicate modern haplotypes ( $n=12$ ; m1–12; see Supplementary Table S3). For both networks, the asterisks (\*) denote those haplotypes that are common to both ancient and modern populations.

**Table 4** Pairwise  $F_{ST}$  values based on a portion of the mitochondrial DNA D-loop between UK roe from six contemporary populations and ancient north and south samples

|   | 1           | 2           | 3           | 4           | 5           | 6     | 7            |
|---|-------------|-------------|-------------|-------------|-------------|-------|--------------|
| 1. Norfolk                              |             |             |             |             |             |       |              |
| 2. Hamps, Somerset, Wilts and Berkshire | <b>0.88</b> |             |             |             |             |       |              |
| 3. North York, Durham and Carlisle      | <b>0.88</b> | <b>0.58</b> |             |             |             |       |              |
| 4. Perth and Moray                      | <b>0.79</b> | <b>0.34</b> | <b>0.52</b> |             |             |       |              |
| 5. Ayrshire                             | <b>0.89</b> | <b>0.63</b> | 0.007       | <b>0.56</b> |             |       |              |
| 6. Lancashire                           | <b>0.86</b> | <b>0.29</b> | <b>0.41</b> | <b>0.25</b> | <b>0.44</b> |       |              |
| 7. Ancient south                        | <b>0.66</b> | <b>0.24</b> | <b>0.51</b> | <b>0.10</b> | <b>0.53</b> | 0.08  |              |
| 8. Ancient north                        | <b>0.79</b> | <b>0.26</b> | <b>0.2</b>  | <b>0.17</b> | <b>0.25</b> | 0.017 | <b>0.167</b> |

Values in bold indicate significance after Bonferroni adjustment.

(see Table 1 and Supplementary Table S1, for details) from which we identified 24 haplotypes ( $h/N=0.279$ ). From our 279 modern UK samples, 12 haplotypes were detected (Figure 5 and Table 2;  $h/N=0.043$ ). Six haplotypes were common to both ancient and modern populations and most of these occurred in northern United Kingdom. Some haplotypes common to both time points were sampled at very low frequencies and found in isolated populations (for example, m7, m8 and m11). Six haplotypes, unique only to modern populations, may represent haplotypes that have either gone undetected in historical populations or that have been introduced from nonnative locations. One population, which likely reflects the latter scenario, is that of Norfolk, which is known to be a site of nonnative reintroduction, and distinctly exhibits the single unique haplotype m3 (Figure 5b and Table 3). Genetic differentiation between all ancient and UK contemporary populations was significant based on mitochondrial data (Table 4). The most closely related contemporary population to ancient UK populations was Lancashire based on mitochondrial data (the only nonsignificant difference). In general, mitochondrial data showed that contemporary populations

found in the north were more closely related to ancient UK populations than those found in the south. Specifically, the southern population of Norfolk was most distantly related to ancient populations.

## DISCUSSION

### Phylogeography, genetic diversity and expansion signals

Based on a 750-bp region of the mtDNA, Randi *et al.* (2004) identified the existence of three main roe deer lineages in Europe; central, eastern and western together with the subspecies *C. c. italicus*, which each likely represent independent glacial refugia. Royo *et al.* (2007) later described the existence of a Celtic–Iberian group. When the UK data were combined with these European data sets, and a reduced consensus region (388 bp) examined, the same structure was maintained (Figure 2). The inclusion of the UK ancient and modern genetic data revealed that 22 haplotypes were unique to Britain. The finding that all UK haplotypes cluster with the central lineage strongly supports its origins from this lineage (see Table 1 and Figure 2). Colonisation from northern Europe near the land

bridge, where the central lineage is common, would be expected based on geographic proximity.

The location of the refuge from which the central lineage originated is still unknown, although a Carpathian or further eastern origin is supported by recent molecular and fossil data (see Randi *et al.*, 2004; Lorenzini and Lovari, 2006; Sommer and Zachos, 2009). A colonisation route from this area would correlate well with patterns of broad-leaf forest expansion (Petit *et al.*, 2003) that is the preferred habitat of the roe deer (Putman and Langbein, 2003). Our comparisons shown in Table 1 support the affinity between the UK samples and the central lineage illustrated in the network (Figure 2), and this seems strongest for the ancient sample set. A stronger association between the ancient UK and modern European sample sets may reflect the greater inclusion of samples from translocated and bottlenecked populations among the modern UK samples. Although studies based exclusively on mtDNA data run the risk of misinterpretation because of looking only at matriline history or only a single gene tree, roe deer comparative analyses tend to show good agreement between nuclear and mtDNA indications of population structure, as seen both on a fine geographic scale in the United Kingdom (Baker and Hoelzel, 2013) and on a broader European scale (Randi *et al.*, 2004; Lorenzini and Lovari, 2006; Sommer and Zachos, 2009). As of yet, there are no nuclear DNA-based studies that compare roe deer populations in the United Kingdom with European populations.

Both neutrality tests and mismatch distributions suggested strong expansion events for all European lineages combined, the ancient UK sample alone, and the ancient and modern UK samples combined. Calculating tau ( $\tau$ ) from the mismatch distributions and using the substitution rate calculated in BEAST, there was evidence of expansion at 13 500 YBP for the combined European lineage, and 6300 YBP for the UK population. The expansion date for the UK population was consistent with the BSP graph that showed that UK roe populations expanded over a similar timescale (Figure 4).

Mismatch analyses detected possible expansion events (or selective sweeps) for roe deer following the LGM (see Figure 3). The LGM (23 000–18 000 YBP; Kukla *et al.*, 2002) confined roe and other temperate species to separate southern glacial refugia. This was because of the permafrost and Arctic tundra ecosystems that were widespread in central Europe down to a latitude of 45° (Andersen and Borns, 1997). Following the LGM, species were able to recolonise by expansion into formerly glaciated regions. According to the fossil record, it was not until the period of warming, between 14 700 and 11 600 YBP, that roe deer were able to rapidly expand into much of Europe (Sommer *et al.*, 2009; Sommer and Zachos, 2009). This is consistent with the estimated expansion date for the European lineages (12 000 YBP; Figure 3).

For northern European lowlands (such as the United Kingdom), fossil evidence suggests that roe did not recolonise until the early Holocene (Sommer and Zachos, 2009). For the United Kingdom, the earliest evidence for postglacial recolonisation originates from Thatcham in southern England where bones were radiocarbon dated to 9439 ± 100 YBP (Sommer *et al.*, 2009). During this time, the central European lowlands were apparently being slowly recolonised by birch and pine woods (Usinger, 2004) that would have in turn improved the environmental conditions for roe deer. However, it was not until 6000 YBP that the vegetation pattern broadly resembled that of today (Hewitt, 1999), and the expansion signal for ancient UK roe populations at 6300 YBP (Figure 3) may have been a response to the improved environmental conditions.

Taken together, our results indicate that new habitat was quickly exploited by expanding roe deer populations following the end of the last glaciation. Randi *et al.* (2004) previously proposed two expansion events for continental European populations (based on 750-bp segments of the mtDNA control region), scaling values of  $\tau$  from their mismatch analyses with a 'phylogenetic rate' of 4–6% per million years. The resulting expansion times were estimated to have coincided with the penultimate (*c.* 250 Ka) and the last (*c.* 130 Ka) interglacials. Fossil evidence suggests that roe deer have been present in Europe through at least 600 000 years, since the Middle Pleistocene (Lister *et al.*, 1998). Using the same data but with the new substitution rate calculated in this study (37% per million years), it is estimated that expansions instead occurred at 13 300 (HPD; 8400–22 900) YBP and 8400 YBP (HPD; 5300–17 100), respectively. Both of these expansion dates are consistent with the European expansion signals proposed above, based on the same data, but with a reduced sequence length (388 bp).

The results of this study are consistent with a number of recent studies that have replaced 'phylogenetic rates' with substitution rates directly calibrated using aDNA (Ho *et al.*, 2005), more relevant to the shorter time frame of the Holocene. For example, the substitution rate from our study ( $3.69 \times 10^{-7}$  s.s.yr<sup>-1</sup>) is similar to that for the ancient brown bear (Saarma *et al.*, 2007) and bison (Shapiro *et al.*, 2004) ( $3.2 \times 10^{-7}$  and  $3.0 \times 10^{-7}$  s.s.yr<sup>-1</sup>, respectively). In fact, reviews show broad consistency among a wide range of studies (Ho *et al.*, 2005, 2008). It is unlikely that all aDNA data sets are confounded by error, as these errors would need to have been made systematically and substantially (Ho *et al.*, 2007). The use of the roe deer substitution rate that we calculated suggested that divergence and population expansions occur over much shorter timescales than previously proposed. As for previous studies, the more recent dates (based on higher substitution rates) are consistent with expectations based on historical environmental events (see de Bruyn *et al.*, 2011). For the roe deer in Europe, the expansion date is consistent with expected postglacial expansions. For the UK sample, it is consistent with the separation of the United Kingdom from the European landmass.

### Demographic analyses

For the UK sample, expansion signals from IM, mismatch distributions and the BSP plot are around the time of the separation of the United Kingdom from the European landmass. When the Scandinavian and British ice sheets reached their maximum extent, and the North Sea as a consequence receded to its lowest level, Britain was connected to the continent by a land bridge (Fairbanks, 1989). This dry land, referred to as Doggerland, would have allowed roe deer to migrate from central Europe. Doggerland likely existed between 8000 (Shennan *et al.*, 2000; Sturt *et al.*, 2013) and 5800 YBP (van der Molen and van Dijkstra, 2000) when its loss resulted from warming, unlocking large quantities of water from ice caps and causing sea levels to rise. A channel restricting movement likely developed early in this period. The split may have coincided with increasingly suitable habitat in the United Kingdom, resulting in expansion of a population recently isolated from the source population in Europe (Sommer and Zachos, 2009).

### Direct comparison between ancient and modern DNA

Direct comparisons between ancient and modern populations in the United Kingdom revealed an overall loss in genetic variation (see Table 2 and Figure 5) that could be attributed to a recent period of bottlenecking caused by overhunting and deforestation between the

late fourteenth and eighteenth centuries (Whitehead, 1964), followed by the establishment of new populations based on small founder groups. Conservation biology is often concerned with preserving native populations to secure large-scale genetic diversity and preserve possible local adaptations (Nielsen *et al.*, 1999). In this respect, it appears that populations in the most northern parts of the United Kingdom (for example, Scotland; Perth/Moray/Glasgow) would be most important to conserve. These populations have retained high number of 'native' haplotypes (that is, those present in historical populations; see Figure 5 and Supplementary Table S3), show a close relationship with historical populations (Table 4) and exhibit high levels of both microsatellite DNA and mtDNA variability relative to other populations (see Baker and Hoelzel, 2013).

This result is concordant with the historical record that suggested that medieval bottlenecking was less intense in Scotland. For example, Whitehead (1964) claimed that roe never went extinct in this region and may have retained appreciable numbers. The other northern populations, such as those in northern England (for example, Carlisle, Durham, North York, Lancashire), appear to be primarily native, but lower representative numbers of haplotypes (see Supplementary Table S3) may reflect near extinction or extinction in these areas followed by later re-establishment through expansion (see Baker and Hoelzel, 2013). This inference is also supported by the historical record (Bewick, 1790). Other areas of the United Kingdom that appear to harbour populations based on native stock include extant southern populations (for example, Dorset, Wiltshire, Somerset, Berks) that were founded from stock translocated from Scotland (Whitehead, 1964) after the medieval bottleneck that extirpated the original southern populations. The genetic record is concordant with this scenario, as it appears that large losses of haplotypes between ancient and modern periods have occurred in the south (see Supplementary Tables S2 and S3) and that the three native haplotypes currently present in these southern populations (m1, m2 and m4; see Figure 4) are also common to Scotland (see Supplementary Table S3).

For other areas of the United Kingdom, it appears that populations have experienced some level of influence of restocking involving nonnative individuals. The Lancashire population was characterised by three native haplotypes (m1, m4, m6; see Supplementary Table S3) and one potentially nonnative introduced haplotype (that is, absent from ancient populations; see Figure 4). The latter nonnative haplotype may reflect the relicts of an introduction event that occurred when 12 Austrian roe were introduced into this population in 1913 to 'improve the local breed' (Whitehead, 1964; Prior, 1995), although it is also possible that it was present in the ancient population, but not detected. There was evidence for complete lineage replacement in Norfolk, a finding that is consistent with the records of the human translocation of nonnative (German) stock into this area (see Whitehead, 1964). This was supported by the findings that a single, novel haplotype unique to this location (m3; Figure 3) was detected and that this population exhibited the highest levels of differentiation (based on both mitochondrial (see Table 4) and microsatellite  $F_{ST}$  values; see Baker and Hoelzel, 2013) when compared with ancient and other contemporary UK populations.

## CONCLUSION

This study has used ancient and modern DNA to provide otherwise intractable information on the evolutionary events that have shaped European roe deer during its recent postglacial history, including direct data on the impact of a major vicariance event (the closing of the land bridge). Recolonisation of the roe deer across Europe seems to have occurred very rapidly as environmental conditions improved

following the end of the LGM, and our data on this process agree well with inference from the fossil records. Shortly after, roe deer recolonised the United Kingdom but populations became isolated as the land bridge was cut from mainland Europe. Our data provide a plausible time frame for when this occurred, and suggest a postisolation expansion. More recent anthropogenic events led to a pattern of historical bottlenecks and reintroductions, and for these events the genetic data track well with the historical data. Since establishment during the Holocene, British roe deer populations have evolved considerable genetic structure (see Baker and Hoelzel, 2013), reflecting processes associated with both natural environmental and anthropogenic changes. Understanding the integration of specific responses to climatic and anthropogenic change in species of conservation concern can help predict future patterns of diversity (Hadly and Barnosky, 2009) that may be fundamental to long-term conservation and species management planning (Leonard, 2008).

## DATA ARCHIVING

Sequence data have been submitted to GenBank: accession numbers JX971589–JX971615; KF961012–KF961035.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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