Mitochondrial Genomes of Giant Deers Suggest their Late Survival in Central Europe

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The giant deer Megaloceros giganteus is among the most fascinating Late Pleistocene Eurasian megafauna that became extinct at the end of the last ice age. Important questions persist regarding its phylogenetic relationship to contemporary taxa and the reasons for its extinction. We analyzed two large ancient cervid bone fragments recovered from cave sites in the Swabian Jura (Baden-Württemberg, Germany) dated to 12,000 years ago. Using hybridization capture in combination with next generation sequencing, we were able to reconstruct nearly complete mitochondrial genomes from both specimens. Both mtDNAs cluster phylogenetically with fallow deer and show high similarity to previously studied partial Megaloceros giganteus DNA from Kamyshlov in western Siberia and Killavullen in Ireland. The unexpected presence of Megaloceros giganteus in Southern Germany after the Ice Age suggests a later survival in Central Europe than previously proposed. The complete mtDNAs provide strong phylogenetic support for a Dama-Megaloceros clade. Furthermore, isotope analyses support an increasing competition between giant deer, red deer, and reindeer after the Last Glacial Maximum, which might have contributed to the extinction of Megaloceros in Central Europe.

The extinct giant deer Megaloceros giganteus (also Irish Elk), first described by Blumenbach in 1799¹, stands out amongst the Pleistocene megafauna not only due to its sheer body size, but also because of its immense antlers, which spanned up to 4 m in diameter and weighted up to 45 kg². Appearing in the fossil record around 400,000 years ago (ya)³, its populations are thought to have ranged from Ireland to Lake Baikal⁴. Many theories have been proposed to account for its pattern of distribution across Eurasia in the Late Pleistocene and its extinction in the early Holocene. One unresolved question concerns the reason for the absence of giant deer during the Last Glacial Maximum (LGM, 20,000 – 12,500 ya) in Western and Central Europe, implying that these species had completely withdrawn from the region⁵. Before its purported extinction ca. 6,900 ya in western Siberia, Megaloceros recolonized northwestern Europe in the Late Glacial Interstadial⁶; however, no evidence has thus far been found for the presence of giant deer in Southern- and Central Europe.

The phylogenetic position of Megaloceros within the family Cervidae is still debated. The presence of large palmate antlers in extant fallow deer (Dama dama) and Megaloceros giganteus suggests a close relationship between the two species⁶,⁷, whereas postcranial skeletal characters place Megaloceros into a group comprising red deer (Cervus elaphus), cheetal (Axis), and bush antlered deer (Eucladoceros)⁸.

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Previous genetic studies on short regions of mitochondrial DNA (mtDNA) provide evidence for a closer relationship to fallow deer\cite{9,10}; however, the statistical support for the Dama - Megaloceros clade is low, likely due to the partial mtDNA regions studied and the limited availability of modern cervid mtDNA for comparison\cite{11,12}. Here we present two reconstructed nearly complete mitochondrial genomes of large cervid bone fragments found in two cave sites in Southern Germany dating to the late glacial period (ca. 12,000 ya). Phylogenetic analyses reveal that both specimens derive from *Megaloceros giganteus*. Comparison against mtDNAs from 44 extant deer species provides furthermore strong support for a Dama - Megaloceros mtDNA clade.

**Results and Discussion**

**Morphological Analyses and Dating.** During an excavation at the Hohlenstein Stadel cave at the Lone Valley (Baden-Württemberg, Southwestern Germany), an accumulation of large cervid bones was discovered including an almost complete atlas, two scapulae and two pelvic fragments, two ribs, a tooth (M3) and six fragments of a tibia. Apart from these finds, a metatarsus shaft fragment from a large cervid was obtained from the Hohle Fels cave at Schelkingen (Baden-Württemberg, Southwestern Germany). We genetically analyzed one of the six tibia fragments (ST/213/203/144, Fig. 1a) recovered from Hohlenstein Stadel cave, dated to 12,175 ± 50 uncal ya (ETH-41223), and the metatarsus fragment (HF/65/100, Fig. 1b) from Hohle Fels cave, which was dated to 12,370 ± 30 uncal ya (MAMS-16557). Radiocarbon ages were calibrated based on IntCal13 curve and calculated using the Calib 7.0 program.

**mtDNA assembly.** In order to reconstruct the mitochondrial genomes of our ancient cervid specimens, we isolated the total DNA, turned it into DNA sequencing libraries and enriched for mtDNA using bait generated from modern roe deer with specific primers (table 1). Illumina sequencing on a HiSeq2500 produced 944,648 and 6,123,389 merged reads, for Hohlenstein Stadel and Hohle Fels, respectively. These reads were mapped to the mitochondrial reference sequence of a roe deer (NC_020684.1) and a fallow deer (JN632629.1). Consensus sequences were generated for each sample based on at least 3-fold coverage. Both ancient samples produced identical consensus sequences for overlapping regions, demonstrating mitochondrial similarity. However, using fallow deer\cite{13} as a mapping reference, created an almost complete mtDNA sequence (91.52% for Hohlenstein Stadel and 99.99% for Hohle Fels), with 7634 unique mapping fragments for the Hohlenstein Stadel sample and 1,009,775 unique mapping fragments for Hohle Fels (table 2). The consensus sequence generated from positions with at least 3-fold coverage would reveal the phylogenetic relationships between the ancient and extant cervids, providing insights into the evolutionary history of the species.
DNA damage patterns. To authenticate the sequenced fragments as ancient, the frequency of terminal substitutions was analyzed. It has been suggested that C to T substitutions at the 5’ end and G to A substitutions at the 3’ end are likely caused by deamination of cytosine causing miscoding lesions; these accumulate over time, and hence are characteristic of ancient DNA\(^{14}\). DNA fragments with frequencies of at least 20% for both types of substitutions at the 5’ and 3’ end can be regarded as authentic ancient DNA\(^{13}\). We observed a substitution frequency of 27% for C to T changes at the 5’ ends and at least 20% for both types of substitutions at the 5’ and 3’ ends can be regarded as authentic ancient astragalus from Killavullen in Ireland\(^{10}\). However, 7 bases in the Hohlenstein Stadel M. giganteus from a sequence, average mitochondrial genome coverage, average read length, and frequency of C to T substitutions at 5’ end.

Phylogenetic Analysis. The reconstructed mtDNAs from both ancient cervid bones were aligned with 44 publicly available full mitochondrial genomes of extant cervids. The hypervariable D-loop region was excluded from the cervid mtDNA alignment due to its fast evolutionary rate that may decrease the phylogenetic resolution. We used MEGA 6.0.6 to construct both a maximum-likelihood (ML) tree (Fig. 3a) and a maximum-parsimony (MP) tree (Fig. 3b) and robustness of both methods was tested with 1000 bootstrap replicates. The best-fit substitution model for ML was identified with MEGA 6.0.6 to be the General Time Reversible (GTR+G+I) model (BIC score = 215112.733). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3366)). The rate variation model allowed for sites to be evolutionarily invariable ([+I]). Both tree reconstructions were based on a total of 14,147 positions. Alpine musk deer (Moschus chrysogaster, KC425457.1) was chosen as an outgroup. Both ML and MP topologies place the mtDNA sequences reconstructed from our two ancient cervid bones (Hohlenstein Stadel and Hohle Fels) in a completely resolved clade with both extant fallow deer subspecies (Dama dama and Dama mesopotamica), and exclude them from the group comprising Pere David’s Deer (Elaphurus davidianus), Rusa sp. and red deer (Cervus elaphus sp). The other large cervid present in Europe during the Pleistocene, the European elk, Alces alces, can also be excluded as the source of our ancient cervid bones.

The relationship of our ancient cervids to both Dama species within the Dama clade is, however, not completely resolved. As Dama was only introduced to Europe in the Medieval period\(^{16}\) and is absent in the palaeontological record of Western Eurasia, and since both ancient cervid bones derive morphologically from a large cervid, which is not elk (Alces alces) based on phylogenetic evidence, we conclude that both specimens originate from Megaloceros giganteus. To test this hypothesis we further compared our reconstructed ancient cervid mtDNAs to cytochrome b (cytb) regions of Megaloceros mtDNA previously published (table 3). We observe 1 nucleotide difference (mismatch) for the Hohlenstein Stadel sample compared to the previously published cytb sequence from a complete M. giganteus skeleton from the Kamyshlov site in western Siberia\(^9\) and no nucleotide differences compared to the cytb sequence obtained from a M. giganteus astragalus from Killavullen in Ireland\(^{10}\). However, 7 bases in the Hohlenstein Stadel cytb sequence could not be resolved because the coverage was too low at those positions. We find only 1 mismatch between the Hohle Fels sample and each of the previously published M. giganteus cytb sequences. For the same region we find 89 mismatches between the Hohlenstein Stadel sample and fallow deer\(^9\) and 87 mismatches compared to red deer\(^{17}\), whereas for the Hohle Fels sample we observe 104 mismatches compared to fallow deer and 97 mismatches to red deer, respectively.

Table 2. Mapping results for the Hohlenstein Stadel sample (ST/213/203/144) and the Hohle Fels sample (HF/65/100). EB: Extraction blank, LB: library blank. Columns from left to right: Sample, calibrated radiocarbon date, number of merged reads, number of unique mapped reads to the fallow deer mtDNA sequence, average mitochondrial genome coverage, average read length, and frequency of C to T substitutions at 5’ end.
The small nucleotide distance of our ancient large cervids and both previously determined giant deer cytb sequences confirm the attribution of our samples to *Megaloceros giganteus*. The close genetic relationship between the large cervid bone from Hohle Fels and the giant deer skeleton from Kamyshlov in western Siberia (AM072744.1) and the giant deer astragalus from Killavullen in Ireland (AM182645.1), respectively, suggests furthermore a close maternal relationship and low genetic diversity between Northwestern European, Eastern and Central European giant deer populations.

**Stable Isotopes.** To evaluate the stable isotope signature of our ancient cervids from the Hohle Fels and Hohlenstein Stadel cave sites, stable isotopes from collagen carbon (\(^{13}C\)) and nitrogen (\(^{15}N\)) were measured and compared to large cervids present in Central Europe during the Pleistocene such as red deer, reindeer, and giant deer\(^{18-20}\). Pre-LGM (ca. 35,000 uncal ya) *Megaloceros* samples from Southern France and Belgium typically show isotopic signatures of collagen comparable to those of red deer (*Cervus elaphus*), while reindeer (*Rangifer tarandus*) provides systematically higher \(^{13}C\) values likely due to the consumption of lichen\(^{18}\) (Fig. 4a). During the Late Glacial period (13,000 – 12,000 uncal ya), the\(^{13}C\)-based distinction among larger cervids from the Swabian, French, and Swiss Jura decreases. Stable isotope signatures of our both ancient cervid bones from the Hohle Fels cave and the Holenstein Stadel cave fall inside the red deer-reindeer cluster reflecting a potential overlap in diet and habitat (Fig. 4b).

**Discussion**

We obtained nearly complete mtDNA sequences from two ancient cervid bones from the Swabian Alb dated to 12,175 ± 50 uncal ya (13,904 – 14,215 cal ya) and 12,370 ± 30 uncal ya (14,153 – 14,681 cal ya), respectively. Both sequences are distinct from 44 mtDNAs of extant cervids. The phylogenetic analyses suggest that the reconstructed mtDNAs are maternally closely related to fallow deer (*Dama*). Based on the phylogenetic position of our reconstructed ancient mtDNAs, their close genetic relationship to the previously determined partial *cytb* sequence from a complete giant deer skeleton from western Siberia\(^9\).
and to the complete cytb sequence from a giant deer astralagus from Ireland and due to the absence of fallow deer in Europe in the Pleistocene as well as due to the size of the bones, we conclude that both specimens derive from *Megaloceros giganteus*. We find strong support for a close maternal relationship to both *Dama* species. The maternal relationship within the *Megaloceros-Dama* clade however could not be resolved in our phylogeny suggesting an almost equal genetic distance of the two fallow deer species and giant deer. Our results disagree with the morphological conclusions that *Megaloceros* is closer related with a group comprising *Cervus*, *Axis*, and *Eucladoceros* and that the occurrence of palmate antlers in *Megaloceros* and *Dama* must be the result of homoplasy. Our results also disagree with the conclusions derived from short mtDNA sequences such as partial cytb reported by Kuehn and colleagues which suggested a *Cervus-Megaloceros* clade and attributed the palmate antlers in *Megaloceros* and *Dama* to

**Table 3.** Number of nucleotide differences between the reconstructed and previously published cervid cytb sequences.

| Sequence                      | *M. giganteus cytb* Kamyshlov (AM072744.1) | *M. giganteus* cytb Killavullen (AM182645.1) | Fallow Deer cytb (AJ000022.1) | Red Deer cytb (AB924664.1) |
|-------------------------------|-------------------------------------------|---------------------------------------------|--------------------------------|---------------------------|
| Hohlenstein Stadel cytb      | 1                                         | 0                                           | 89                             | 87                        |
| Hohle Fels cytb               | 1                                         | 1                                           | 104                            | 97                        |

**Figure 3.** Phylogenetic trees of full mtDNA sequences from 44 extant cervid species and two ancient mtDNA sequences from two ancient cervid bones likely representing *Megaloceros giganteus*. Each tree is based on 14,147 positions. Bootstrapping was performed with 1000 bootstrap replicates. Only bootstrap values different from 100 are indicated at inner nodes. (a) Maximum-likelihood tree based on the General Time Reversible (GTR+G+I) model. (b) Maximum-parsimony tree. Branch-numbers in the Parsimony tree indicate the accumulated steps of genetic change (base substitutions) for each species after the divergence from its most recent common ancestor. Both topologies place *Megaloceros giganteus* together with fallow deer (*Dama* sp.) into a distinct clade from red deer (*Cervus elaphus*). Both trees were rooted with musk deer (*Moschus chrysogaster*) as outgroup. The deer drawings were kindly prepared and provided by Kerttu Majander.
homoplasy. Instead, our results support the hypothesis of a Megaloceros-Dama clade suggested by previous studies based on morphological features and phylogenetic analyses from cytb2,3,9,10.

Megaloceros is traditionally considered a species adapted to open-areas that could have suffered from the development of forest in the early Holocene, most likely because the huge antlers would have restricted the movement of males in dense woodland5. However, anatomy and distribution suggest that Megaloceros was a mixed feeder5, and carbon-13 results on enamel of Megaloceros of previous interglacial periods support the possibility of a boreal habitat23. Isotope signatures of our two Megaloceros samples reveal that the diversity in habitat and diet decreased after the LGM between Megaloceros, red deer, and reindeer, probably resulting in an increased competition among the deer species in Central Europe. The overlapping niches and thus the increased competition with other deer species could explain, at least in part, the local extinction of Megaloceros in Southern Germany.

In conclusion, we generated two almost complete mitochondrial genomes from large cervid bones from the Hohle Fels and Hohlenstein Stadel caves in Southwestern Germany that date back to 12,000 uncal ya. Phylogenetic comparisons to contemporary deer mtDNA and previously determined ancient cytb DNA suggest that both mtDNA genomes derive from Megaloceros giganteus, which demonstrates its presence in Central Europe after the LGM. The close maternal relationship with the two fallow deer species resulting in a near polytomy in the Dama-Megaloceros clade questions the morphology-based grouping of giant deer and fallow deer in two separate genera. To date there has been no evidence that Megaloceros recolonized Central Europe after the LGM5; our findings provide support that Megaloceros returned to central parts of Europe even if the presence of humans might have hindered the re-colonization. In addition stable isotopes from our ancient cervid bones suggest a direct competition with other cervid species at the onset of the Holocene, potentially due to the lack of niche partitioning. Thus enviromental factors may have played an important role in the final extinction of the giant deer.

Material and Methods

Extraction of ancient DNA. Bone samples were exposed to UV-light overnight to remove surface contamination. A sample of 50mg was removed from the inside of the longbone of each bone using a dentistry drill. DNA extraction was carried out using a guanidinium-silica based method24. For each sample a DNA library was prepared according to published protocols25. Sample-specific indexes were added to both library adapters to allow differentiation between individual samples after pooling and
multiplex sequencing. Indexed libraries were amplified in 100 µl reactions followed by purification over Qiagen MinElute spin columns (Qiagen, Hilden, Germany) and quantification using Agilent 2100 Bioanalyzer DNA 1000 chip. Target enrichment of mitochondrial DNA was performed by capture of the pooled libraries using bait generated from modern roe deer (Capreolus capreolus) mitochondrial DNA. The bait was generated by use of three primer sets (table 1) designed with the Primer3Plus software package. All extractions and pre-amplification steps of the library preparation were performed in clean room facilities and one negative control was included for each reaction.

Sequence Processing, Assembly, Duplicate Removal. Library pools were sequenced on the Illumina HiSeq 2500 platform using two index reads (2*100 + 7 + 7 cycles) following the manufacturer’s protocol. De-indexing was performed by sorting all sequences corresponding to their p7 and p5 combinations using the CASAVA software version 1.8. Forward and reverse reads were merged into single sequences if they overlapped by at least 11 bp. Unmerged reads were discarded and merged reads were filtered for a length of at least 30 bp. Mapping of the length-filtered reads and removal of duplicate reads was performed using a custom mapping iterative assembler (MIA) which was developed to take into account sequence errors which commonly occur from ancient DNA damage. Reads were mapped to a full mitochondrial genome reference sequence of the roe deer, Capreolus capreolus (NC_020684.1). To achieve a higher resolution in the topology, in a second round sequence reads were mapped to a full mitochondrial genome of the fallow deer Dama dama (JN632629.1), and in a third round to the consensus sequence of our ancient putative Megaloceros giganteus sample HF/65/100, which was generated by mapping to the Dama dama mitochondrial reference sequence as described.

Analysis of ancient DNA damage patterns. C to T and G to A substitution patterns were obtained from the sequences using a custom software developed as an extension package to handle the output format of the mapping iterative assembler.

Multiple Sequence Alignment and Molecular Phylogenetic Analyses. A multiple sequence alignment was generated from 44 full mitochondrial genome Genbank sequences of extant cervid taxa together with the assembled mitochondrial genome sequences of our putative Megaloceros samples using ClustalW (Larkin, M.A. et al. ClustalW and ClustalX version 2. Bioinformatics 23: 2947–2948 (2007)). Alignments and phylogeny constructions were conducted in MEGA 6.0.6. The mitochondrial D-Loop region was excluded using the BioEdit sequence alignment editor (Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41:95–98 (1999)) and phylogenies were constructed from a total of 14,147 positions. Both maximum-likelihood and maximum-parsimony topologies were generated for all positions for which coverage was at least three-fold in each of the ancient sequences. Alignment columns with gaps or missing data were eliminated. Bootstrap support values were obtained over 1000 replicate data sets, using alpine musk deer as an outgroup (Moschus chrysogaster, KC425457.1). The phylogenetic trees were edited in FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree).

Pairwise Comparison of Cytochrome b Sequence Differences. To identify cytochrome b (cytb) coordinates within our reconstructed mitochondrial sequences, these were aligned to previously published cytb sequences of Megaloceros giganteus (AM072744.1, AM182645.1), fallow deer (AJ000022.1), and red deer (AB924664.1) using MEGA 6.0.6 and sequences outside of the aligned regions were discarded. Nucleotide sequence differences were then calculated by pairwise alignment between each of our ancient cytb sequences and each of the previously published cytb sequences using BLAST.

Stable Isotope Analyses. To study the habitat pattern revealed by stable isotopes, collagen was extracted from both bone fragments and carbon (13C) and nitrogen (15N) were measured. The results were combined with stable isotope data obtained from ancient reindeer (Rangifer tarandus) and red deer (Cervus elaphus) remains of the Swabian, French, and Swiss Jura dating 13,000 to 12,000 uncal year ago, which corresponds roughly to the GI-1e interstadial. The results were compared to stable isotopes from morphologically defined deer specimens including giant deer (Megaloceros giganteus) dating to the pre-LGM from southwestern France (SW France) and Belgium.

Isotopic analysis (δ13Ccoll, δ15Ncoll) was conducted at the Department of Geosciences of Tübingen University using a Thermo Quest Delta+XL mass spectrometer coupled to a NC2500 CHN-elemental analyzer, which provides elemental analysis (Ccoll, Ncoll). The international standards used include marine carbonate (V-PDB) for δ13C and atmospheric nitrogen (AIR) for δ15N. Analytical error, based on within-run replicate measurement of laboratory standards (albumen, modern collagen, USGS 24, IAEA 305A), was ±0.1‰ for δ13C values and ±0.2‰ for δ15N values. Reliability of carbon and nitrogen isotopic values was established by measuring the chemical composition, with C/N atomic ratio within the range of 2.9 to 3.6.
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Author Contributions
A.I. and J.K. conceived and designed the research. T.K.J., S.C.M. and C.J.K. provided the bone samples and conducted initial morphological analyses. M.B. and V.J.S. performed the extraction of mtDNA and the preparation of sequencing libraries. A.I. and A.H. performed the bioinformatic analyses and D.G.D. conducted the stable isotope analyses. A.I. wrote the manuscript and J.K. and D.G.D. mostly contributed to the discussion. All authors reviewed the manuscript.

Additional Information
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