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SCIENCE DIRECT
Molecular Phylogenetics and Evolution 40 (2006) 285–291
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Short communication

Molecular phylogeny of the extinct giant deer, *Megaloceros giganteus*

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Received 13 July 2005; revised 2 February 2006; accepted 7 February 2006
Available online 23 March 2006

1. Introduction

DNA sequences of many species of extinct megafauna have now been determined allowing a re-evaluation of their phylogenetic positions (Orlando et al., 2003), although the most spectacular extinct cervid species, the giant deer (so-called 'Irish elk'), had not been sequenced until very recently (Lister et al., 2005). *Megaloceros giganteus* (Blumenbach, 1803), one of the largest deer ever to have existed, appeared in the fossil record about 400,000 years ago (Lister, 1994). In the Late Pleistocene its range extended across the middle latitudes of Eurasia, from Ireland to east of Lake Baikal. It is now known to have survived to at least ca. 6900 ^{14}C years BP (ca. 7700 years ago) in western Siberia (Stuart et al., 2004). With its enormous palmate antlers, together spanning up to 4 m and weighing up to 45 kg—the largest of any deer, living or fossil (Gould, 1974)—it was one of the most striking members of the Late Pleistocene Palaearctic fauna. Moreover, *M. giganteus* is one of the key megafaunal species to have become extinct in the Late Quaternary in northern Eurasia (Stuart et al., 2004).

In view of its importance, the considerable gaps in our knowledge of *M. giganteus* are perhaps surprising (Aaris-Sørensen and Liljegren, 2004). In particular, until recently, on the basis of morphological data only, its origins and phylogenetic position within the Old World deer (Cervinae) have so far remained unresolved. *M. giganteus* has been placed in the tribe Megalocerini, which comprises at least 14 species of giant deer, including island dwarf forms (Lister, 1994). On morphological grounds, most authors consider that the closest extant relative of *M. giganteus* is the fallow deer (*Dama dama*) (Gould, 1974; Kitchener, 1987; Lister, 1994). This has been confirmed very recently by a phylogenetic analysis of 69 informative morphological (dental, cranial, and post-cranial) characters studied on 11 Cervinae species (Lister et al., 2005). However, the closer relationship between *Megaloceros* and *Dama* has been disputed by some authors (see Gould, 1974 and references therein; Geist, 1987), and notably a recent morphological study (Pfeiffer, 1999) argues for a closer relationship with red deer and wapiti.

With the aim of furthering our understanding of the phylogenetic position of *M. giganteus* in relation to extant cervids, we analysed mtDNA sequences of several specimens from different geographical origins (Ireland, Belgium, and Siberia).

2. Material and methods

2.1. Samples and dating

Nine samples all from different individuals were used for this study. Four Irish samples were provided by the National

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1055-7903/$ - see front matter
doi:10.1016/j.ympev.2006.02.004

History Museum of Ireland: two teeth in mandibles (NMING19 1968/358, NMING19 1968/326) recovered from Late Glacial lake marls beneath a peat bog at Ballybetagh near Dublin, an astragalus from Kilavullen Cave (NMING F21746 KVI60) and a phalanx from Newhall Cave (NMING F21747 NH161, a humerus from probably the same context was ^{14}C dated to 11,750 ± 90 years BP, OxA-3723; Woodman et al., 1997). Five other samples were provided by the Royal Belgian Institute of Natural Sciences: a maxillary fragment and tooth (2769-31) from Goyet Cave (Bone Horizon 2), Belgium (^{14}C dated to 23,840 ± 260 years BP, OxA-1167), two left tibiae (DM69, DM171) from Dendermonde, a fluvial site in Belgium, and a fragment of humerus (H4-4, ^{14}C dated to 39,290 ± 350 years BP, OxA-12116) from an early Upper Palaeolithic open-air site, Kamenka (Complex A), Buryatia, Siberia (Germonpré and Lbova, 1996). Two additional AMS dates are available for Kamenka (Complex A): 41,350 ± 450 (OxA-12117) on a shed *Megaloceros* antler, and $37{,}350 \pm {}^{410}_{390}$(GrA-5435) on a cut-marked tibia from a Mongolian gazelle (*Procapra gutturosa*).

2.2. Ancient DNA analysis

2.2.1. Extractions and amplifications

Extractions and amplifications were made in the ancient DNA facilities at the laboratory in Lyon (UMR CNRS 5534, France) using standard procedures (Gilbert et al., 2005). In most cases, the samples were extracted separately using the protocol detailed by Orlando et al. (2002). In cases where more than two samples of *Megalo-ceros giganteus* were extracted together, a sample from another species (bear, goat) was also co-extracted. The protocol of Yang et al. (1998) was used once for a second DNA extraction of both Kamenka and Kilavullen samples. Primers for nine overlapping fragments (F1–F9, 149–247 bp long) were used to amplify the entire cytochrome *b* (Table 1). Between two and four independent PCR amplifications were done for each fragment and individual. Three different blanks, including one serving as a cross-contamination control, were performed to monitor contamination (Loreille et al., 2001). As a control, a further sample of the Kamenka specimen was sent directly by the Brussels Museum to the ancient DNA laboratory in Dijon (UMR CNRS 5561, France). At this laboratory the DNA of this sample was independently extracted, using a commercial kit (Dneasy, Qiagen), and the F2 fragment amplified.

2.2.2. Consensus sequences

Each amplified fragment was cloned, using TOPO-TA for sequencing (Invitrogen). Size of inserts was tested by PCR using M13 universal primers and the PCR products were subsequently sequenced. Several clones of each fragment and sample were sequenced and consensus sequences were determined using all sequences from independent PCRs. The entire sequence of cytochrome *b* was reconstructed for the Kamenka and Kilavullen specimens by merging the nine overlapping fragments since no differences between the overlapping zones were detected. All sequences are deposited in EMBL (Accession Nos. AM182644–AM182647).

2.2.3. UNG-treatment

Some of the fragments amplified showed degradation, principally transitions of thymine to cytosine. This could result from deamination of cytosines that transformed cytosines into uracyls (Hofreiter et al., 2001). As a check on the substitutions, treatment with Uracyl-*N*-Glycosylase

Table 1
List of the primers used

Fragment amplified	Position on the cytochrome *b*	Primer	Sequence
F6	14106–14254	6F	GACTAATGATATGAAAAACCAT
		6R	CAAAATTTCATCAGGATGA
F7	14196–14410	7F	AAACAACGCATTYATTGATC
		7R	CGTTTGCGTGYATRTATC
F1	14344–14550	1F	TCCTCTGTCACCCATATCTG
		1R	GACGTATCCTACGAATGCTG
F2	14500–14692	2F	GGAGTAATTCTCCTATTYACAGTT
		2R	CGAAGAATCGAGTTAAGGTTGCTT
F3	14638–14792	3F	GAATGAATCTGAGGRGGCTT
		3R	GATGGRATTCCTGTTGGGTT
F4	14722–14897	4F	GCAGCACTCGCTATAGTAC
		4R	TCTGGTGCGAATARTACTAGT
F5	14827–14994	5F	ACCATTAAAGATATYTTAGG
		5Fb	ACGATCAAAGACATTYTAGG
		5R	GATTGCGTATGCAAATAGGAA
F8	14927–15173	8F	CAGCAAAYCCACTCAACACA
		8R	CCTCCRATTCATGTRAGTGTT
F9	15127–15350	9F	TTCTGARTCTTAGTAGCAGA
		9R	TTGTTCTTCATCTCTGGTTTACAAGAC

The primer 5Fb has been designed according to the sequence obtained for the Kamenka specimen of *Megaloceros giganteus*. Only this primer F5b has permitted an amplification of the fragment F5 for the Kilavullen sample. Positions of each fragment (including primers) are given according to the complete mitochondrial genome sequence of *Muntiacus muntjack* (acc. number: NC_004563).

(UNG), an enzyme that removes uracyl residues, was performed on the Kamenka and Kilavullen ancient DNA extracts. 1 μl of the enzyme was added during the PCR preparation and an incubation step of 10′ at 37 °C was carried out before starting the amplification process by PCR.

2.3. Phylogenetic analyses

The cytochrome *b* sequence of the Kamenka sample of *Megaloceros* was added to a dataset of 48 complete cytochrome *b* sequences of other cervids (Pitra et al., 2004). Using this large dataset, the position of *Megaloceros* within the subfamily Cervinae might be first tested and then, more clearly determined. On the basis of preliminary saturation analyses, three data sets were considered: (1) complete nucleic data, (2) data where all substitutions for the first and second codon positions were taken into account, but only transversions on the third, and (3) data restricted to Cervinae sequences, i.e., 33 taxa. Phylogenetic inferences were carried out using Bayesian and maximum likelihood (ML) methods. Three Bayesian reconstructions were done with MrBayes v3.ob4 (Ronquist and Huelsenbeck, 2003) using the appropriate model (see below) and the following parameters: four Markov chains using the Metropolis-coupled Markov chain Monte Carlo algorithm, run for 10^6 generations, random starting tree, tree sampling every 100 generations, MrBayes' defaults on Bayesian priors and burn-in value determined after empirical check of stationarity. ML reconstructions were performed using Paup* 4.0b10 (Swofford, 2000) with the model of evolution and model parameters selected by the AIC criterion implemented in Modeltest (Posada and Crandall, 1998). Inferences of robustness were assessed by 500 bootstrap replicates. AU-tests (Shimodaira, 2002) were finally performed to first test that *Megaloceros giganteus* is a member of the Cervinae, and then to compare five alternative topologies/affinities for this species within this group.

2.4. Molecular dating

We used the Bayesian relaxed molecular clock approach developed by Thorne et al. (1998) and Kishino et al. (2001) in the Multidivtime software to estimate times of divergence within cervids. This method has the advantage of taking into account various time constraints and can compare sequences of different ages. The protocol followed was identical to that described by Hassanin and Douzery (2003). Based on fossil evidence, the first split within the Cervidae is thought to have occurred in the Early Miocene, i.e., between 16.4 and 23.8 Myr (Ginsburg, 1988), and we took this figure as a time constraint (i.e., Rttm = 2.01; Rttmsd = 0.37; for a time unit of 10 Myr). We included three additional constraints: a minimum age of 5 and 7 Myr for the origin of the New World Odocoileinae (Webb, 2000) and the Muntiacinae/Cervinae split (Han, 1985; Dong et al., 2004), and the date of the Kamenka *M. giganteus* sequence (39,290 ± 350 years BP) used in this study. All the other parameters have been adjusted following the Multidivtime author instructions (Rtrate = 0.054525; Rtratesd = 0.054525; Brownmean = 0.746269; Brownsd = 0.746269; Bigtime = 10), and two independent runs were performed.

3. Results

3.1. Sequences obtained from fossils of Megaloceros giganteus

We successfully amplified mitochondrial DNA in four out of nine samples of *M. giganteus* from Siberia, Belgium, and Ireland. There were a humerus from Kamenka, Baïkal region (^{14}C dated to 39,290 ± 350 years BP), a tooth from Goyet Cave, Belgium (23,840 ± 260 years BP) and a phalanx and an astragalus from two Irish caves, Newhall (County Clare) (a related sample was dated to 11,750 ± 90 years BP) and Kilavullen (County Cork). The complete sequence of the cytochrome *b* gene (1140 base pairs) was determined for the Kamenka and Kilavullen specimens using nine overlapping fragments (F1–F9). A short sequence from each of the Goyet and Newhall specimens (115 bp, F3; Table 1) was also obtained. The five other samples could not be amplified, possibly because the environmental conditions in which they were preserved were unsuitable (lacustrine marls, fluvial site), while the successful samples came from a cold environment (Kamenka), and cave deposits (Kilavullen, Newhall, Goyet); situations where conditions are known to be suitable for good preservation of DNA (Smith et al., 2003).

3.2. Degradation observed

At least two independent amplifications were performed for each of the nine fragments, and all the amplified products were cloned (Supplementary data S1). Analysis of the differences observed between the clones sequenced for Kamenka and Kilavullen, shows that more than 80% of the degradation was due to deamination of cytosines (Supplementary data S1), as expected from ancient DNA substrates (Hofreiter et al., 2001). The most important example of degradation was observed for the first amplification of the fragment 5 (F5) for Kamenka that shows more than 25 differences (T instead C) between clones for a total fragment size of 127 sites (Supplementary data S2). To remove the deaminated cytosines (uracyls), we used UNG before the amplification process. However, this treatment reduces the quantity of substrate available for the amplification by PCR. A positive amplification was possible at least once for nearly all the fragments (8/9) for the Kamenka specimen, but only two fragments have been amplified after the UNG-treatment from the Kilavullen sample. As expected from this treatment, no differences between clones (T instead of C or A instead of G) were observed on the F5 for the Kamenka specimen.

3.3. Differences between individuals from different geographical origins

The consensus sequence obtained for the complete cytochrome *b* of the Kilavullen specimen shows less than 1% of divergence compared with Kamenka (nine transitions), almost always in the third codon position (Supplementary data S1). This is within the range of intraspecific variation observed for other cervids, for example $2.77 \pm 0.30\%$ within *Cervus nippon*, $1.86 \pm 0.22\%$ and $1.27 \pm 0.20\%$ within western and eastern red deer respectively (Ludt et al., 2004). A DNA fragment of 115 bp (F3) was also successfully amplified independently for two other giant deer samples (Goyet, Newhall) and both gave an identical sequence. This sequence showed one substitution from that of Kamenka specimen but was identical to the one from Kilavullen.

While this paper was in the process of submission, two publications have provided partial cytochrome *b* sequences of *Megaloceros giganteus* (Kuehn et al., 2005; Lister et al., 2005). The short sequences (137 and 167 bp; AY485666, AY347756, AY347754, AY244492–AY244495) published by Kuehn et al. (2005) are identical, or show only one substitution, with sequences of *Cervus elaphus* from Europe. Given that on the basis of morphological characters *M. giganteus* is clearly a distinct species from *Cervus elaphus* (Lister et al., 2005), one might expect that the pairwise difference between cytochrome *b* sequences of *Cervus* and *Megaloceros* would be much greater, as we observed in our results (7.9–8.9% of divergence). Thus, it seems more than likely that these sequences result either from contaminations by modern DNA from red deer or, but with low probability, from an inaccurate identification of the bones from which the DNA was extracted (see the Supplementary data of Lister et al., 2005). This is confirmed by the observation that both the sequence for the Kilavullen specimen obtained in this study and that independently obtained by Lister et al. (2005) (AM072745; 755 bp amplified) are identical. It is interesting to note that both of these *M. giganteus* samples originate from Ireland. Thus, the 1% of divergence observed between Kilavullen and Kamenka specimen probably results from geographical distance rather than from the difference in age (Kamenka specimen is about 30,000 years older than the dated specimen (Lister et al., 2005) identical in sequence to Kilavullen, but this is not enough to explain the nine substitutions observed).

3.4. Authenticity

We are confident in the authenticity of the sequences of *Megaloceros giganteus* that we obtained, given that: (i) two individuals gave complete sequences of cytochrome *b* which are identical at 99% (i.e., within the range of usual intra-specific variation in the family) and three individuals gave the same short sequence of 115 bp; (ii) all the experiments were carried out in ancient DNA facilities where modern DNA is never handled; (iii) contamination by *Cervus* or *Dama* was never detected in the controls performed (blank, mock, and cross-contamination); (iv) the same sequence was independently obtained for a fragment (F2; see Table 1) in another laboratory (UMR 5561, Dijon) from a bone sample of the Kamenka specimen sent directly to Dijon by the museum; (v) all the amplifications were independently duplicated and cloned and the consensus sequences were obtained from several clones; (vi) the pattern of degradation observed is in agreement with that generally observed for ancient DNA substrates; (vii) the complete cytochrome *b* sequence of the Kamenka and Kilavullen samples can be translated into amino acids and do not result in a truncated protein, furthermore, their base composition is similar to that of other cervids.

3.5. Comparison with other cervids

We compared the sequences that we obtained from the extinct giant deer to complete cytochrome *b* sequences of 48 species of extant cervids (Pitra et al., 2004). Only one sequence representative of each species, or subspecies, was taken into account. Given the weak variability observed between the sequences obtained for the Kamenka and Kilavullen specimens, only Kamenka was included in the further analyses. The Kamenka cytochrome *b* clearly differs from all other cervid sequences (Supplementary data S3) by between 7% (*Elaphurus davidianus*, Pere David's deer) and 14% (*Blastocerus dichotomus*, marsh deer) and does not show a significantly different evolutionary rate, except when compared with *Alces alces* (moose) and *Mazama sp* (brocket deer).

3.6. Phylogenetic analyses and divergence times

The topology obtained from the Bayesian analyses of the complete dataset is given in Fig. 1. To reduce artefacts induced by saturation or long branches, posterior probabilities, bootstraps proportions, and *p* values presented on Fig. 1 were computed on complete data for nodes outside Cervinae and on data restricted to Cervinae for nodes within the Cervinae group. Since the classification of *Cervus elaphus* is debated, red deer and wapiti have been considered in this analysis as a single species even if both appear clearly separated in the tree shown here, suggesting two different species; a result previously reported by Randi et al. (2001), Polziehn and Strobeck (2002), and Ludt et al. (2004).

All the topologies place *M. giganteus* within the Cervinae (node A, Fig. 1), but exclude it from the group comprising the genus *Cervus* and its synonyms (nodes C, D). Although tests correcting for long branch attractions (not shown) or comparison with alternative topologies (AU test, Fig. 1) cannot totally exclude it from a group including *Rucervus* sp. (node E) or in a basal position among Cervinae (node A), the analyses clearly place *M. giganteus* within the same clade as *Dama* sp. (node B).

According to the estimated divergence times obtained, the split between *Dama* and *Megaloceros* (node B, Fig. 1)

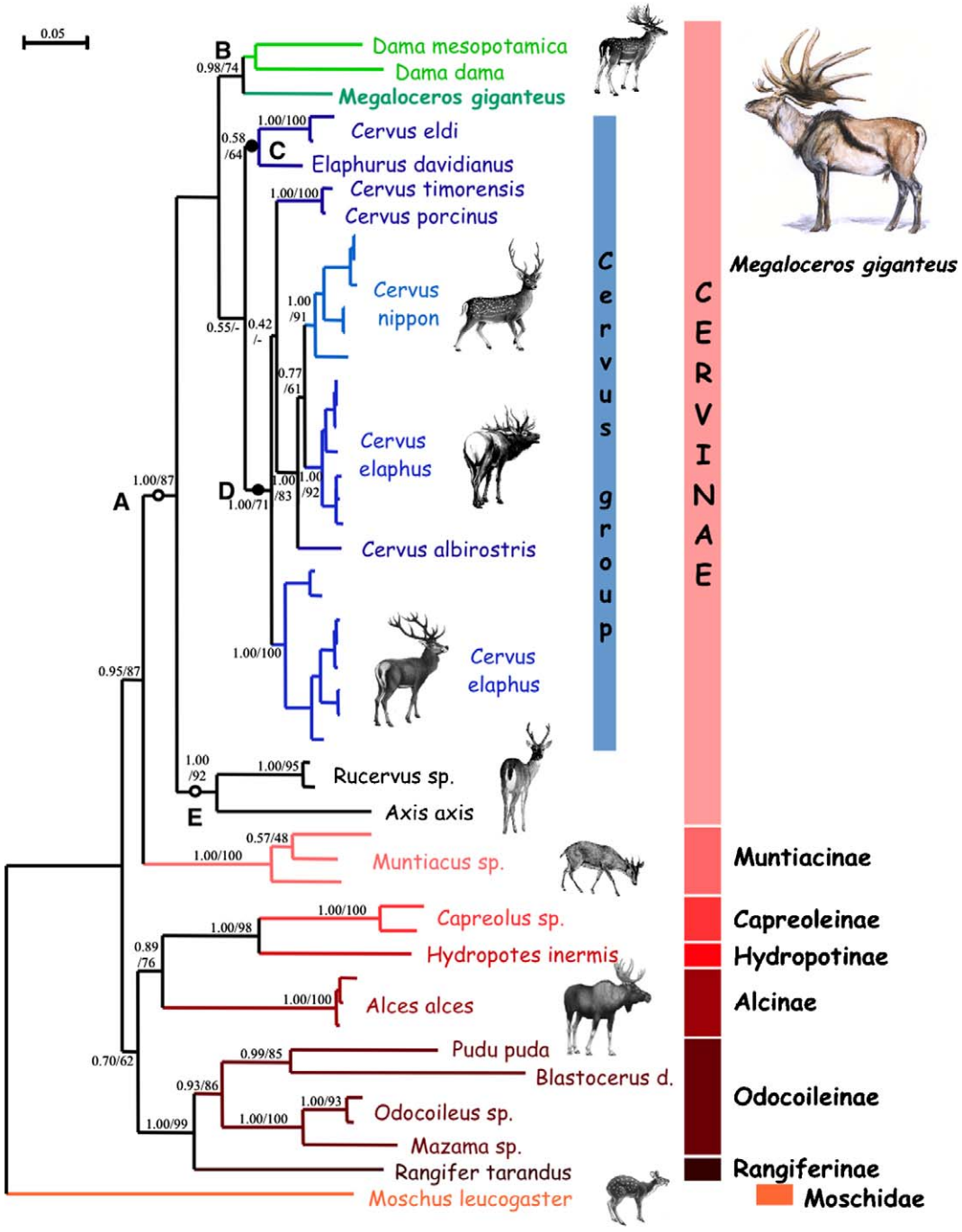

Fig. 1. Phylogenetic tree of cytochrome *b* sequences from 48 extant cervid taxa and *M. giganteus* obtained from Bayesian reconstruction with a GTR+I+Γ model (see Section 2). *Moschus leucogaster* was used as outgroup. Posterior probabilities (Bayesian reconstruction) followed by bootstrap supports (ML) are given (see Section 3.6). A dash instead of a bootstrap-value indicates that the branch was not supported by ML methods. All methods agree that *M. giganteus* falls within the Cervinae, and does not group with other cervid species (AU-test, *p* value 0.054). Among Cervinae, the open circles indicate alternative phylogenetic positions for *M. giganteus* that cannot be totally excluded according to the AU-tests (*p* values of 0.424 and 0.226 for A and E, respectively) and the black circles those that can be excluded (0.062, 0.02 for C and D). Thus, a basal position of *M. giganteus* among Cervinae or clustered with the *Rucervus*/*Axis* clade cannot be eliminated entirely. Nevertheless, the analyses together with morphological evidences more strongly support *M. giganteus* as a member of the same clade as *Dama* spp., i.e., in a sister group to the genus *Cervus*.

would have occurred 10.7 Myr ago ($CI_{95\%}$: 6.4702–16.4973 Myr), whereas the split of the Cervinae group (node A, Fig. 1) is estimated around 15.6 Myr ago ($CI_{95\%}$: 10.0774–22.7221), and the divergence of the two *Dama* species at 9.9 Myr ($CI_{95\%}$: 5.8465–15.4883). These last estimates are older than the previous dates proposed by Pitra et al. (2004), but in agreement with those of Hassanin and Douzery (2003) and Randi et al. (2001). Finally, the analyses performed suggest a split of recent red deer (node D, Fig. 1) around 6.8 Myr ($CI_{95\%}$: 3.8360–11.4417), compatible with

previous results obtained by Ludt et al. (2004). The differences between our results and those of Pitra et al. (2004) are probably because the present study does not use the same time constraints, i.e., a split of the Cervids in the Early Miocene. The sensitivity of the estimates of the divergence times within the Cervidae to the palaeontological calibration points are well known (Lister, 2004). Otherwise, the dates obtained in this study are compatible with palaeontological data (Vrba and Schaller, 2000).

4. Discussion

4.1. Phylogenetic position of the M. giganteus

The complete sequences of cytochrome *b* were obtained from two different giant deer individuals, including one dated to around 40,000 years BP. The two sequences show less than 1% of difference, which can be explained by intraspecific variability, and the fact that one individual comes from eastern Siberia and the other from Ireland. These sequences were clearly distinct from 48 other known sequences of extant cervids. The Bayesian and ML phylogenetic analyses performed on this dataset confirmed that *M. giganteus* is clearly excluded from cervids that are not Cervinae. Moreover, the topology obtained (Fig. 1) suggests that the fallow deer is the closest extant relative of *M. giganteus*, and that both lineages would have diverged around 10.7 Myr ago ($CI_{95\%}$: 6.4702–16.4973 Myr). This argues for the hypothesis of an older divergence of a group that would include the extant *Dama* and the European *Dama*-like forms with the Megacerines, as suggested in Lister et al. (2005). Thus, mitochondrial DNA supports a hypothesis that has often been suggested on the basis of phenotypic traits (Gould, 1974; Kitchener, 1987; Lister, 1994).

However, the AU-tests performed have not been able to totally eliminate some alternative topologies, such as a basal position of *M. giganteus* among Cervinae. Future sequencing of nuclear genes for *M. giganteus* could thus complete this analysis. For example, the exon 4 of the κ-casein, that is already known in many cervids (Cronin et al., 1996) and has a lower evolutionary rate than the mitochondrial control region, could be a good marker to settle this point.

Our results disagree fundamentally not only with the conclusions of Pfeiffer (1999), which are based on morphology, but also with the short DNA sequences on *Megaloceros giganteus* published in Kuehn et al. (2005). Both of these studies suggest a closer relationship with *Cervus elaphus*. With regard to the molecular data, as explained previously, perhaps the sequences of Kuehn et al. (2005) result either from contamination during the DNA amplification process or from misidentification of bones. In contrast our conclusion that *M. giganteus* is most closely related to extant *Dama* species agrees with a recently published study on both molecular and morphological characters (Lister et al., 2005). Significantly, the sequence that we obtained for Kilavullen is identical to that published by Lister et al. The reasons for the discrepancy between the morphological analyses of Pfeiffer (1999) and Lister et al. (2005) were discussed in the latter paper (see Lister et al. Supplementary data). The differences are mostly due to the choice of the morphological characters analysed.

4.2. Perspectives for the phylogeography of the M. giganteus and its extinction

The greatest concentration and most of the best-preserved specimens of *M. giganteus* have been found in Ireland, where it became locally extinct about 10,600 ^{14}C years BP, at the onset of the Younger Dryas cold phase, well before the earliest record of humans (ca. 9000 ^{14}C years BP) on the island (Stuart et al., 2004). Together with evidence from other parts of North Western Europe, the Irish record has often been used to argue that the global extinction of *M. giganteus* was due to climatic fluctuations and consequent vegetation changes in the Late Glacial (Barnosky, 1986; Moen et al., 1999). However, we now know that some populations much further east survived well into the Holocene and disappeared at a time of more stable climate suggesting that the demise of this species may have multiple causes (Stuart et al., 2004). In this context, the determination of sequences, such as the highly variable mitochondrial DNA control region ones, from different giant deer populations through time, will allow assessment of the evolution of the genetic diversity within this species, and could help to test some of the hypotheses proposed to explain the final demise of this species.

Acknowledgment

We thank N. Monaghan and the National Museum of Ireland, W. Clarke for the *Megaloceros* drawing, L. Orlando, M. Pages, S. Calvignac, and M. Duffraisse for their help in some experiments. This work was supported by a President's Award from UCD, Université Lyon 1 and CNRS (ECLIPSE program).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.02.004.

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