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Research Article

Phylogeny and diversity of moose (*Alces alces*, Cervidae, Mammalia) revealed by complete mitochondrial genomes

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Abstract

Mitogenomes are valuable data sources for phylogeographic and evolutionary studies of relatively closely related organisms. Here we describe eight complete sequences of moose mitogenomes belonging to the three clades of the European lineage and compare them with those of the Asian lineage. Mitochondrial genomes of moose and other cervids were used to infer highly resolved phylogenetic relationships and estimate divergence times. The analyses clearly distinguished two mtDNA lineages of moose and supported the division of the European lineage into three clades: East, West, and Central. The divergence of the European and Asian mtDNA lineages occurred in the Middle Pleistocene (ca. 443 kya), which significantly exceeds the fossil records of modern *Alces alces* dated to 100–200 kya. It indicates that the evolutionary history of the moose is more complex and could encompass inheritance of the ancestral variation. Our estimates also showed rapid diversification of present-day clades of the European lineage, which coincided with the transition from the Penultimate Glacial Period (MIS 6) and the Eemian interglacial (MIS 5) approximately 100 kya. Despite the strong division of the nucleotide sequences, we detected no evidence for the divergence of amino acid sequences between Clade Central and Clade East. The recent diversification of *A. alces* clades, in combination with their evident reciprocal monophyly, could be a result of low effective population size over its evolutionary history, augmented by severe bottlenecks during the Last Glacial Period. Our results are in agreement with the presence of different glacial refugia recently proposed for three identified clades of the European lineage and suggested the relict character of the Scandinavian (from Clade West) and the Biebrza moose population in northeastern Poland (belonging to Clade Central).

Introduction

The present-day phylogeographic pattern of most European species is an outcome of Quaternary climatic oscillations and ice ages (Hewitt, 2000). Several lines of evidence suggest that populations of European temperate species went extinct over large parts of their range and that some of them survived in well-identified, separated refugia in southern areas during the Last Glacial Maximum (LGM) or in certain cryptic northern refugia (Taberlet et al., 1998; Provan and Bennett, 2008; McDevitt et al., 2012). Molecular studies have proved to be a powerful tool for revealing the trajectories of postglacial range expansions and accompanying demographic processes (Davison et al., 2011; Matosiuk et al., 2014a; Niedziałkowska, 2017). The moose (*Alces alces* L. 1758) was one of the first large mammals which has recolonized areas of Central Europe after the LGM (Schmölcke and Zachos, 2005). The earliest fossil records found throughout Eurasia (Netherlands, Kola peninsula, southeast of Western Siberia, and the Enissey river basin; Boeskorov, 2005) support the first appearance of *A. alces* in the late Middle Pleistocene approximately 150,000 years ago (150 kya). Using molecular data, Hundertmark et al. (2002) showed that the modern diversity of this species arose within the last 100 kya at most and have identified three well-recognized evolutionary mtDNA lineages of moose in the world: Asian, European and American. Within the European range of moose, three highly supported mtDNA clades, separated geographically in part, have been detected: Central, East and West (Kholodova et al., 2014; Niedziałkowska et al., 2014, 2016a,b; Kangas et al., 2015; Wennerström et al., 2016). Today, moose representing Clade East are widely distributed over almost the whole Eurasian range (Fig. 1), while the other clades have narrow or scattered distributions and occur mainly in Scandinavia and central Poland (Clade West) as well as eastern Poland and western Belarus (Clade Central; Świsłocka et al., 2013; Niedziałkowska et al., 2014; Niedziałkowska, 2017), suggesting that they experienced isolation and bottlenecks and thus underwent genetic drift (Rowe et al., 2004). Indeed, mtDNA control region (*mtDNA-cr*) studies of moose populations occupying the northern and western edges of the species range have postulated the relict character of the moose populations in Sweden (Hundertmark et al., 2002) and Poland (Świsłocka et al., 2008, 2013), as very distinct moose mtDNA haplotypes were found in these peripheral areas.

In this study we used complete mitogenomes of the European and Asian moose lineages in order to: (1) reveal phylogenetic relationships among the moose lineages and clades, (2) re-estimate divergence times, and (3) describe the general features of moose mitogenome diversity in isolated, relict populations in a comparison with their continuous distribution areas, to verify evidence of their relict character.

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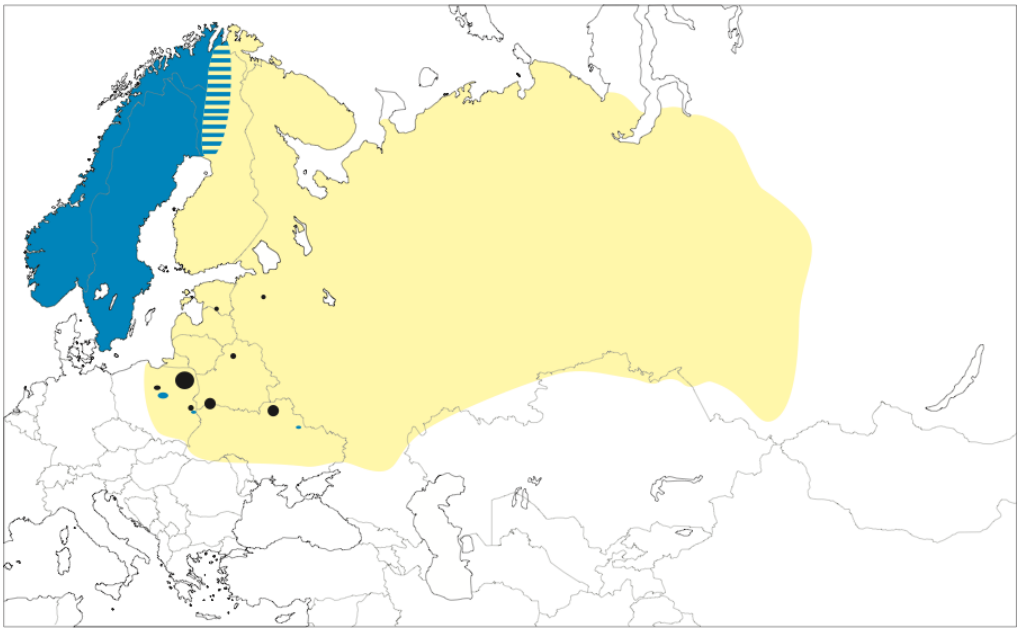

Figure 1 – Distribution of three mtDNA clades (East, West and Central) of European lineage of moose in Eurasia (after Świsłocka et al., 2013; Niedziałkowska, 2017). Clade East in yellow (light), Clade West in blue (dark) and Clade Central in black.

Materials and Methods

Sample collection and DNA extraction

This study used samples from eight different animals representing all of the mtDNA phylogenetic clades (Central, East and West) forming the European lineage of *A. alces* previously identified using the *mtDNA-cr* and cytochrome b sequences (Hundertmark et al., 2002; Świsłocka et al., 2008, 2013; Niedziałkowska et al., 2014, 2016a,b). Seven muscle samples were collected from dead animals found in northeastern Poland, whereas one hair sample came from a Swedish individual translocated into "Moose Valley" animal park located in northern Poland. Table 1 includes the sampling localities for the analyzed specimens. Genomic DNA was extracted with a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

PCR and Sequencing

PCR thermal cycling was performed in 5 µL reaction volumes containing 2 µL genomic DNA (≈20 ng), 1.7 µL Qiagen Multiplex PCR Master Mix (1×), 0.3 µL primer mix (0.2 µM of each primer), and 1 µL RNase-free water. The reaction conditions were: initial denaturation step at 95 °C for 15 min and 35 cycles with denaturation at 94 °C for 30 s, annealing for 90 s (for details of the annealing temperature for different primer pairs see Supporting information Table S1), extension at 72 °C for 60 s, and final elongation for 30 min at 60 °C. We applied a set of 19 primer pairs for rapid amplification of *A. alces* mitochondrial genomes belonging to the European lineage, and to generate overlapping reads. Supporting information Table S1 shows the primers used in this study, including 10 primer pairs newly designed in FastPCR software (Kalendar et al., 2009) on the basis of available mitogenomes of moose (*A. alces*, JN632595, Hassanin et al., 2012; *A. alces cameloides*, KP405229, Liu and Jiang, 2016). The primer pair 16290a_F/16410a_R was designed, due to the presence of a long homopolymer (13 bp long) in the *mtDNA-cr*. Amplified PCR products were purified with shrimp alkaline phosphatase (SAP) and Exonuclease I (Thermo Scientific) in an enzymatic reaction following the manufacturer's protocol. They were then processed for cycle sequencing PCR with a BigDye^{TM} Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems, Foster City, CA, USA). Additional sequencing primer (4300a_F2) was applied to amplify the fragment containing two homopolymers located between base pairs 4950 and 5300. Unincorporated dideoxynucleotides were eliminated from the sequencing reaction with an ExTerminator Kit (A&A Biotechnology, Gdynia, Poland). Bi-directional sequencing was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Data analysis

The obtained mitogenomic sequences were manually revised and aligned with the BioEdit sequence-editing program (Hall, 1999). Two data sets were prepared: whole mitochondrial genome alignment (16418 bp) and alignment restricted to the 13 protein-coding mitochondrial genes (11373 bp). The former was used to estimate basic sequence diversity indices and create a nucleotide network; the latter served for protein network construction. All of the protein-coding genes, as well as tRNA and rRNA genes, were identified using the MITOS online mitochondrial genome annotation server (http://mitos.bioinf.uni-leipzig.de/index.py, Bernt et al., 2013) and the reference gene sequences of *Bos taurus* (NC_006853). To avoid nuclear DNA sequences of mitochondrial origin (pseudogenes/numts), we also checked all coding regions for open reading frames.

We used DnaSP v.5.10 (Librado and Rozas, 2009) to calculate the measures of sequence variation, including the number of segregating sites (S) and the number of synonymous and nonsynonymous substitutions; to perform a sliding window analysis of nucleotide diversity of the individual moose from Clade Central, East and West of the European lineage as well as from the European and Asian lineages across the whole mitogenomes (window size 100 bp, step size 25 bp).

Phylogenetic analyses

Phylogenetic analyses were performed on two sets of complete mitogenomic sequences. One set contained 10 sequences of *A. alces* (*Alces* set; Tab. 1) and the other set contained 38 sequences of Cervidae in addition to representatives of Moschidae and Bovidae, used as an outgroup (Cervidae set; Supplementary Table S2). All of the mitogenomic markers were included: control region, 13 protein-coding genes, 12S and 16S rRNAs, and 22 tRNAs. The sequences were aligned in MAFFT using an accurate algorithm L-INS-i with 1000 cycles (Katoh and Standley, 2013). The alignments were edited in JalView (Waterhouse et al., 2009). Poorly aligned and nonconservative parts of control regions were removed. The partitions that did not show any sequence variation were excluded from the *Alces* set for the purpose of phylogenetic and molecular dating analyses. The final concatenated alignments of Cervidae and *Alces* sets consisted of 16098 bp and 11924 bp respectively.

Table 1 – List of the eight mitochondrial genomes of *Alces alces* obtained in this study, with their sampling locality and assignment to haplotypes and the European mtDNA phylogenetic clades, according to Niedziałkowska et al. (2014).

No	Symbol of nucleotide haplotype	Symbol of amino acid haplotype	Sample type	Locality	Coordinates	Clade	Length [bp]	GenBank acc. num.
1	H1	A1	muscle	Goniądz, Poland	53°32′ N, 22°45′ E	Central †	16418	MF784597
2	H13	A1	muscle	Gostynin-Włocławek Forest, Poland	52°25′ N, 19°27′ E	Central†	16418	MF784602
3	H12	A1	muscle	Rzędziany, Poland	53°9′ N, 22°51′ E	Central †	16418	MF784601
4	H6	A2	hair	Koszowatka, Poland	54°15′ N, 18°8′ E ‡	West †	16418	MF784600
5	H17	A3	muscle	Srokowo Forest, Poland	54°14′ N, 21°29′ E	West †	16418	MF784603
6	H22	A3	muscle	Srokowo Forest, Poland	54°14′ N, 21°29′ E	West †	16418	MF784604
7	H2	A4	muscle	Srokowo Forest, Poland	54°14′ N, 21°29′ E	East †	16418	MF784598
8	H3	A5	muscle	Rzędziany, Poland	53°9′ N, 22°51′ E	East†	16418	MF784599
9	Aa1	A6	muscle	Kazakhstan	-	Asian lineage	16417	JN632595.1
10	Aa2	A6	muscle	Hanma National Nature Reserve, China	-	Asian lineage	16417	KP405229.1

†according to Niedziałkowska et al., 2014.
‡sample of ranch moose taken from "Moose Valley" (Poland), but the individual was previously translocated from Sweden.

Three phylogenetic approaches were applied: the maximum likelihood method in IQ-TREE (Nguyen et a., 2015), Bayesian inference in MrBayes (Ronquist et al., 2012), and PhyloBayes (Lartillot and Philippe, 2004). We considered all potential partitions to find the best substitution models, i.e. three codon positions for individual protein-coding gene and separate partitions for individual RNA genes and the control region.

ModelFinder in IQ-TREE (Chernomor et al., 2016; Kalyaanamoorthy et al., 2017) was used to select the best fitting scheme of substitution models (Supplementary Tables S3 and S4). In IQ-TREE, we applied the Shimodara-Hasegawa-like approximate likelihood ratio test (SH-aLRT) with 10000 replicates and non-parametric bootstrap with 2000 replicates. In the tree search, we used a more thorough NNI search.

In MrBayes, we assumed the partitioned scheme of substitution models according to the results of PartitionFinder (Lanfear et al., 2012; Supplementary Tables S3 and S4). Nevertheless, we applied mixed models to specify appropriate substitution models across the large parameter space (Huelsenbeck, 2004). The models describing heterogeneity rate across sites were adopted according to the PartitionFinder results. Two independent runs using 8 Markov chains were applied. The trees were sampled every 100 generations for 10000000 generations. When the runs reached convergence, we generated a posterior consensus based on the trees from the last 6806000 and 6560000 generations for the Cervidae and *Alces* sets respectively.

In PhyloBayes, we applied the CAT-GTR+Γmodel with parameters inferred from the data. Two independent Markov chains were run for 35000 and 3000 generations for the Cervidae and *Alces* sets respectively. The last 5000 and 1000 trees respectively, were collected from each chain to compute a posterior consensus after reaching convergence.

The consensus of trees obtained in the three approaches was calculated in IQ-TREE. Three tree topologies assuming different relationships between moose clades were tested in IQ-TREE applying one million resamplings. Site-wise log-likelihoods for the trees were also compared using Wilcoxon signed-rank test in R package (R Core Team, 2018). Additionally, we compared the competitive topologies using Bayes Factor based on the mean marginal likelihood estimated by the stepping-stone method in MrBayes, using 10 independent runs with 8 Markov chains and 50 steps of the sampling algorithm.

The median-joining method available in Network v.4.6.1.3 (Bandelt et al., 1999) was used to visualize the networks between moose mitogenomes, based on whole mitogenomic sequences, and concatenated amino acid sequences of the coded proteins.

Molecular dating

Divergence times for the Cervidae and *Alces* sets were estimated using the BEAST 1.10.4 (Drummond et al., 2012). We assumed substitution models as proposed by PartitionFinder (Supplementary Table S5). In the Cervidae set, we introduced five calibration points. The split between Cervidae and Bovidae+Moschidae was assumed based on the recent estimations including fossil data (Mennecart et al., 2017), which provided 21.5-–35.0 Mya as the 95% Highest Posterior Density (HPD) interval in three methods. We applied the normal distribution prior on this range with the average 28.25 Mya. On the age of the crown Cervidae, we assumed two types of distribution prior: the lognormal one with the minimum age bound 14.2 Mya of *Euprox minimus* regarded as the earliest crown deer (Aiglstorfer et al., 2014; Mennecart et al., 2017) and the normal distribution with 95% HPD interval 14.1–25.2 Mya as obtained in three methods by Mennecart et al. (2017). The lognormal distribution with the minimum age bound 8 Mya and 4 Mya was also applied for respectively the Cervini-Muntiacini split and the Odocoileini divergence, based on the age of corresponding fossils (Vislobokova, 1980; Dong, 2007; Petronio et al., 2007; Hassanin et al., 2012). Additionally, we used normal distribution prior with the average 0.810 Mya for the most recent common ancestor of *Capreolus capreolus* and *C. pygargus*, based on the molecular estimations using control region and cytochrome b, which gave the range 0.510–1.110 Mya (Matosiuk et al., 2014a). We applied the Yule model and the lognormal relaxed clock model rather than the strict clock because the coefficient of variation of the relaxed clock was quite big (>0.2).

For the *Alces* set, we applied two versions of the normal distribution for the age of the root with 95% HPD 374–725 kya or 309–610 kya as obtained in the dating for the Cervidae set assuming the lognormal or normal distribution prior for the age of the crown Cervidae respectively. We used the coalescent (constant size) model and selected the strict clock model for the *Alces* set because the data showed the clock-like evolution.

For two data sets, posterior distributions of parameters were estimated for 1 billion generations with a sampling frequency of 1000 steps. The convergence and sufficient sampling were checked using loganalyzer and Tracer 1.7 (http://beast.bio.ed.ac.uk/Tracer). All parameters had the Effective Sample Size (ESS) exceeding 200. The phylogenetic trees were summarized in TreeAnnotator with a 10% burn-in and assuming common ancestor heights. The final trees were visualized in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

Results and discussion

Organization and diversity of *Alces alces* mitogenome

This study describes complete mitogenomic sequences of moose belonging to the three clades of the European lineage: Central, East, and West. The eight mitogenomes obtained in this study (GenBank accession no. MF784597–MF784604) were selected from sixteen recently recognized mtDNA-cr haplotypes within the European moose lineage (Tab. 1; Świsłocka et al., 2013; Niedziałkowska et al., 2014. They were compared to previously published mitogenomes of the Asian moose

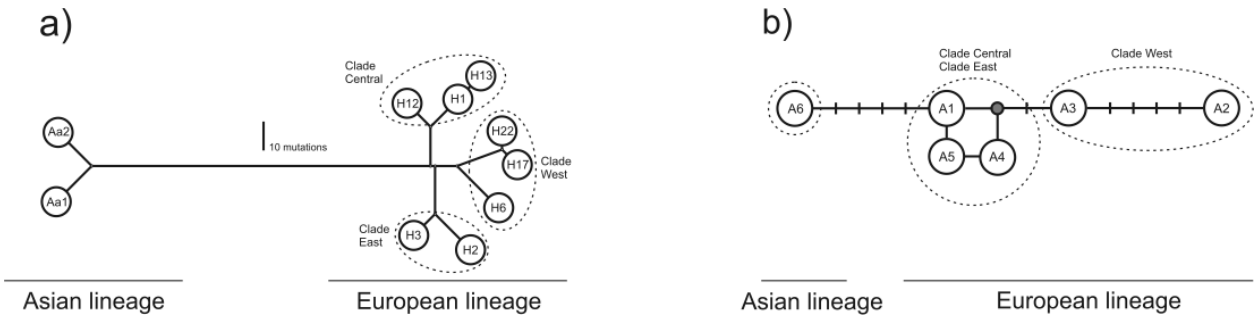

Figure 2 – Median-joining networks based on (a) nucleotide sequences of the whole mitogenome and (b) amino acid translations of 13 concatenated protein-coding genes of *Alces alces*.

lineage. The complete moose mitochondrial genomes are 16418 bp in length and include 13 protein-coding genes, two rRNA genes and 22 tRNA genes, and show the typical organization of metazoan mitochondrial genomes (Bernt et al., 2013). Out of the 89 (0.54%) segregating sites identified in the whole mitogenomic sequences of the European moose lineage, 58 were parsimony-informative and 31 were singletons. Eight nonsynonymous mutations resulting in six amino acid variants (Fig. 2b) and 40 synonymous polymorphisms were identified in the concatenated fragment consisting of 13 protein-coding genes (3791 codons).

A sliding window analysis performed to estimate nucleotide diversity (π) revealed a very high proportion of invariable sites in moose mitogenomes belonging to the European lineage. Substantial variation above 2% was identified only in the first hypervariable domain of the mitogenomic control region (*mtDNA-cr*) (Fig. 3). This variation increased to 5% when two mitogenomes of Asian lineage were included in the analysis. The abovementioned results are in agreement with previous molecular reports based on control region sequences (Hundertmark et al., 2002; Świsłocka et al., 2013; Niedziałkowska et al., 2014, 2016a,b). They indicate that the *mtDNA-cr* is an effective marker for studying the phylogenetic relationships between different *A. alces* mtDNA lineages and clades. On the other hand, mitogenomic diversity within the European moose lineage is limited. This is particularly apparent when compared with other Capreolinae (Odocoileinae) species such as the European roe deer (*C. capreolus*), in which a larger number of highly divergent regions was detected, including protein-coding genes in the mitogenome (Matosiuk et al., 2014b). Based on the whole mtDNA sequences, the phylogenetic clades of the European moose lineage differed in 13 (for Clade Central-Clade West comparison) to 18 (for Clade East-Clade Central comparison) fixed nucleotide substitutions. This result indicates that through its extensive range in Europe the moose gained considerably lower diversity within the whole mtDNA sequence than other hoofed mammals, which is in agreement with previous studies of the species (Hundertmark et al., 2002; Świsłocka et al., 2013; Niedziałkowska et al., 2014; Kangas et al., 2015).

Phylogenetic relationships of *Alces* with other cervids

The mitogenomes of moose and other cervids were used to infer phylogenetic trees (Supplementary Figures S7 and S8). Maximum likelihood inference and two Bayesian approaches provided almost the same topology. The relationships between the analyzed taxa are congruent with those obtained in other studies based on complete mitogenomes or its large parts (Hassanin et al., 2012; Immel et al., 2015; Zurano et al., 2019). However, our approaches provided much stronger and in most cases maximal support of the main groups and relationships within them. Two subfamilies with their tribes are clearly determined: Cervinae (with Muntiacini and Cervini) and Capreolinae (with Odocoileini, Capreolini, and Alceini). Our results confirm the legitimacy of the taxonomic shift of *Przewalskium albirostre* to *Cervus albirostris* (Mattioli, 2011) and *Rucervus eldii* to *Panolia eldii* (Groves and Grubb, 2011). Consequently, the monophyly of these lineages was recovered. Still, it does not refer to *Rusa* and clearly polyphyletic *Pudu* and *Mazama*, for which taxonomic revision and resolving phylogenetic relationships remain challenging (Heckeberg et al., 2016). *Alces* mitogenomes were grouped with Capreolini in three approaches undertaken in this study, which is in line with other studies (Randi et al., 1998; Pitra et al., 2004; Hughes et al., 2006; Agnarsson and May-Collado, 2008; Hassanin et al., 2012; Immel et al., 2015; Martins et al., 2017; Mennecart et al., 2017; Zurano et al., 2019). However, our analyses provide more robust support for such grouping (Supplementary Figures S7 and S8). Phylogenetic position of *Alces* inferred in other studies was not always consistent and strongly supported usually due to the use of a single approach or shorter molecular markers (Polziehn and Strobeck, 1998; Gilbert et al., 2006; Hoffmann et al., 2015; Immel et al., 2015; Heckeberg et al., 2016; Gupta et al., 2018).

Table 2 – Results of tests comparing tree topologies assuming different relationships between the clades of the European mtDNA lineage of moose. The topology t1 corresponds to the best tree found in phylogenetic analyses. The table includes: p-values from an approximately unbiased test (AU) and Bayes factor (BF) expressed in natural logarithm units as differences between marginal likelihoods of the given and the best topology (t1).

Topology	logL (IQ-TREE)	ΔlogL	AU	Marginal logL	BF
t1:West+Central	-16889.658	0.000	0.9441	-16926.21	0.00
t2:West+East	-16890.195	0.537	0.0637	-16939.25	13.04
t3:Central+East	-16890.202	0.543	0.0648	-16933.44	7.23

Phylogenetic relationships among moose lineages and clades

The phylogenetic analyses (Supplementary Figures S7 and S8) revealed the presence of the Asian lineage, which included *A. alces* from Kazakhstan along with *A. alces cameloides*, as well as the European lineage, which was divided into three evolutionary clades: Central (with haplotypes H1, H12, H13), West (H6, H17, H22) and East (H2, H3). This finding is in line with earlier studies (Świsłocka et al., 2013; Niedziałkowska et al., 2014; Kangas et al., 2015). The Asian and European lineages create significantly supported groups in the global Cervidae tree (Supplementary Figures S7 and S8). The relationships within the European clades are also well resolved. However, grouping between these clades is not consistent across the methods applied. In IQ-TREE and MrBayes trees, Clade West is clustered with Clade East, whereas in PhyloBayes with Clade Central. Unfortunately, none of these groupings was significantly supported. Therefore, we conducted phylogenetic analyses of only *Alces* mitogenomes additionally including a more variable part of *mtDNA-cr* in the concatenated alignment (Fig. 4). As a result, all three methods produced consistently the same topology, in which Clade West was grouped with Clade Central. Although posterior probabilities were weak, the bootstrap support was high.

To further assess the significance of this grouping, we performed a test of the competitive three tree topologies assuming different relationships between the European clades (Tab. 2). The log likelihood value of the best tree (t1), assuming Clade West + Clade Central grouping, was bigger than that for other topologies: t2 (assuming Clade West + Clade East) and t3 (assuming Clade Central + Clade East). *p*-values produced

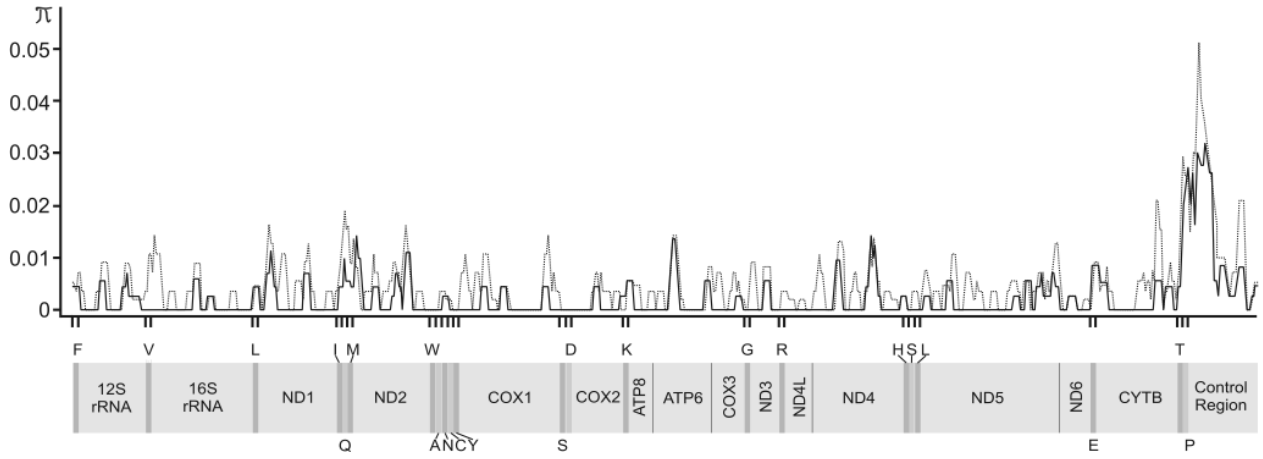

Figure 3 – Organization of mitochondrial genomes in *Alces alces* and sliding window analysis of nucleotide diversity within the European lineage (solid line) and pooled European and Asian lineages (dotted line; window size 100 bp, step size 25 bp).

by the AU test were only slightly greater than the 0.05 threshold for the alternative hypotheses, implying their weak acceptance. However, Bayes factor greater than 7 indicates overwhelming support for the best topology; the threshold for such interpretation is 5 (Kass and Raftery, 1995). Analyses of site-wise log-likelihoods also supported this view, with 53 more alignment sites (columns) that favour the t1 topology than t2, and 384 more sites that support the t1 than t3. *p*-value obtained in the Wilcoxon signed-rank test was smaller than 2.2×10^{-16}.

The phylogenetic network based on the complete mtDNA sequences (16418 bp) revealed the presence of the same *Alces* lineages and clades (Fig. 2a). The two moose mtDNA lineages, Asian and European, were separated by at least 142 mutation steps. The level of mitogenomic divergence observed between moose lineages resembles those detected among present-day clades of other cervid species (Skog et al., 2009; Matosiuk et al., 2014a). The European mtDNA lineage of moose diversified further into three young clades, which already attained reciprocal monophyly.

The phylogenetic analyses corroborated the results obtained from the nucleotide network (Fig. 2a) and confirmed that the European moose mitogenomes are divided into three strongly supported clades (Niedziałkowska et al., 2014, 2016a). This result differs somewhat from the outcome of the recent analysis of the short fragment of the *mtDNA-cr* (Niedziałkowska, 2017), which identified only Clade East and Clade Central-West in the European lineage. However, that analysis was based on very short DNA sequences (about 400 bp) and may have delivered much lower phylogenetic resolution.

The evolutionary relationships produced by the phylogenetic trees and the network based on the complete mitogenome was not in agreement with the network based on amino acid variants of mitochondrial protein-coding genes. We found six amino acid haplotypes among the moose mitogenomes in the two evolutionary lineages (Tab. 1, Fig. 2b). The amino acid variants of the Asian and European moose lineages differed in 5–11 amino acid substitutions. The divergence of protein variants between the Asian lineage and two of the European clades (Central and East) is comparable with the protein variation identified within Clade West of the European lineage. The haplotypes from Clade Central and Clade East differed in as few as 1–2 amino acid substitutions and consequently were grouped (Fig. 2b). We detected no evidence of divergence between Clade Central and Clade East with respect to amino acid sequences, although the strong division of these clades is visible in their nucleotide sequences. Protein evolution does not always follow the diversification of DNA sequences, especially in evolutionarily young groups (Fink et al., 2004). We also found no signs of positive or negative selection in protein-coding genes of moose mitochondrial genomes (data not shown).

Estimates of divergence times and evolutionary history of moose

Using five calibration points for the Cervidae set, we estimated the divergence time of individual lineages in two approaches, assuming the lognormal (Supplementary Figure S9) and normal (Fig. 5) distribution prior for the age of the crown Cervidae. The first approach provided estimations on average 2 Mya (0.1–3.6 Mya) older than the latter (Supplementary Table S6). In further description and discussion, we considered the results of the second approach. The calibration points were based on the latest results by Mennecart et al. (2017), who unambiguously attributed the fossil *Euprox furcatus* (with the age of 13.8 Mya) to crown Cervidae. As a consequence, our estimations pushed back the origin of crown Cervidae and other lineages on average 3 Mya in comparison to previous analyses (Pitra et al., 2004; Gilbert et al., 2006; Hassanin et al., 2012; Bibi, 2013; Chen et al., 2019). Our estimations are comparable with those by Mennecart et al. (2017) and Zurano et al. (2019), who assumed other calibration points in the global time-calibrated molecular phylogeny of Cetartiodactyla using mitochondrial data. The split between Cervidae and Moschidae+Bovidae estimated by us is also in the range of calculations based on 110 nuclear protein-coding genes of 21 Cetartiodactyla species (Zhou et al., 2011). Estimates dating 3.5 to 5.5 Mya further back than ours were obtained only on cytochrome b for selected cervid lineages (Randi et al., 1998). Our analyses included the largest number of calibration points for Cervidae, and the recent palaeontological discoveries and calculations by Mennecart et al. (2017). Therefore, we think that they are the most reliable.

The divergence time of *Alces* lineages was estimated in the Cervidae time-calibrated phylogeny to the median value of 443 kya with 95% HPD interval 309–610 kya. This result was used to calibrate the phylo-

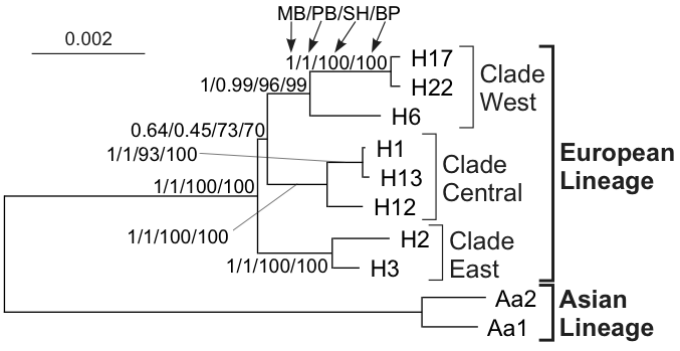

Figure 4 – The MrBayes tree obtained for the *Alces* set. The numbers at nodes, in the order shown, correspond to posterior probabilities estimated in MrBayes (MB) and PhyloBayes (PB) and the support values obtained by the approximate likelihood ratio test based on a Shimodaira–Hasegawa-like procedure (SH) and bootstrap method (BP) calculated in IQ-TREE.

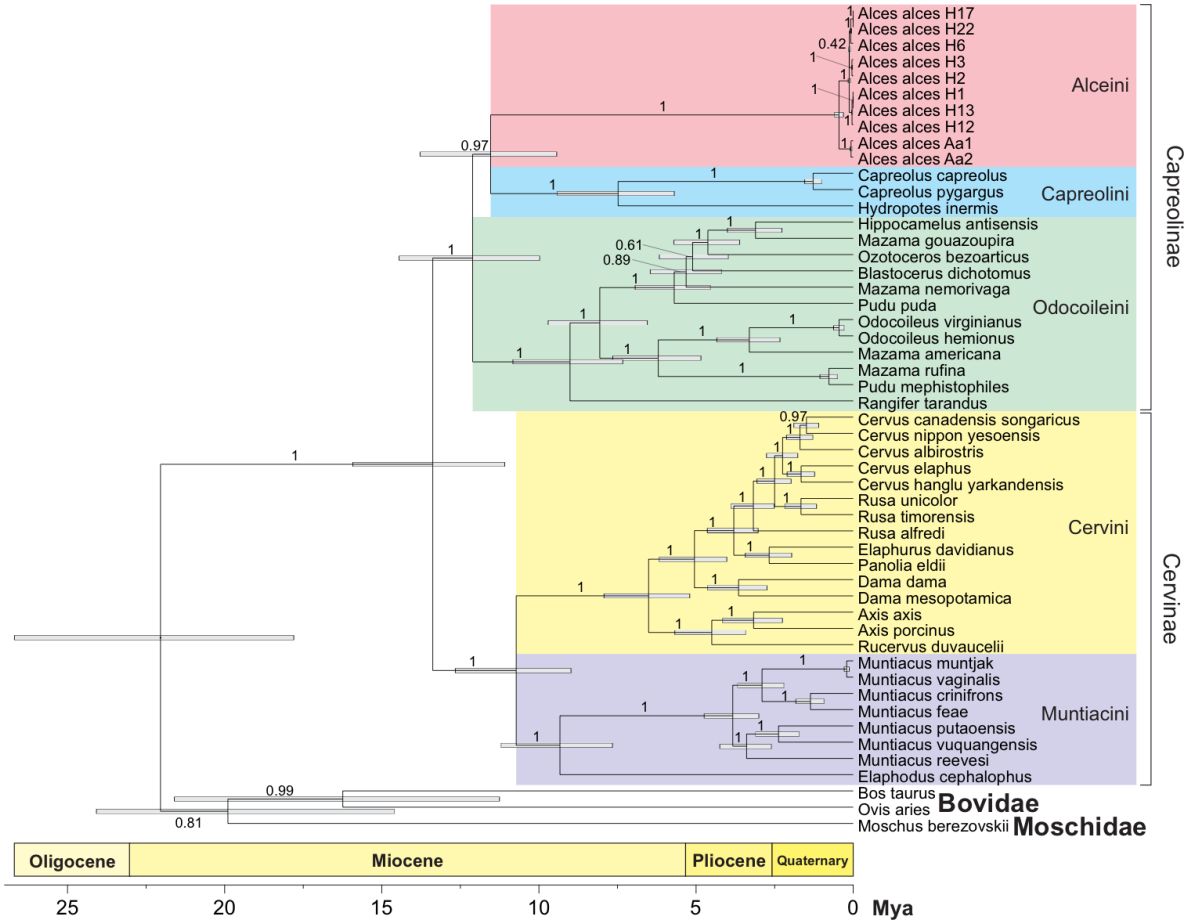

Figure 5 – The chronogram for the Cervidae set assuming the normal distribution prior for the age of the crown Cervidae. Posterior probabilities are shown at nodes. Bars correspond to 95% HPD range. The average, median and 95% HPD of main splitting events were included in Table S6.

geny of the *Alces* set, in which this estimate was tuned up to the median value of 437 kya with 95% HPD interval 253–621 kya (Fig. 6; see Supplementary Figure S10 for estimations assuming other priors, which gave slightly older divergence times). Our result suggests that the split of the Asian and European moose lineages occurred in the Middle Pleistocene. It is later than the estimate by Pitra et al. (2004), i.e. ca. 873 kya and considerably earlier than reported by other authors from 21.5–150 kya (Hundertmark et al., 2002; Udina et al., 2002; Niedziałkowska et al., 2014; Niedziałkowska, 2017). It is worth noting that Mikko and Andersson (1995) estimated this time to 165–350 kya, which overlaps with 95% HPD interval obtained by us. This variability in estimated divergence times may result from differences in calibration methods and sequences used. The previous analyses were based on selected markers with shorter sequences, while the complete mitogenomes used here.

Our estimates for the split of the present-day clades of the European moose lineage indicate that three clades diverged in a quick succession: first, the Clade East at 110 kya with 95% HPD 58–169 kya and next Clades Central and West at 100 kya (52–154 kya) (Fig. 6, see also Supplementary Figure S10). The diversification of these clades coincides with the transition from the Penultimate Glacial Period (MIS 6) to the Eemian interglacial (MIS 5) and can be explained by the contraction-expansion model (Taberlet et al., 1998; Lister, 2004; Hofreiter and Stewart, 2009). This concept assumes that during glacial periods, larger populations were fragmented and isolated in refugial areas, where they were subjected to bottleneck and genetic drift. Consequently, it caused the evolution of distinct lineages with different genetic pools, which expanded during warmer periods (interglacials). Each clade of the European moose lineage is at a different stage of the lineage sorting process. Clade West diversified at ca. 68 kya (34–109 kya), while Clades Central and East at about 35–37 kya (14–64 kya). Diversification of the most abundant Clade East could be underestimated due to the small sample size, although two sequenced mitochondrial genomes (H2 and H3), which belong to this clade, came from the most distant subclades (E2 and E3) defined by Niedziałkowska (2017). The recent divergence of *A. alces* European clades, in combination with their reciprocal monophyly found in our study, could be a result of low effective population size over their evolutionary history, affected by severe bottlenecks during the Last Glacial Period (Weischelian glaciation, 115–11.7 kya). Nevertheless, more samples and calibration points directly associated with *Alces* should be included to verify the estimates presented here. Ancient DNA proved to be extremely helpful in investigating the evolutionary history of numerous species (Soubrier et al., 2016; Ciucani et al., 2019). Thus, future analyses of ancient DNA from moose fossil records collected throughout Eurasia (i.e. Sommer and Nadachowski, 2006) could help in refining phylogenetic relationships in moose and shed more light on its past genetic variability and distribution, both before and after the LGM.

Although the time of the *Alces* divergence estimated by us exceeds the emergence of *Alces alces* postulated previously between 100 kya and 200 kya (Lister, 1993), the molecular dating can be easily reconciled with new palaeontological records of moose and their interpretation (Fig. 6). A predecessor of the modern moose is *Alces* (*Cervalces*) *latifrons*, which was widely distributed in Eurasia from MIS 31/30 (1.1 Mya) to MIS 7/6 (0.2 Mya) and in North America since the Middle Pleistocene until the end of the Late Pleistocene (Boeskorov, 2005; Breda and Marchetti, 2005; Stefaniak et al., 2014; van der Made et al., 2014). *A. latifrons* was highly diversified and this ancestral variation could be inherited by modern *Alces*. Between MIS 7 (200 kya) and MIS 6 (170 kya) in Eurasia there existed a form described as its subspecies *Alces* (*Cervalces*) *latifrons postremus* or as a separate species *Alces* (*Cervalces*) *postremus*. It is likely that this species spread into North America through the Beringian Bridge in the late Middle Pleistocene (Boeskorov, 2005). This moose is regarded by some authors as

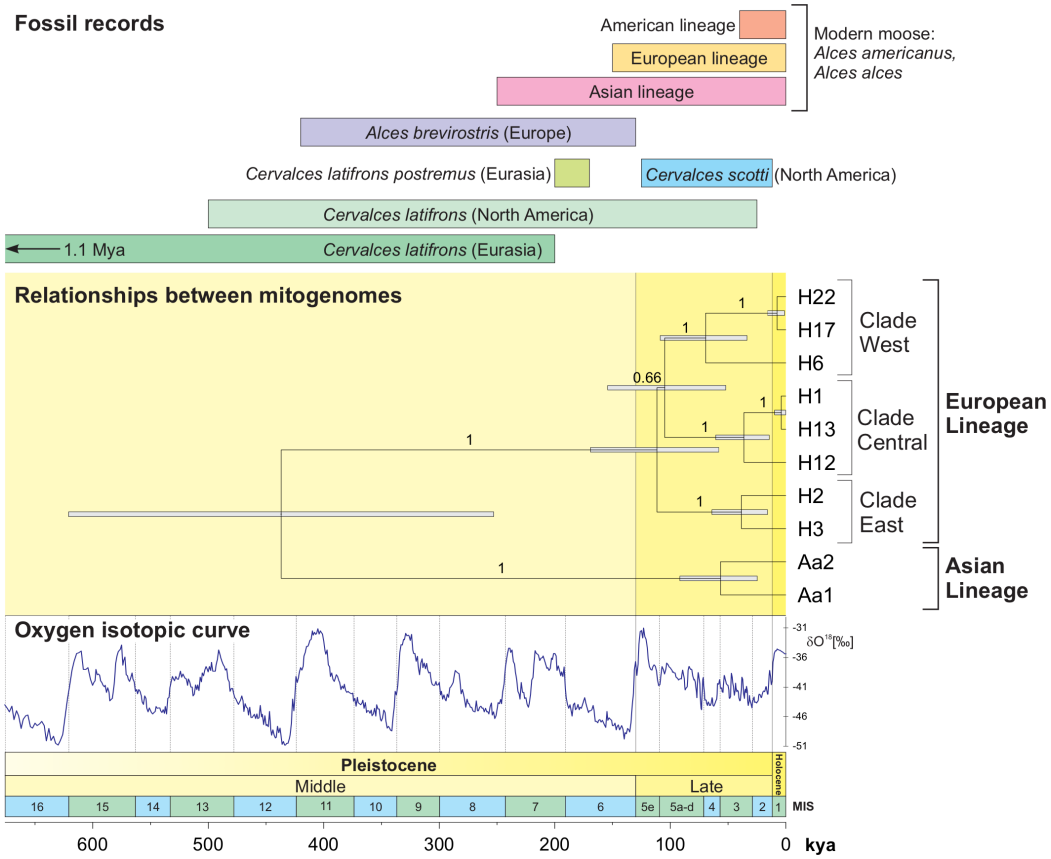

Figure 6 – The chronogram for the *Alces* set compared with the distribution of fossil records and the oxygen isotopic curve. Posterior probabilities are shown at nodes. Bars correspond to 95% HPD range. MIS means Marine isotope stages. The δ18O curve was compiled from several sources depending on four time slices: 0–9750 yrs. BP from NGRIP1 (Rasmussen et al., 2014; Seierstad et al., 2014), 9770–10,630 yrs. BP and 56,070–122,230 yrs. BP from NGRP2 (Rasmussen et al., 2014; Seierstad et al., 2014), 10,650-56,050 yrs. BP from Combined Cariaco and Greenland Ice Core Chronology 2005 (GICC05) (Cooper et al., 2015) and 123,000–5,320,000 yrs. BP from the benthic curve by Lisiecki and Raymo, 2005. This tree was obtained assuming for the age of the root 95% confidence interval 309–610 kya as obtained in the dating for the Cervidae set assuming the normal distribution prior for the age of the crown Cervidae.

an intermediate form leading to the modern *Alces* (Heintz and Poplin, 1981; Breda and Marchetti, 2005). Taking into account its stratigraphic age and our molecular dating, *Alces postremus* predates the divergence of the European clades and could be an ancestor of this lineage. The oldest remains of true *Alces* are dated to MIS 6 (150 kya) and become more abundant in MIS 5 sediments in Eurasia (Breda and Marchetti, 2005), which perfectly corresponds to the molecular divergence time of European clades.

According to another hypothesis, *Alces brevirostris* found in Hungary was the intermediate form between *Alces latifrons* and *Alces alces* (Vörös, 1985; Kahlke, 1990; Breda and Marchetti, 2005; Nikolskyi, 2010). This species could be an ancestor of both the European and Asian lineages because its stratigraphic age, MIS 11 to 6 (424–130 kya; Breda and Marchetti, 2005; Stefaniak, 2015), is in the 95% HPD range of the split time between these lineages obtained in molecular dating.

In contrast to the above-mentioned views, Boeskorov (2006) found no evidence supporting a direct phyletic link between the evolutionary lineage of true moose and *Alces latifrons*. Therefore, he postulates that the evolutionary lineage of true moose most likely developed in parallel, starting even as early as the end of the Late Pliocene or the beginning of the Pleistocene. Yet, remains of a real ancestral form of *Alces* have not been found.

The emergence of the modern *Alces* occurred most likely in Eastern Europe or Asia (Breda and Marchetti, 2005), likely in Western Siberia and the Far East as indicated by genetic data (Hundertmark et al., 2002; Udina et al., 2002; Boeskorov, 2003). Such origin is also supported by the large number of primitive features in teeth of Asian moose (Nikolskiy and Boeskorov, 2012) and moose remains in Eastern Siberia dated to ca. 250 kya (MIS 8/7), which are older than those from Europe or Western Asia (Nikolskyi, 2010). The age of these fossils agrees with our estimates, indicating that the Asian lineage already diverged at that time. Nevertheless, the phylogenetic position of the third North American mitochondrial lineage of moose (identified by Hundertmark et al., 2002), presently distributed in North America and some regions in Eastern Asia, is unclear. Future mitogenomic and nuclear DNA analyses are necessary to fully unravel the phylogeny of three apparent mtDNA lineages of moose in the context of two species, *Alces alces* and *Alces americanus*, distinguished by some authors (Boeskorov, 1999, 2003; Grubb, 2005).

The relict character of European moose populations

The moose populations from Poland and Sweden are genetically impoverished and were dramatically reduced in size in the last century (Dzięciołowski and Pielowski, 1993; Charlier et al., 2008). Noteworthy, Chen et al. (2019) described a much earlier population declines in a ruminants genome survey. Currently, moose possessing relict haplotype H6 (Clade West) are found from southern Norway over Sweden to northern Finland, while haplotypes H1 and H13 (Clade Central) are restricted to the central and northeastern parts of Poland, though occasionally identified in Belarus (Hundertmark et al., 2002; Świsłocka et al., 2008, 2013; Niedziałkowska et al., 2014). Both clades (Central and West) are restricted to the edge of the western range of the moose in Europe. On the other hand, haplotypes H2 and H3 represent the most widespread Clade East, which is spread across most of the continuous moose distribution range in Eurasia and has undergone recent expansion in Poland (Fig. 1; Świsłocka et al., 2013).

Our phylogenetic analyses based on nucleotide and amino acid sequences yielded evidence of the relict status of the moose population inhabiting Norway, Sweden, and northern Finland, characterized by the predominance of haplotype H6 (amino acid variant A2) in several hundred thousand moose that live there (Wennerström et al., 2016). It is strongly differentiated from the other haplotypes of Clade West (H17 and H22, both representing the A3 amino acid variant; Fig. 2a and Fig. 2b). It is possible that this relict moose population repres-

ents descendants that survived the Last Glacial Maximum (LGM) in the Italian Peninsula refugium and recolonized Fennoscandia at about 10 kya (Kangas et al., 2015; Niedziałkowska, 2017). However, the relict character of the haplotypes found in moose populations from the Biebrza River valley and the Gostynin-Włocławek Forest (H1, H13) is not so striking or unequivocal. Those haplotypes did not differ in their amino acid variant (A1) from haplotype H12 of Clade Central, and they differed by as few as 1-2 amino acid substitutions from the variants found in Clade East (A4, A5; Fig. 2b). Phylogeographic data suggest that Clade Central recolonized Central Europe from the Carpathian refugium after the LGM (Niedziałkowska, 2017). Our molecular dating is congruent with this assertion. This result, combined with the monophyly of all three European clades and the very limited distribution of the present populations characterized by H1 and H13 haplotypes, supports the autochthonous and therefore relict status of these populations. Moreover, clear distinction of Clade Central as a relict lineage is particularly important in the context of its preservation over time. Based on haplotype frequencies (Świsłocka et al., 2013; Niedziałkowska, 2017) we estimate that there are no more than 1500–2500 individuals possessing Clade Central mtDNA, mostly occupying the northeastern Poland. Observed expansion of Clade East and proposed abolition of moratorium under which this game species thrived in Poland since it was almost wiped out in mid/late 1990s could affect the long-term survival of Clade Central relict mtDNA. Thus, our results should be considered while making any policy related to management of moose populations, especially those from Biebrza River Valley and the surrounding areas.

Our study and the available data clearly suggest that the most important factors in shaping the phylogeographic pattern of moose were: isolation, bottleneck and divergence in separate refugia during the last glaciation, as well as rapid post-glacial recolonization of deglaciated areas, which resulted in the presence of relict population at the westernmost edge of the species' range.

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Supplemental Table S1 List of primer pairs used for PCR and sequencing of mitogenomes in *Alces alces*.

Supplemental Table S2 List of ungulate species and GenBank accession numbers of their mitogenomic sequences used in phylogenetic analysis.

Supplemental Table S3 Substitution models and partitions applied for the Cervidae mitogenomic set.

Supplemental Table S4 Substitution models and partitions applied for the *Alces* mitogenomic set.

Supplemental Table S5 Substitution models and partitions applied for two mitogenomic set in molecular dating in BEAST.

Supplemental Table S6 Molecular dating of the main splitting events within Cervidae.

Supplemental Figure S7 The consensus of trees obtained in three approaches for the Cervidae set.

Supplemental Figure S8 Individual trees obtained in three approaches for the Cervidae set.

Supplemental Figure S9 The chronogram for the Cervidae set assuming the lognormal distribution prior for the age of the crown Cervidae.

Supplemental Figure S10 The chronogram for the *Alces* set.