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Ancient DNA sequences from *Coelodonta antiquitatis* in China reveal its divergence and phylogeny

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Ancient DNA data have supported a sister relationship between woolly rhinoceros and extant Sumatran rhinoceros. This relationship has been used to explore the divergent times for the woolly rhinoceros from their relatives. Complete and partial ancient DNA sequences of the mitochondrial cytochrome *b* (*cyt b*) gene were retrieved from bones of the late Pleistocene *Coelodonta antiquitatis* excavated from northern and northeastern China. The newly obtained sequences together with the European and northern Asian *Coelodonta antiquitatis* sequences from GenBank were used to estimate the evolutionary divergence time. Phylogenetic analyses showed the exchange of genetic information between the Chinese individuals and *Coelodonta antiquitatis* of north Asia, which also indicated a more recent evolutionary timescale (3.8–4.7 Ma) than previous molecular estimations (17.5–22.8 or 21–26 Ma) for woolly rhinoceros based on the fossil calibration of outgroups. This new timescale was more consistent with the fossil record of the earliest known genus *Coelodonta*.

***Coelodonta antiquitatis*, cytochrome *b* gene, divergence time, phylogeny**

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As one of the most well-known periglacial extinct animals, *Coelodonta antiquitatis* was highly adapted to life on cold grasslands during the late Pleistocene in the Northern Hemisphere from 72°N to 33°N (Zhou, 1978; Jiang, 1982; Álvarez-Lao et al., 2006; Kahlke et al., 2008; Nie et al., 2008). In Asia, the earliest representative of the genus, *Coelodonta thibetana* (3.7 Ma), has been found in the Zanda Basin in southwestern Tibet (Deng et al., 2011), while another fossil species, *Coelodonta nihowanensis* (2.5 Ma), has been found in the Linxia Basin in Gansu Province, northwestern China (Deng, 2002). Comparably, the earliest fossil species in northern Asia, *Coelodonta tologoijensis,* has been found around the early middle Pleistocene in Buryatia, Russian Federation, where is nearby China. The European woolly rhinoceros appeared around 400–460 ka, such as one of the early fossil sites in Romania, eastern Europe (Kahlke et al., 2008). Therefore, some researchers suggested that the woolly rhinoceros may have originated in China (Deng, 2002; Deng, 2008; Elias et al., 2008; Kahlke et al., 2008; Deng et al., 2011). However, it is difficult to infer when woolly rhinoceros reached Europe due to the incompleteness of its early fossil records (Kahlke et al., 2008; Deng et al., 2011). In late Pleistocene, the extensive fossil records showed little variation between European and Asian woolly rhinoceros in terms of morphological characteristics, leading to the suggestion that there was only one species of

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woolly rhinoceros i.e., *Coelodonta antiquitatis,* during this period (Zhou, 1978).

Both as typical members of the "*Coelodonta-Mammuthus* Fauna" in late Pleistocene, the woolly rhinoceros and the woolly mammoth have received unequal attentions at the molecular level. The phylogenetic position of the woolly mammoth has been fully resolved by multiple sampling locations (Yang et al., 1996; Noro et al., 1998; Lister et al., 2001; Debruyne et al., 2003; Krause et al., 2006; Rogaev et al., 2006; Yang et al., 2006; Gilbert et al., 2008; Miller et al., 2008; Enk et al., 2009), whereas DNA studies on *Coelodonta antiquitatis* were focused only on very limited sampling sites in Europe and northern Asia (Orlando et al., 2003; Binladen et al., 2006; Willerslev et al., 2009; Lorenzen et al., 2011). Among the interested issues of the woolly rhinoceros, the close phylogenetic relationship between this species and the modern Sumatran rhinoceros was accepted by previous ancient DNA studies (Orlando et al., 2003; Willerslev et al., 2009). As to the divergent time of the woolly rhinoceros from its relatives, both Orlando et al. (2003) and Willerslev et al. (2009) obtained very similar results of 21–26 and 17.5–22.8 Ma, respectively, based on the fossil calibration dates of 56 Ma (the split time between the equids and ceratomorpha) (Xu et al., 1996) and 60 Ma (the split time between Artiodactyla and Cetacea) (Arnason et al., 1996). However, based on the same calculations, they failed to recover the date of about 2 Ma for the emergence of the *Equus* genus (Orlando et al., 2003). Moreover, these molecular dating results were not consistent with the earliest known fossil records of 3.7 Ma (Deng et al., 2011). Therefore, issues remain with the woolly rhinoceros regarding their origin, phylogeography, dispersion, and divergence time (Orlando et al., 2003; Scott, 2007; Elias et al., 2008; Kahlke et al., 2008; Willerslev et al., 2009; Deng et al., 2011). Clearly, more sequences from individuals at various locations are needed for a better understanding of these evolutionary history questions.

In China, not only the middle Pliocene fossils but also a large number of late Pleistocene fossils have been found from various locations. Fossil findings indicated that the *Coelodonta antiquitatis* was widely distributed in Northeastern and Northern China Plain in late Pleistocene deposits (Zhou, 1978; Huang, 1979; Luo et al., 1983; Jin et al., 1984; Lu et al., 1986; Jiang, 1991; Cai et al., 1992; Zheng et al., 1992; Pei, 2001; Li et al., 2007; Nie et al., 2008). However, no ancient DNA studies have taken the advantage of these materials from China, and thus the phylogenetic and phylogeographical issues of *Coelodonta antiquitatis* remain insufficient in terms of the fossil distribution.

In this study, we obtained *cyt b* sequences from *Coelodonta antiquitatis* bones excavated from Salawusu (Inner Mongolia), Zhaodong and Qinggang (Heilongjiang Province). Our studies aimed at revealing the detailed taxonomic status of *Coelodonta antiquitatis* from China and providing insights into the evolutionary history of this extinct species.

1 Materials and methods

1.1 Sample information

Five late Pleistocene femur, radius, and ischium bones of *Coelodonta antiquitatis* are included in this study: (1) two samples (*C.a.*_SL1, *C.a.*_SL4) from Salawusu, Inner Mongolia Autonomous Region, northern China; they were collected from the same stratigraphy, the AMS radiocarbon age of one sample (*C.a.*_SL4) is 42230±370 a BP; (2) two samples (*C.a.*_HS12, *C.a.*_HS14) from Zhaodong County, Heilongjiang Province, northeastern China, and they were collected from the same stratigraphy and one sample (*C.a.*_HS14) was dated as 39625±250 a BP; (3) one sample (*C.a.*_Qg13) from Qinggang County, Heilongjiang Province, northeastern China, and it was dated as 35085±180 a BP (Figure 1, Table 1). The AMS radiocarbon dating of these specimens was carried out in the Archaeological Geochronology Laboratory of Peking University.

1.2 DNA extraction, PCR, and cloning

Ancient DNA extractions and PCR reactions were set up in the Molecular Biology Laboratory at the China University of Geosciences in Wuhan, which is dedicated to ancient DNA research in a building physically separated from post-PCR facilities. Ancient DNA was extracted from about 200 mg of bone powder following the improved silica method (Rohland et al., 2007a, 2007b). To prevent possible contamination, we followed vigorous contamination control measures for bone preparation and DNA extraction (Yang et al., 2005; Rohland et al., 2007a, 2007b). To all samples: the outer layer of the bone was removed using graphite blades and discarded, a cut-off piece of the bone were ground into powder in a mortar. Negative extraction controls were always included throughout the extraction.

We attempted to generate 1140 bp of the mitochondrial *cyt b* gene, using twenty-one newly designed overlapping primer pairs specifically for this study. Multiple PCR amplifications were carried out as described in Krause et al. (2006). PCR products were purified using the QIAEX II Gel Extraction Kit gel (Qiagen, Germany) and cloned into pMD-T18 vector (Takara, Japan) following the supplier's instructions. The recombinant plasmids were transformed into competent *E. coli DH5α*. White transformants obtained from LB plates containing Amp (0.1mg/mL), X-Gal (0.04 mg/mL), and IPTG (0.024 mg/mL) were screened by PCR with the M_{13} primer pair. For each fragment, a minimum of eight clones, four from each of two independent primary amplifications, were sequenced at Shanghai Sangon Ltd. Company using an ABI 3700 sequencer following manufacturers' instructions. When consistent differences were found between the first and the second PCR products, due

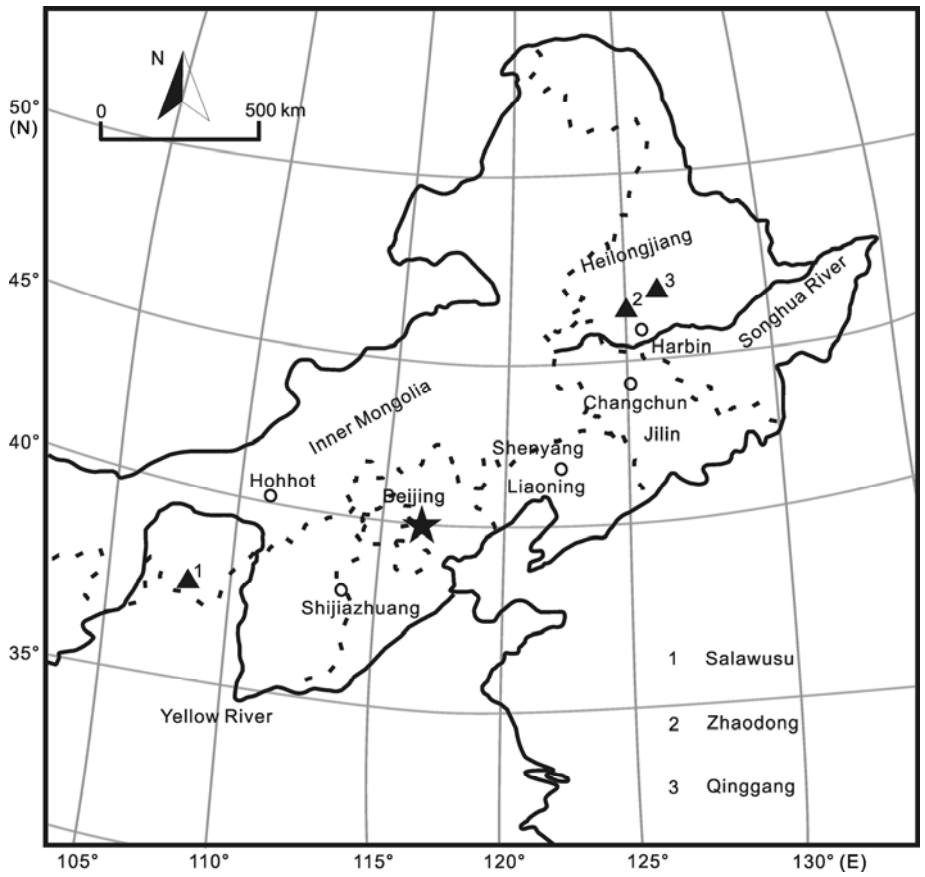

Figure 1 Locations of the *Coelodonta antiquitatis* sampling sites in this study. The samples *C.a.*_SL1 and *C.a.*_SL4 were collected from Salawusu, Inner Mongolia, northern China; the samples *C.a.*_HS12 and *C.a.*_HS14 were collected from Zhaodong County, Heilongjiang Province, northeastern China; the sample *C.a.*_Qg13 was collected from Qinggang County, Heilongjiang Province, northeastern China.

to sequence errors resulting from template damage, a third amplification was performed to determine which sequence was reproducible (Hofreiter et al., 2001).

1.3 Sequence replication

The repeated experiments were carried out at the Centre for Ancient Genetics, University of Copenhagen, which include the samples of *C.a.*_SL1, *C.a.*_SL4, *C.a.*_HS12, and *C.a.*_HS14. Just as the above described extraction operations, first we removed the outer layer of the samples and ground them into powder (about 500 mg each sample). Then added 10 mL extraction buffer for each sample (extraction buffer contains 0.02 mol/L Tris/HCl, 0.01 mol/L DTT, 0.025 mg/mL Prot. K, 10 mg/mL SDS, 0.5 mol/L EDTA), rotated them overnight at 55°C, took the supernatant, and purified them with the PCR purification kit. PCR amplifications were carried out in 25 μL volumes and the PCR mixture consists of 0.08 mg/mL RSA, 1.25 U HiFi Taq, 0.25 mmol/L dNTPs, 2 μmol/L Mg^{2+}, 1×PCR buffer, 0.8 μmol/L each of primer and extraction solution 5 μL. Thermal cycling conditions were 50 cycles of 95°C, 30 seconds/49–53°C, 30 seconds/68°C, 40 seconds. Then PCR products were purified, cloned, and sequenced.

1.4 Phylogenetic reconstruction and estimation of divergence time

In order to investigate the precise phylogenetic position of the Chinese late Pleistocene *Coelodonta antiquitatis*, we analyzed *cyt b* sequences obtained in this study together with three European and northern Asian *Coelodonta antiquitatis* samples (*C.a.*_Orlando, *C.a.*_Willerslev, *C.a.*_Binladen), five extant rhinoceroses (*Dicerorhinus sumatrensis*, *Rhinoceros sondaicus*, *Rhinoceros unicornis*, *Ceratotherium simum*, and *Diceros bicornis*), and various outgroup sequences (*Sus scrofa*, *Pecari tajacu*, *Balaenoptera physalus*, *Bos taurus*, *Herpestes auropunctatus*, *Panthera tigris*, *Ursus arctos*, *Tapirus indicus*, *Phoca vitulina*, *Equus grevyi*, *Equus caballus*, *Artibeus jamaicensis*, *Chalinolobus tuberculatus,* and *Pteropus scapulatus*) retrieved from GenBank (Table 1). We used three datasets to initiate different analyses: (1) a first dataset included *C.a.*_HS12, *C.a.*_HS14, *C.a.*_SL4, *C.a.*_Orlando, *C.a.*_Willerslev, *C.a.*_Binladen, *Dicerorhinus sumatrensis*, *Rhinoceros sondaicus*, *Rhinoceros unicornis*, *Ceratotherium simum,* and *Diceros bicornis* for which 668 bp of the *cyt b* gene were available. This dataset was used for phylogenetic analyses and molecular dating between rhinoceros populations, with

Table 1 List of the woolly rhinoceros and extant rhinoceroses sequences used in this study

Sample No.	Taxa	Age (a BP)	Total sequence length (*cyt b*)	GenBank No.	Reference	Geographic origin
*C.a.*_HS12[a)]	*Coelodonta antiquitatis*	About 39000	1130 bp	GU371439	This study	Zhaodong, northeastern China
*C. a.*_HS14	*C. antiquitatis*	39625±250	1140 bp	GU371440	This study	Zhaodong, northeastern China
*C. a.*_Qg13	*C. antiquitatis*	35085±180	490 bp	JQ974919	This study	Qinggang, northeastern China
*C. a.*_SL1[a)]	*C. antiquitatis*	About 42000	651 bp	JQ974920	This study	Salawusu, northern China
*C. a.*_SL4	*C. antiquitatis*	42230±370	1100 bp	JQ974921	This study	Salawusu, northern China
*C. a.*_Orlando[b)]	*C. antiquitatis*	60000−70000	668 bp	AY178623/AY178624/ AY178625	Orlando et al., 2003	Belgium
*C. a.*_ Binladen[c)]	*C. antiquitatis*	>49000	1140 bp	DQ318533	Binladen et al., 2006	Siberia, Russia
C. a._Willerslev	*C. antiquitatis*	N.A.	1140 bp	NC_012681	Willerslev et al., 2009	Yakut, Russia
Dicerorhinus sumatrensis	*Dicerorhinus sumatrensis*	Modern	1140 bp	AJ245723	Tougard et al., 2001	N.A.
Rhinoceros sondaicus	*Rhinoceros sondaicus*	Modern	1140 bp	AJ245725	Tougard et al., 2001	N.A.
Rhinoceros unicornis	*Rhinoceros unicornis*	Modern	1140 bp	NC_001779	Xu et al., 1996	N.A.
Ceratotherium simum	*Ceratotherium simum*	Modern	1140 bp	Y07726	Xu et al., 1997	N.A.
Diceros bicornis	*Diceros bicornis*	Modern	1140 bp	X56283	Irwin et al., 1991	N.A.

a) The age of specimen based on the stratigraphy; b) the age of the sample is from Orlando et al. (2003); c) the age of the sample is from Binladen et al. (2006). N.A.= not available.

outgroups chosen following Orlando et al. (2003); (2) a second dataset of 289 bp of the *cyt b* gene included eight woolly rhinoceros samples: *C.a.*_HS12, *C.a.*_HS14, *C.a.*_Qg13, *C.a.*_SL1, *C.a.*_SL4, *C.a.*_Orlando, *C.a.*_Willerslev, *C.a.*_Binladen; five extant rhinoceroses: *Dicerorhinus sumatrensis*, *Rhinoceros sondaicus*, *Rhinoceros unicornis*, *Ceratotherium simum,* and *Diceros bicornis*; two outgroup samples: *Balaenoptera physalus* and *Equus grevyi*; (3) a third dataset of 1100 bp of the *cyt b* gene included *C.a.*_HS12, *C.a.*_HS14, *C.a.*_SL4, *C.a.*_Willerslev, *C.a.*_Binladen and *Dicerorhinus sumatrensis*, and the dataset was used for molecular dating between rhinoceros populations.

We performed the first dataset for minimum-evolution (ME) analyses using MEGA Version 4.0 (Tamura et al., 2007). The statistical confidence of each node was estimated by 1000 random bootstrap runs. Divergence time was estimated by using 56 Ma as the split time between the equids and ceratomorpha, or 60 Ma as the split time between Artiodactyla and Cetacea (Xu et al., 1996; Arnason et al., 1996). We then carried out Bayesian analyses for the second dataset using BEAST vl.1.6.1 (Drummond et al., 2007). We selected the substitution model HKY+G and site heterogeneity model Gamma+; the MCMC analyses were run for 100000000 iterations; the length of chains was chosen 100000000, and the parameter of Burn In was set at 1000. Using 56 Ma as the split time between the equids and ceratomorpha (Xu et al., 1996), we estimated the divergence time between woolly rhinoceros and Sumatran rhinoceros. Lastly, we analyzed the third dataset using the software Network 4610 (Bandelt et al., 1999) to estimate the divergence times between rhinoceros populations. Due to the uncertainty of reliable fossil data of woolly rhinoceros, dating of the divergence time between woolly rhinoceros and extant Sumatran rhinoceros was estimated based on the evolution rate of the *cyt b* gene as 2% per million years (Brown et al., 1979).

2 Results

2.1 Fragmented sequences from the Chinese late Pleistocene *Coelodonta antiquitatis*

In comparison with the temperate cave samples from Belgium (Orlando et al., 2003) and permafrost samples from Siberia or northern Asia (Binladen et al., 2006; Willerslev et al., 2009; Lorenzen et al., 2011), specimens used in this study were not well preserved (Lai et al., 2005). The primers used to amplify longer fragments (>200 bp) of the *cyt b* gene (Orlando et al., 2003) showed no positive results in our samples, while the specimens produced positive amplifications when only shorter fragments (95–175 bp) were targeted. In details, twenty-one overlapping fragments of a 1140 bp- long region of the mitochondrial *cyt b* gene were amplified from sample *C.a.*_HS14 collected from Zhaodong County. Fourteen overlapping fragments of a 762 bp and six overlapping fragments of a 378 bp, total 1130 bp-long region of the *cyt b* gene were amplified from sample *C.a.*_HS12 collected from Zhaodong County. There were a 1100 bp-long fragment amplified from one sample (*C.a.*_SL4) and a 651 bp-long fragment from the other sample (*C.a.*_SL1) which was collected from Salawusu. Only a 490 bp- long fragment could be recovered from the sample (*C.a.*_Qg13) collected from Qinggang County.

2.2 Phylogenetic analyses and estimate of divergence time

Phylogenetic results using MEGA and Bayesian analyses show that all ancient *Coelodonta antiquitatis* samples analyzed are clustered together, with the extinct *Coelodonta antiquitatis* group sharing the closest relationship with the extant Sumatran rhinoceros (Figures 2 and 3), which are consistent with previous phylogenetic analyses (Orlando et al., 2003; Willerslev et al., 2009). Notably, our results also reveal that the *Coelodonta antiquitatis* samples from Salawusu together with one sample from Zhaodong County appear at one sub-clade of the *Coelodonta antiquitatis* clade. However, the other samples from Heilongjiang Province (*C.a.*_HS12 and *C.a.*_Qg13) are grouped with the sample from northern Asia (*C.a.*_Willerslev). The samples from Europe (*C.a.*_Orlando) and from Siberia (*C.a.*_Binladen) form another sub-clade (Figures 2 and 3).

We have obtained two sets of divergent timescales for woolly rhinoceros from Sumatran rhinoceros. Firstly, an old timescale that is very similar to previous studies has been calculated based on the calibration time points of outgroups. We obtained 24.5–27.6 Ma via MEGA 4.0 (Figure 2) and 22.5 Ma by BEAST software (Figure 3) when we used the calibration times of 56 Ma between the equids and ceratomorphs or 60 Ma as the split time between Artiodactyla and Cetacea (Xu et al., 1996; Arnason et al., 1996). Secondly, we obtained a much younger split time of 3.8–4.7 Ma when we applied the software Network 4610 (Figure 4) with the evolution rate of the *cyt b* gene as 2% per million years (Brown et al., 1979). Moreover, we estimated that the separation of Siberia sample from Chinese individuals occurred approximately 303–486 ka (Figure 4).

3 Discussion

3.1 Sequence authentication

Authentication of sequences remains the central issue in any ancient DNA studies (Pääbo et al., 2004). According to the

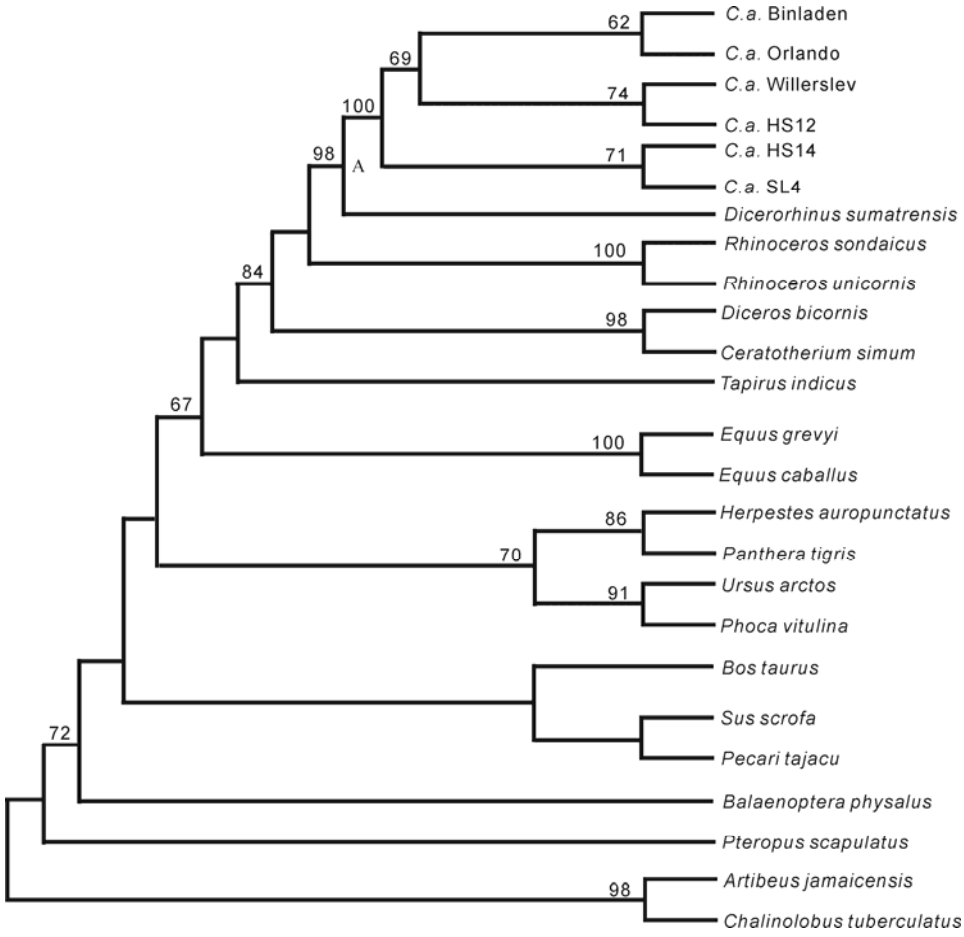

Figure 2 Phylogenetic relationship between *Coelodonta antiquitatis* and extant rhinoceroses. Phylogenetic tree based on 668 bp partial *cyt b* of *Coelodonta antiquitatis* and extant rhinoceroses using MEGA 4.0 with bootstrap values indicated at the branches, derived from 1000 replications, outgroups were chosen following Orlando et al. (2003). For the node A, the divergence time between woolly rhinoceros and Sumatran rhinoceros was estimated at 24.5–27.6 Ma by using 56 Ma as the split time between the equids and ceratomorpha, or 60 Ma as the split time between Artiodactyla and Cetacea (Xu et al., 1996; Arnason et al., 1996).

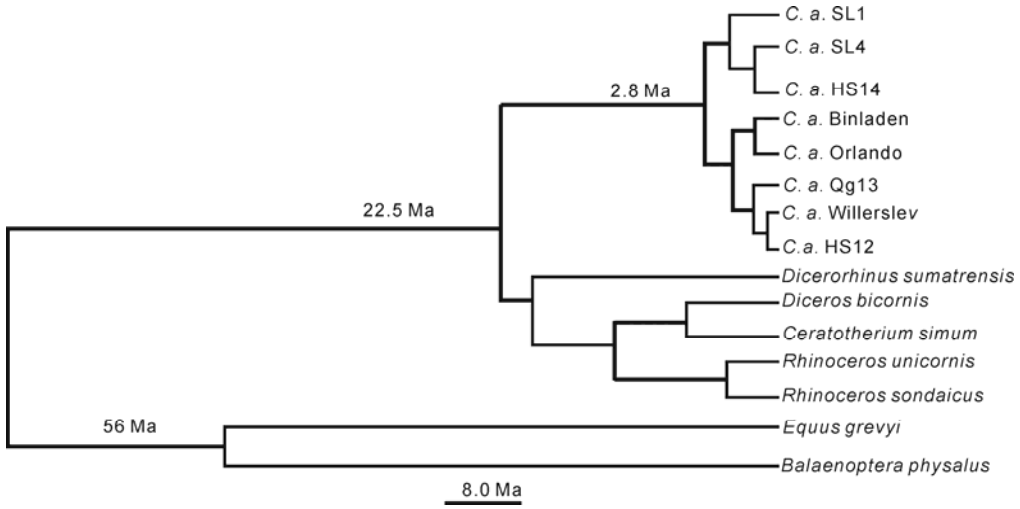

Figure 3 Phylogenetic relationship between woolly rhinoceros and extant rhinoceroses. Phylogenetic tree was reconstructed using BEAST vl.1.6.1 based on 289 bp partial *cyt b* of *Coelodonta antiquitatis* and extant rhinoceroses, the divergence times were indicated at the branches by using 56 Ma as the split time between the equids and ceratomorpha (Xu et al., 1996).

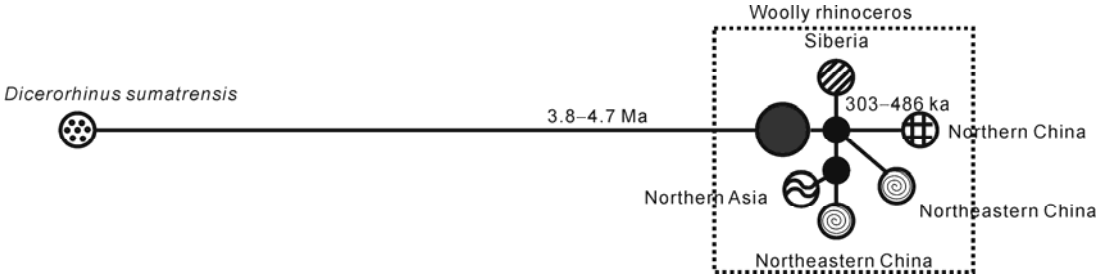

Figure 4 Median Joining network based on 1100 bp *cyt b* sequences calculated in Network 4610. The divergence times were estimated by the evolution rate of *cyt b* gene as 2% per million years (Brown et al., 1979).

international standards, we believe that the sequences obtained from our Pleistocene samples belong to *Coelodonta antiquitatis* for the following reasons: (1) DNA extractions and pre-PCR procedures were carried out in an isolated ancient DNA laboratory, and PCR amplifications and post-PCR analyses were carried out in another laboratory. (2) Complete analysis from extraction to sequencing was carried out at least twice independently for each fragment of all the samples. (3) Contamination was monitored throughout the experiment with extraction and PCR blanks which remained negative. (4) The data were analyzed using BLAST and phylogenetic analysis, and the results indicated that the obtained sequences belong to *Coelodonta antiquitatis*. (5) Most importantly, the molecular experiments for four out of five samples (*C.a.*_HS12, *C.a.*_HS14, *C.a.*_SL1 and *C.a.*_SL4) were independently duplicated at the Centre for Ancient Genetics, University of Copenhagen, and identical results were obtained.

3.2 Phylogenetic status and dispersal events

To investigate phylogenetic status of *Coelodonta antiquitatis* from China, we analyzed *cyt b* sequences of different aged individuals from different locations in Salawusu, Zhaodong County, and Qinggang County. All the phylogenetic trees indicated that the woolly rhinoceros and Sumatran rhinoceros are sister groups, and the results were similar with previous ancient DNA studies in which the complete12S rRNA, partial *cyt b* gene, or whole mitochondrial genome were used for phylogenetic analyses (Orlando et al., 2003; Willerslev et al., 2009). Moreover, our study provides additional clues for describing the phylogeny of the Chinese *Coelodonta antiquitatis*, which gives us new information on its disperse and evolutionary history in late Pleistocene at the molecular level.

Fossil records strongly imply that this genus originated in Tibet (Deng et al., 2011). Phylogenetic analyses based on fossil morphological traits demonstrated that *Coelodonta thibetana* (3.7 Ma) from Zanda Basin in southwestern Tibet occupies the most basal position of the *Coelodonta* lineage which contains three later fossil species: *Coelodonta nihowanensis* (the late Pliocene) from northern China, *Coelodonta tologoijensis* (the early middle Pleistocene) from eastern Siberia, and *Coelodonta antiquitatis* (the late Pleistocene) from northern Eurasia (Deng et al., 2011). The influence of Quaternary ice ages and the uplift of the Qinghai-

Tibet Plateau on the geographical distribution and migration of mammalian fauna have been intensively discussed (Zhou, 1964; Zheng et al., 1992; Deng et al., 2011). It has been suggested that the *Coelodonta thibetana* gradually evolved to be adapt to life in the cold tundra and steppe when the Ice Age began about 2.8 million years ago (Deng et al., 2011). It should be possible for the Chinese woolly rhinoceros adapted to the cold and dry climate and then dispersed to high latitudes, such as northern Asia and Europe, during the middle Pleistocene and /or later time (Deng, 2006). *Coelodonta thibetana* gradually evolved into *Coelodonta nihowanensis, Coelodonta nihowanensis* then evolved into *Coelodonta tologoijensis,* and finally gave rise to *Coelodonta antiquitatis* (Deng, 2008; Deng et al., 2011). Therefore, it is highly likely that woolly rhinoceros might evolve in China. However, up to now the origin of *Coelodonta antiquitatis* has not been fully resolved; many researchers thought that *Coelodonta antiquitatis* originated in Central Europe. At the molecular level, more sequences from individuals of different locations are needed to test the hypothesis.

At the same time, in the phylogenetic trees displayed in Figures 2 and 3, we noticed that the intermediate position of the *Coelodonta antiquitatis* from Heilongjiang Province may reflect the exchange of genetic information, and thus may provide clues to reveal the spread of *Coelodonta antiquitatis* population in Asia during the late Pleistocene. In these phylogenetic trees, samples from Heilongjiang Province belonged to different sub-clades. One sample (*C.a.*_HS14) is clustered with samples from Salawusu, whereas the other two individuals (*C.a.*_HS12 and *C.a.*_Qg13) clustered together with *C.a.*_Willerslev to form another sub-clade. Lorenzen *et al*. (2011) reconstructed the demographic history of *Coelodonta antiquitatis* based on mitochondrial DNA control region sequences, and the results suggested that the species was deeply affected by climate change. In the late Pleistocene strata of northeastern China, pollen analyses showed that ancient floras were composed of *Pinus*, *Pioea*, *Artemisisa*, and members from *Comopositae* and *Chenopodiae*, which reflect a cold periglacial climate at that time (Jiang, 1982; Sun *et al.*, 1983). The cold and dry climate was well-suited for woolly rhinoceros, consistent with a large number of the late Pleistocene *Coelodonta antiquitatis* fossils (Zhou, 1978; Jiang, 1982; Jin et al., 1984; Lu et al., 1986; Xu, 1986; Wei, 1990; Jiang, 1991; Cai et al., 1992). Northeastern China is the highest latitude region in China, and there are no geographic barriers between northern China, northeastern China, and northern Asia. The geography makes it possible for the *Coelodonta antiquitatis* population in northern/northeastern China dispersed to northern Asia in interglacial period, and retreated back to northern/northeastern China in glacial period (Zhou, 1978; Jiang, 1982).

3.3 Evolutionary time of the woolly rhinoceros

We initially obtained woolly rhinoceros and *Dicerorhinus sumatrensis* diverged in Oligocene by using MEGA phylogenetic analyses (24.5–27.6 Ma) and BEAST phylogenetic analyses (22.5 Ma) using the time point of 60 Ma as the radiation of Cetartiodactyla or 56 Ma as the split time between equids and Ceratomorphs (Figures 2, 3). This timescale is similar to the results of previous ancient DNA studies according to partial mitochondrial DNA sequences (21–6 Ma) (Orlando et al., 2003), or 17.5–22.8 Ma based on complete mitochondrial DNA sequences (Willerslev et al., 2009) by using the same fossil calibrations. In this study, we also derived an alternative estimate of the divergence time between woolly rhinoceros and Sumatran rhinoceros considering the evolution rate of the *cyt b* gene as 2% per million years (Brown et al., 1979). The result showed a more recent evolutionary timescale than previous molecular estimations, indicating the separation between woolly rhinoceros and Sumatran rhinoceros occurred at about 3.8–4.7 Ma (Figure 4).

We consider that the older timescale may have overestimated the event in terms of the molecular dating approach of using the fossil calibration of the outgroups. Recent studies suggested that the rates of molecular evolution are time-dependent (Ho et al., 2005; Burridge et al., 2008; Subramanian et al., 2009), and the recent divergence event would be overestimated according to older calibration since the substitution rates were actually much lower than mutation rates (Ho et al., 2011). For example, Burridge et al. (2008) analyzed the freshwater fish vicariance, which suggested a decline in mtDNA evolutionary rates with an increasing age of calibration. They observed rates of 0.031–0.125 changes/site/Ma from river isolation events younger than 200 ka in galaxiid fishes. In contrast, galaxiid rates derived from older events are slower and less variable, in the order of 0.011–0.026 changes/site/Ma. Interestingly, Orlando and colleagues (2003) also found that their calculations were inconsistent with the emergence time of the *Equus* genus (2 Ma) based on the calibration dates of 56 Ma (Xu et al., 1996) and 60 Ma (Arnason et al., 1996).

Hence, we suggest that the younger timescale is more consistent with the convincing fossil records and thus is more likely. Firstly, Deng et al. (2011) reconstructed phylogenetic trees of five extant and thirteen extinct Rhinoceratini taxa based on the morphological characteristics. The result indicates that the 3.7 Ma-old *Coelodonta thibetana* from southwestern Tibet nested within a clade of Miocene-Pleistocene species of *Stephanorhinus*, and is considered to be the earliest representative of woolly rhinoceros. Obviously, the gap between the older molecular dating (17.5–27.6 Ma) and the fossil dating (3.7 Ma) could not be explained by the earlier divergence of the gene tree than the population tree, which suggested that the timing of an evolutionary divergence event can be assumed to be older than

the earliest appearance of its descendant lineages according to morphological analysis (Ho et al., 2011). Secondly, the known fossil records of *Dicerorhinus sumatrensis* are also consistent with the younger time frame (Zin et al., 2008; Tong et al., 2009).

However, our analysis needs further exploration. We noticed that only partial *cyt b* gene sequences were used to carry out the molecular estimation in our study, which may bring some deviation to the result. Moreover, even the evolution rate of the *cyt b* gene as 2% per million years has been widely accepted (Brown et al., 1979; Birungi et al., 2001; Ma et al., 2010; Geng et al., 2011), there is still debate among researchers who hold the opinion that different species may evolve at different rates even for the same gene (Triant et al., 2006). However, even the *cyt b* gene evolution rate of rhino is not exactly 2% per million years, and the bias of the calculations based on this rate will not exceed the gap of 3.7 Ma to around 17.5−27.6 Ma.

At the present, it is generally believed that the true woolly rhinoceros species originated in Central Europe, and the earliest European *Coelodonta antiquitatis* occurred about 400−460 ka according to fossil records (Kahlke et al., 2008). The age of origin for this species should not be younger than this timescale, and then it dispersed to northern Asia and China during the cold and dry period. In our estimation, the separation of true woolly rhinoceros between Siberia sample and Chinese individuals occurred approximately 303−486 ka ago (Figure 4), perhaps shortly after the origination of the true woolly rhinoceros in Europe, and it rapidly diffused to northern Asia and China.

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