AMPLIFICATION OF NUCLEAR MARKERS OF EARLY MEDIEVAL PIG ANCIENT DNA FROM SOUTH MORAVIA

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Abstract

Ancient DNA (aDNA) is a DNA molecule older than 75 years. This molecule is characterized by many special properties. The main characteristics include its fragmentation. The short fragment length is often a limitation of the analysis. In this study, we analyzed historical animal samples from the Pohansko site (South Moravia, Czech Republic), which is dated to the 10th century. Two pig tooth samples were selected for aDNA extraction. After successful isolation, three nuclear DNA markers were amplified by PCR. These markers were selected due to the length of their amplification products (134 bp, 209 bp, and 312 bp, respectively). The amplification of the markers was successful for the *HAL* gene in one sample, and for the *TEAD3* gene in both samples, the *CYP2E1* gene was not successfully amplified in any sample.

Key Words: pig aDNA, ancient DNA, Middle Ages, PCR

Research on aDNA samples covers many fields including paleoanthropology, paleobotany, paleo- or achaeolozoology. Animal bones or teeth are often excavated together with human skeletal remains or as archaic waste. The animal skeletal remains are found in human graves for many reasons – as part of clothing, amulets, sacrificial offerings, as posthumous companions, and aDNA analysis can provide species determination (e.g., PORR and ALT, 2006; GRÜNBERG, 2013). On the other hand, the results of animal aDNA studies provide important information, for example, on livestock breeding, migrations, and domestication (see, e.g., HAN *et al*., 2010, BOSSE, 2018, WANG *et al*., 2022).

The Early Middle Ages in our region are marked by the settlement and colonization of the area by the Slavs. Agriculture formed the basis of their economic throughout the territories they occupied. They bred livestock primarily for the procurement of meat, milk, and leather, as well as for agricultural purposes. The predominant species included cattle and pigs (BARFORD, 2001).

The aDNA molecule differs from recent DNA molecules, making analysis difficult or impossible. The *postmortem* degradation process causes fatal damage to the nucleic acid structure (PÄÄBO *et al*., 2004). Autolysis and the action of cellular enzymes begin immediately after the death of the organism, followed by hydrolysis and oxidation. The result is strand breaks, baseless sites, base alteration, crosslinks, and overall molecule fragmentation (WILLERSLEV and COOPER, 2005). Fragment lengths varied between 100 and 500 bp (PÄÄBO, 1989; HANDT *et al.* 1994; HÖSS *et al.* 1996), not exceeding 300 bp (KEFI, 2011) but most typically between 50 and 200 bp (HUMMEL, 2003). The main causes of damage are exogenous such as ambient temperature and its stability over time, humidity, soil pH, and exposure to sunlight (BURGER *et al*., 1999). These factors are more important for the preservation of aDNA than the age of the sample.

Given that fragmentation is a predominant characteristic of aDNA, we hypothesize that shorter fragments of porcine nuclear aDNA are more amenable to amplification compared to longer fragments.

Material and Methods

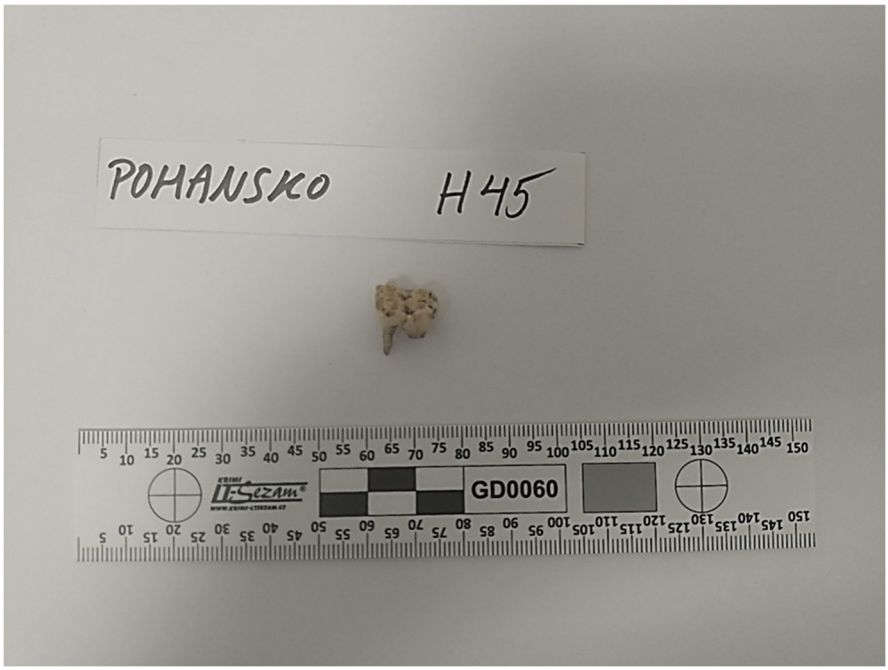
The porcine teeth (Fig. 1 and Fig. 2) originate from the archaeological locality Pohansko near Břeclav (South Moravia) in the Czech Republic. This locality is one of the most important sites of the Slavic era and is dated to the 8^{th}-10^{th} century AD. The bones and teeth of the animals were excavated together with human skeletal remains from graves (DROZDOVÁ, 2005).

The aDNA from the samples was extracted using the protocol optimized in Laboratory of Biological and Molecular Anthropology for extraction from bones/teeth and using the MinElute® PCR Purification Kit (Qiagen). The yield of aDNA was quantified by fluorometer Qubit 2.0 using Qubit™ dsDNA HS Assay Kits (Thermo Fisher) for extreme low amount of extracted DNA.

The amplification process was performed by polymerase chain reaction (PCR) utilizing specific primers targeting the nuclear genes *HAL* (BRENING and BREM, 1992), *CYP2E1* (MÖRLEIN *et al*., 2012), and *TEAD3* (ROBIC *et al*., 2012). The reaction mixture and the amplification conditions were derived from the mentioned publications and their respective optimizations (FALKOVÁ, 2020). The reaction conditions were refined to enhance the amplification of aDNA by increasing the cycle count to 50. Recent porcine DNA served as a positive control for amplification. A negative control was included in each PCR reaction to confirm the absence of contamination. The separation of the amplified products was executed by electrophoresis utilizing a 3% agarose gel. Following this, the PCR products were purified and sequenced using Sanger sequencing. For comparative analysis with a reference sequence of *Sus scrofa*, the software tools BLAST and UniproUGENE (OKONECHNIKOV *et al*., 2012) were employed.

Figure 1. The sample Po H9, mandibula with teeth

Figure 2. The sample Po H45, tooth fragment



Results and Discussion

The isolated aDNA exhibited concentrations of 0.15 ng/µl for sample Po H9 and 0.18 ng/µl for sample Po H45, corresponding to aDNA samples that are generally concentrated in the range of tenths to units of ng/µl.

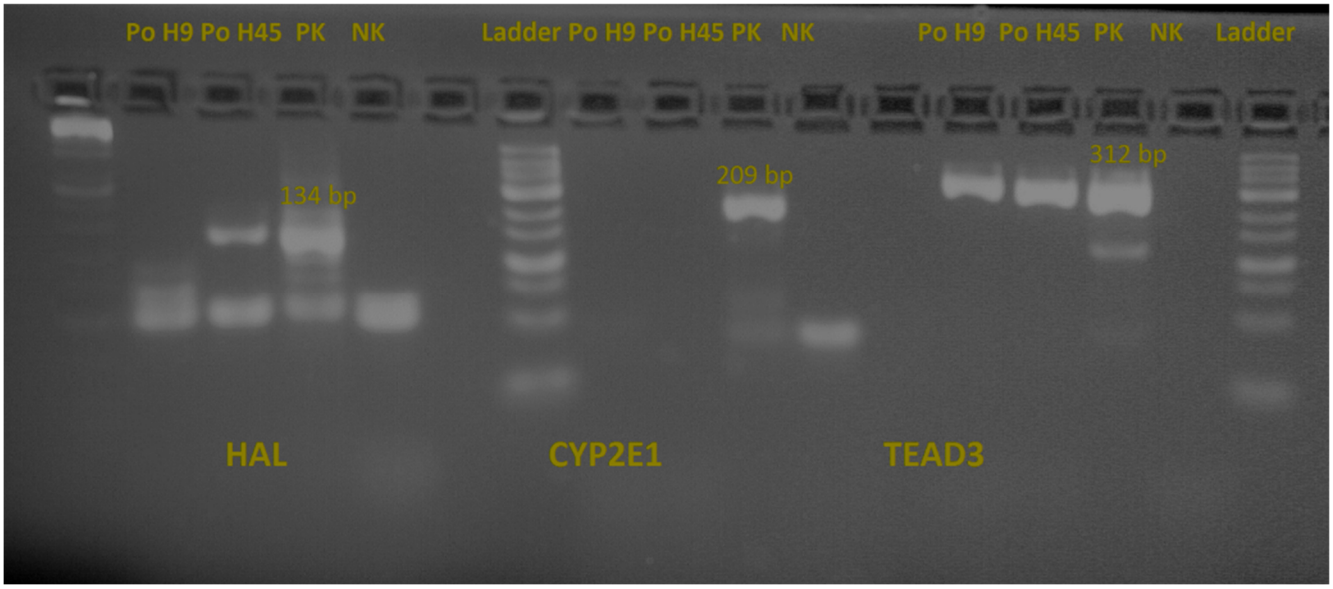
The nuclear genes selected for amplification are important markers for the analysis of health and economical traits in commercial breeds as well as pig genetic resources (e.g. FUJII *et al*., 1991; DVOŘÁK, 1994; HORÁK *et al*., 2004; FALKOVÁ and VRTKOVÁ, 2019). They were chosen for the fragment length of their amplification products (134 bp, 209 bp, and 312 bp, respectively). The *HAL* fragment length of 134 bp could be suitable for aDNA (HUMMEL, 2003). The fragment size of 209 bp of *CYP2E1* is in the middle of the range (e.g. PÄÄBO, 1989) or at the upper limit typical for aDNA (HUMMEL, 2003). The *TEAD3* fragment of 312 bp is relatively long for aDNA molecule (KEFI, 2011).

In the Po H45 sample, the *HAL* marker amplification was performed with a positive result. Visualization on agarose gel showed a 134 bp fragment (Fig. 3), while in the Po H9 sample this fragment was not amplified. In both tested samples, there was no amplification result of the *CYP2E1* marker. On the contrary, in the *TEAD3* marker, the amplification fragment was found in both samples, although the fragment length of 312 bp is quite large for aDNA samples.

Amplification failure may be caused by reasons other than the fragment lengths. The presence of inhibitors such as humic or fulvic acids may prevent PCR amplification (TURROSS, 1994). Since amplification was successful in both samples in *TEAD3*, at least this reaction was not inhibited. On the other hand, PCR inhibition may be due to base modification (LINDAHL, 1993) caused by *postmortem* mutation. These may of course vary between samples, so amplification of one sample may have been successful compared to another sample, such in our study for *HAL* marker, or/and in both samples for *CYP2E1*.

After sequencing, the sequencing data was compared with the *Sus scrofa* reference sequence. The sequence of the *HAL* gene amplification product from Po H45 matched the *HAL* gene from *Sus scrofa*. As well as the sequences of both amplification products of samples Po H9 and Po H45 in the *TEAD3* gene matched the *Sus scrofa* reference sequence in the *TEAD3* gene.

Figure 3. The visualisation of HAL, CYP2E1 and TEAD3 amplification products in porcine aDNA samples



Po H9, Po H45 – the ancient porcine DNA samples; PK – positive control of recent porcine DNA; NK – negative control (water blank); Ladder – Low Range Ladder (Thermofisher Scientific)

Conclusion

The amplification of porcine aDNA samples was successful in the nuclear genome. The amplification yielded the product of the *HAL* gene in one sample, the *TEAD3* gene in both samples. It is noteworthy that the length of the *TEAD3* fragment is relatively long for the aDNA molecule, but amplification was successful in both samples. In conclusion, it could be said that at least in these porcine aDNA samples it is possible to analyze nuclear genome markers older than a thousand years.

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