

# Using DNA markers to reconstruct the lifetime morphology of barley grains from carbonized cereal crop remains unearthed at Usvyaty Settlement

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## Abstract

Research on taxonomic and genetic diversity of cultivated plants provides valuable data that help to clarify how the flora of cultivated plants was formed in a particular region. Paleogenetics, a discipline that developed at the intersection of molecular biology, archeology and genetics, helps to explore plant origin and changes in plant genotype during evolution. By the 12<sup>th</sup> century, the economy of medieval Northern Russia was based on a developed farming culture. Tracing the origin of its constituent agricultural traditions is an important fundamental task. The objective of this work was to study and identify the species affiliation of carbonized fossil remains of plants that grew in the Russian Northwest in the 12<sup>th</sup> century using morphological and molecular genetic methods. The results of the morphological analysis of grain fossils showed that most of the unearthed plant material was barley. Other cereals, such as wheat, rye and oat, were also observed. Molecular genetic studies helped to clarify the species affiliation of partially destroyed grains and reconstruct their lifetime morphology.

**Keywords:** *H. vulgare*, barley, *Nud*, aDNA, naked

## Introduction

Barley has a number of morphological features that predetermine its intended uses. One of the important traits is the presence of glumes in the grain. Hulled barley has a firm glume which is not easily removed; it protects the embryo from mechanical damage (Yu et al., 2016). Glumes in naked barley are loose and easily detached from the kernel during threshing, leaving a clean grain (Vavilov, 1924b; Zheleznov, Kukoeva, and Zheleznova, 2013). The areas of hulled barley cultivation stretch from Greenland to the Sahara and rise to mountainous areas up to 5,000 m above sea level. The Asian Sino-Japanese center gave origin to all naked, awnless or half-awned barleys (Vavilov, 1924a). Naked two-row barley is common for the eastern half of European Russia; it is sporadically cultivated in the black-earth region of European Russia and in Semirechye (Regel, 1922). Harlan (1920) found out in his studies that the nakedness or hulledness of barley grain was controlled by the presence of a cuticular lipid layer which regulates the adhesion of the hull to the pericarp epidermis (Harlan, 1920). Presently, this character is known to be associated with the *Nud* (*nudum*) gene mapped on chromosome 7HL (Fedak, Tsuchiya, and Helgason, 1972; Kukichi, Taketa, Ichii, and Kawasaki, 2003). This gene encodes the transcription factor AP2/ERF (Apetala2/Ethylene-responsive factor). The AP2/ERF transcription factor belongs to the ethylene response factor family involved in lipid biosynthesis. This gene plays a role in lipid biosynthesis and induces the formation of an additional layer of cells between the pericarp and the glume (Taketa et al., 2008). Of late, the CRISPR/Cas9 technology has been used to obtain a naked phenotype

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from the hulled Golden Promise barley by knocking out the *Nud* gene (Gerasimova et al., 2020), thus unambiguously confirming the role of *Nud*.

There are hypotheses about either polyphyletic (Orlov, 1929; Helback, 1959; Zhukovsky, 1964) or monophyletic origin of naked barley (Taketa et al., 2004; Taketa et al., 2008), based on the data of morphological and molecular genetic analyses. The hypothesis of a multiple independent origin implies the emergence of naked forms independently in Abyssinia, the Far East, Central Asia, and Turkey (Orlov, 1929; Helback, 1959). Studying landraces of Himalayan barley lines made it possible to regard the Himalayas as a separate center of origin for naked barley (Murphy and Witcombe, 1986; Dickin, Steele, Edwards-Jones, and Wright, 2012). Gustafsson (2013), while conducting molecular genetic studies, ascertained the presence of two mutations in the *Nud* gene, associated with hulledness or nakedness, and independent origins of the northern and Asian naked barley forms (Gustafsson, 2013).

The theory of monophyletic origin implies the emergence of cultivated barley forms from a single ancestor. Taketa et al. (2004) confirmed this hypothesis by their molecular analysis of the *sKT7* marker alleles linked to the *Nud* gene locus. Comparing the identical *sKT7* sequences in Ethiopian and Himalayan lines, the authors disproved their independent origin. Later, Taketa et al. (2008) used the cloning of the *Nud* gene locus to prove the presence of a deletion confirming the monophyletic origin of naked barley (Wabila et al., 2019).

The crop composition in the ancient Russian state and its western neighbours in the 10<sup>th</sup>–15<sup>th</sup> centuries incorporated a rich diversity of cultivated cereals. Most of the archeological finds contained mainly rye, wheat, barley, and oat (Pankin and Korff, 2017). At the same time, barley was the main crop in the lands of the Baltic States and Poland until and including the 10<sup>th</sup> century. In the three centuries that followed, barley was replaced with rye. However, barley retained its priority among crops in the lands of Pskov and Smolensk (they were part of the ancient Russian state). That is why both hulled and naked barleys were more frequently identified in the finds within the territory of the ancient Russian state of the 10–13<sup>th</sup> centuries (Kiryanova, 1979; Ereemeev and Furasiev, 2021).

This publication presents a study of carbonized cereal seed remains dating back to the 12<sup>th</sup> century, found during excavations in Usvyaty Settlement. Morphological methods were applied to identify the species composition of the find. In addition, molecular genetic methods were used in the work with damaged cereal grains. PCR markers for the *Nud* gene were employed to distinguish between hulled and naked barley forms: such technique made it possible to clarify the allelic state of *Nud* and thereby specify the lifetime morphology of severely dam-

aged barley grain fossils. Primer sequences for this gene were selected from published sources (Yu et al., 2016).

## Research materials

The target material of the study was a large set of plant seeds found during the excavations in Usvyaty Settlement, Russian Northwest, dated back to the 12<sup>th</sup> century (Ereemeev, 2021; Ereemeev and Furasiev, 2021). About 34,000 carbonized grains had been conserved as a result of a fire. The dried and carbonized ancient plant remains of the 12<sup>th</sup> century were kindly provided by Dr. Ivan I. Ereemeev, Senior Researcher of the Institute for the History of Material Culture.

## Clarification of the species diversity in cereals based on the grain microrelief analysis

Affiliation of cereals to a certain species was established according to the following morphological species-specific traits:

*Hordeum vulgare* L. — Cultivated barley, or common barley. Grains are hulled, adherent to glumes. The shape is elongated-elliptical, pointed at both ends; glumes have longitudinal veins. The groove is wide. The grain surface is smooth or slightly wrinkled. These traits were absent in severely damaged grains.

*Triticum aestivum* L. — Bread wheat, or common wheat. Grains are naked, less frequently hulled, not adhered to glumes, elongated; the grain surface is smooth, and the groove is wide. The shape of the grain is oval-elongated, ovoid, oval or dolioform. The cross section of the grain is more or less rounded, ribbed or roundish-triangular. The grain shape can be oval-elongated, ovoid, oval or dolioform. The grain cross-section is more or less orbicular, costate or roundish-triangular.

*Secale cereale* L. — Common rye. Grains are naked, elongated, pointed towards the base; the surface is finely wrinkled, and the groove is deep. The structure of the rye grain is very close to that of wheat.

*Avena sativa* L. — Common oat. Grains are hulled; glumes are not adhered to the caryopsis but loosely envelop it. Grains of hulled forms are fusiform, strongly pointed towards the top. The surface of the glumes is smooth, and the groove is wide.

## Molecular genetic methods of studying ancient DNA

Published sources were used to select pairs of primers to identify the species and species-specific traits of fossil plant remains (Table 1). The elongation factor 1 $\alpha$  sequence (a housekeeping gene), conservative for the Poaceae family, was chosen to identify the presence of DNA of the studied material in the samples. This gene encodes

**Table 1. List of primers used in the study**

Primer	Forward primer	Reverse primer	Product size, bp	Reference
HvNud2	GAAGAGGAGGGTGTGGTTG	GGAAGTTGGTCTTGGCGTT	132	Yu et al., 2016
Elongation factor (EF) 1 $\alpha$	AAGGATCTCAAGCGTGGG	GTGGGATGTGTGGCAGTC	154	Yu et al., 2016

an isoform of the alpha subunit of the translation elongation factor complex, responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome during protein biosynthesis (Nielsen, Kleinhofs, and Olsen, 1997).

The hulledness/nakedness character in ancient barleys was identified through the analysis of the allelic state of the *Nud* gene.

DNA was isolated under conditions precluding contamination with foreign DNA. DNA isolation was carried out under conditions excluding contamination from other organisms of foreign DNA. In order to prevent the loss of ancient DNA (aDNA) all studies were held in separate laboratory rooms, where no works with modern cereals had been carried out before. The used PCR box was equipped with a UV lamp. All the instruments used were rinsed in 96% ethanol, then in bidistilled water several times. In between works laboratory rooms and instruments were exposed to UV rays. All work was carried out in sterile protective clothing and shoes, double gloves, disposable hats, as well as in masks and goggles. A DNeasy Plant Mini Kit (Qiagen) was used to extract DNA. The process of DNA isolation was in line with the manufacturer's protocol. The stage of aDNA refragmentation (enrichment) was conducted using a Sigma-Aldrich GenomePlex Complete Whole Genome Amplification (WGA) Kit. The DNA fragment enrichment technology through targeted hybridization makes it possible to accumulate a pool of DNA frag-

ments to improve the accuracy of assembling extended genome regions. DNA concentrations were measured using the spectrophotometric method on a NanoPhotometer NanoDrop device.

PCR was performed in a 20  $\mu$ l reaction mixture containing 200 ng of template DNA (after refragmentation), MgCl<sub>2</sub> at a concentration of 12 mM, 1  $\mu$ l of forward and reverse specific primers to a final concentration of 100 mM, dNTP at a concentration of 2.5 mM, PCR buffer B ( $\times 10$ ), and 5 units/ $\mu$ l of Taq-Polymerase, under the following conditions: predenaturation for 2 min at 94°C; 13 cycles consisting of denaturation for 15 s at 94°C, template annealing with primers for 30 s at 65°C (with temperature reduction of 0.7°C/cycle), and polymerization for 45 s at 72°C; 24 cycles consisting of predenaturation for 15 s at 94°C, template annealing with primers for 30 s at 56°C, and polymerization for 45 s at 72°C, and completion of PCR fragments for 10 min at 72°C. The PCR products were separated in 1.5–2% horizontal agarose gel stained with ethidium bromide. Amplification products were visualized on a Biorad Gel Doc<sup>TM</sup>XR+ documenting system.

## Research results

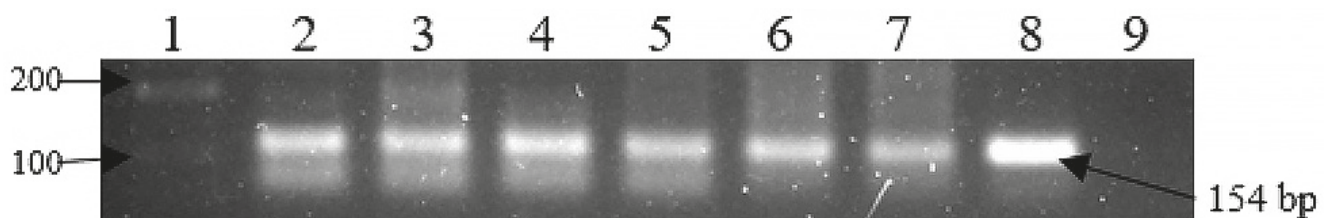
Morphological analysis of carbonized grains helped to determine the species affiliation of most samples (85%, Fig. 1). The initial study of morphological traits and the mi-



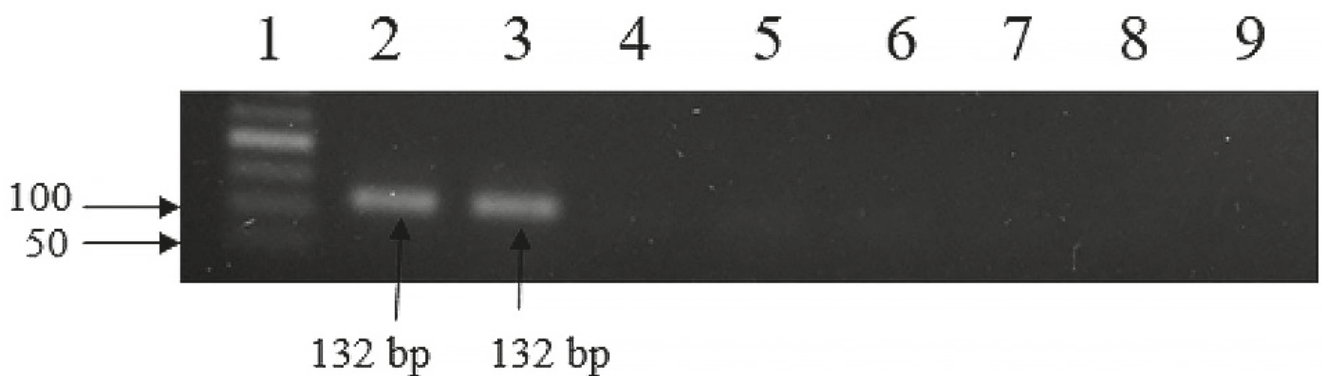
**Fig. 1.** Samples of cereal crop grains found during the excavations at Usvyaty Settlement. (A) *Secale cereale*. (B) *Avena sativa*. (C) *Hordeum vulgare*. (D) *Triticum aestivum*.



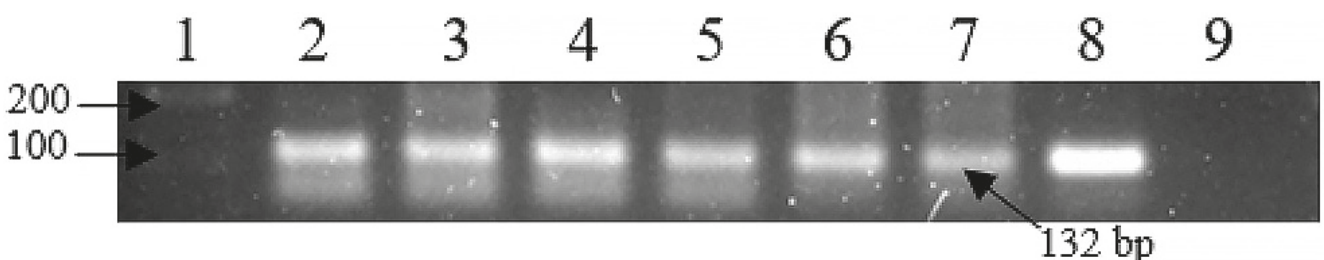
**Fig. 2.** Samples of damaged grains found during the excavations at Usvyaty Settlement



**Fig. 3.** Electrophoretic patterns of amplification products using contemporary or ancient cereal DNA samples as templates with the elongation factor 1 $\alpha$  primers. 1 — molecular weight marker St 100; 2–4 — DNA samples from ancient barley; 5–6 — DNA samples from partially destroyed grains; 7 — DNA sample from contemporary naked barley; 8 — DNA sample from contemporary hulled barley; 9 — negative control.



**Fig. 4.** Electrophoretic patterns of amplification products using contemporary cereal DNA samples as a template with a pair of HvNud2 primers. 1 — molecular weight marker St 50; 2–3 — DNA samples from hulled forms of *H. vulgare*; 4–5 — DNA samples from naked forms of *H. vulgare*; 6 — contemporary DNA sample from *T. aestivum*; 7 — contemporary DNA sample from *A. sativa*; 8 — contemporary DNA sample from *S. cereale*; 9 — negative control.



**Fig. 5.** Electrophoretic patterns of amplification products using control (fresh seeds) DNA samples or aDNA from carbonized seeds (six aDNA samples are shown) as a template, respectively, and the HvNud2 primer pair. 1 — marker of molecular weight St 100; 2–3 — ancient DNA samples from whole grains of *H. vulgare*; 4–6 — ancient DNA samples from partially destroyed grains of *H. vulgare*; 8 — contemporary DNA sample from hulled *H. vulgare*; 9 — negative control.

crorelief of grains clarified that approximately 14% of the studied set of samples were wheat grains, 8% rye grains, 8% oat grains, and less than 1% weed seeds, but the largest part (more than 60%) consisted of hulled barley grains.

It should be mentioned that the techniques of morphological analysis proved ineffective for 10% of grains with severe damage, since external traits were blurred (Fig. 2). Molecular genetic methods could potentially be applied to clarify the species affiliation of such grains. We tested 40 partially destroyed samples that we believed to be barley grains.

The DNA concentrations in the damaged grain samples varied from  $0.95 \pm 0.0095$  ng/ $\mu$ l to  $2.00 \pm 0.02$  ng/ $\mu$ l. After refragmentation, the ancient DNA concentration was equal to  $218.20 \pm 2.1825$  ng/ $\mu$ l.

According to the results of PCR with a pair of EF 1 $\alpha$  primers, amplification products of the expected length (154 bp) were obtained for both the control DNA (from fresh seeds) and aDNA (Fig. 3), which confirmed that the quality of DNA extracted from carbonized grains with subsequent refragmentation was suitable for PCR.

For further studying the allelic state of the *Nud* gene, the selected pairs of primers (HvNud2, Table 1) were first tested on DNA samples of hulled and naked barleys isolated from the accessions of the VIR collection. Amplification products (132 bp) were obtained by PCR from hulled barley samples (as an example, Fig. 4 shows the results of PCR with one of the primer pairs). Such products were not recorded for naked barley forms. Besides, no amplification products were found in other closely related cereal species, so the species specificity of the primers was confirmed (Fig. 4).

In the attempts to apply two primer pairs of the *Nud* gene for aDNA we had to settle on the primer pair that amplified a shorter fragment, since amplification primers failed to procure a product when used on the DNA isolated from carbonized grains, reliably identified by the morphological analysis as hulled barley grains. DNA was then isolated from partially destroyed carbonized grains, presumably (according to the results of the morphological analysis) belonging to barley. For this purpose the pair of HvNud2 primers was used which yielded a 132 bp amplification product (Fig. 5).

As a result, aDNA isolated from all 40 tested samples (partially destroyed carbonized grains presumably related to barley) allowed amplification of a *Nud* gene fragment.

## Discussion

*Hordeum vulgare* belongs to a group of plants that were domesticated about 11,000 years ago in Southwest Asia. Barley was one of the major crops that subsequently spread from Southwest Asia to Europe, Africa, and Asia (Vavilov, 1924a, 1929; Warren et al., 1968; Zohary and

Hopf, 2000; Badr et al., 2000; Lister et al., 2018). Charles and Bogaard (Charles and Bogaard, 2010) registered barley remains during the excavations on an archaeological site in the western part of Central Asia in the south of Turkmenistan. Zohary and Hopf (Zohary and Hopf, 2000) found plant remains in the Caucasian region (5,000 BC). The earliest data on barley domestication in South Asia (Mehrgarh, Pakistan) were collected by Costantini (1984). It is noteworthy that the authors observed the predominance of naked barley forms in this region. The earliest six-row naked forms, which had been cultivated for about 9,000 years, were found at Ali Kosh in Khuzestan (Helback, 1959; Lister et al., 2013). A large number of cereal crop remains were unearthed during the excavations at archaeological sites in the countries of the Old World (Helback, 1959; Mascher et al., 2016; Lister et al., 2018), but such explorations were few in Russia. For example, remains of plants belonging to the Triticeae tribe and dating back to the 8<sup>th</sup>–9<sup>th</sup> centuries were excavated at Goryane Settlement on the Western Dvina (Eremeev and Dzyuba, 2010). The authors continued to analyze this find and discovered the predominance of rye. Rye grain remains were recorded at the Belarussian sites (Zagortsy, Demidovka Village, Bantserovskoye, Bliznaki Village, Staraya Ladoga, and Kamno) dating back to the second half of the 1st millennium AD (Kiryanova, 1979). Rye prevailed in the finds at the sites of the 10<sup>th</sup>/the first half of the 13<sup>th</sup> centuries in Belarus and in Pskov and Smolensk Provinces, while wheat and barley were less common. The 11<sup>th</sup>-century paleontological material from Latvia attested to the dominance of rye among crops (Trofimova, Krylasova, and Sarapulov, 2016).

The material of this study was a set of carbonized grains of the 12<sup>th</sup> century from the excavations at Usvyaty, one of the oldest towns in the Russian Northwest (founded in 1021). Morphological analysis helped to conclude that barley prevailed in the find. The presence of wheat, rye and oat was also observed, but in smaller amounts. The predominance of barley in Usvyaty Settlement pointed to the archaic nature of the grain composition, characteristic of an earlier period, since in the 12<sup>th</sup> century rye was the dominant crop in Russia. Besides, the analysis of grain fossils showed that there were damaged cereal grains that could not be attributed to any species using morphological methods. Disappearance of their external traits prompted the use of modern molecular genetic techniques of paleogenetics. Paleogenetic methods made it possible to analyze the structure of aDNA isolated from biological remains of various ages and origins (Brown et al., 1994; Poinar et al., 2001; Blatter, Jacomet, Schlumbaum, 2002; Bennett and Parducci, 2006; Dabney et al., 2013; Rawlence et al., 2014; Weyrich, Dobney, and Cooper, 2015; Chen et al., 2020). Paleogenetic approaches to the study of archaeological material enabled us to clarify the species affiliation of partially destroyed grains

and study the allelic state of one of the genes responsible for intraspecific variability of barley in grain morphology: the *Nud* gene, determining the hulled or naked grain type.

We have studied and developed a method for isolating ancient plant DNA. Such studies with fossil remains of cultivated grains are unique in Russia. The results of the molecular genetic analysis of aDNA showed:

- species affiliation of the studied grains to *Hordeum vulgare*;
- suitability of the HvNud2 primer pair, amplifying a 132 bp fragment, for the analysis of aDNA;
- absence of naked barley samples among the partially destroyed grains of barley in the studied archaeological find as well as among the whole grains easily identified by morphological analysis.

The prospects of using DNA analysis to specify the characteristics of ancient plant “cultivars” have much greater potential. A new approach to working with plant fossils was proposed in the article, which makes it possible to isolate aDNA and clarify the archaeobotanical data of the destroyed fossil material of plant origin. Currently, the work is particularly relevant. Identification of species, origin, domestication, distribution of cultivated plants and reconstruction of the populations that inhabited a certain area in the past are questions that cannot be answered only with the help of morphological archaeobotanical studies.

## Conclusion

This paper presents the results of a study of carbonized ancient cereal crop grains of the 12<sup>th</sup> century, unearthed during the excavations at Usvyaty Settlement. Analyzing the microrelief of grains helped to find out that most of the material were barleys belonging to *Hordeum vulgare* (and not rye, widespread at that time in this region), which attests to the archaic nature of this find. The outer cover in some grains was destroyed due to their long-term burial in soil layers, thus obstructing their identification. Destroyed grains were studied using molecular genetic methods, because it was difficult to identify them with morphological techniques. It is shown that the aDNA analysis makes it possible not only to clarify the species affiliation but also reconstruct the appearance of the plants whose fragmented carbonized seed were unearthed during archeological excavations, taking into account their intraspecific diversity, including variability in morphological characters. It has been established that only hulled barley forms were present among the material of the 12<sup>th</sup> century excavated at Usvyaty Settlement.

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