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Michał ANISZEWSKI, Michał DROŻDŻEK, Jacek NIEDŹWIECKI, Justyna Anna NOWAKOWSKA, Rafał SOLECKI, Anna TEREBA, Piotr WITOMSKI

THE PARAMETERS OF THE ENVIRONMENT OF DEPOSITION AND MOLECULAR ANALYSES OF WATERLOGGED ARCHAEOLOGICAL WOOD FROM THE EARLY MEDIEVAL SITE OF CZERMNO IN EASTERN POLAND

The following paper presents an environmental examination and the results of the selected analyses of archaeological waterlogged oak wood (Quercus sp.) obtained from excavations carried out at the early medieval site of Czermno in eastern Poland near the Ukrainian border. Due to the good state of preservation of the wood tissue (De Jong's classification class III – maximum moisture content Umax < 185%) and its deposition in near anaerobic layers (reed peat and calcareous gyttja) an attempt was made to obtain the DNA sequence from samples acquired from the uncovered historical construction. In the course of the research, a DNA sequence was obtained from one sample derived from the trial pit W4/2014, the radiocarbon dated to 776-982 calAD. A comparison of the DNA data extracted from the historical wood to two sequences obtained from the trees (Quercus robur L.) growing near the site of Czermno, indicates clear similarities between each of them. The DNA sequence obtained from the archaeological oak wood confirms the assumption that proximate anaerobic layers, under specific conditions, can inhibit the degradation of DNA structure.

Keywords: waterlogged archaeological wood, soil science, environmental analysis, ancient DNA analyses, nuclear microsatellites, chloroplast DNA, archaeological site of Czermno

Michał ANISZEWSKI✉ (*m.aniszewski@post.pl*), Scientific Association of Polish Archaeologists, Warsaw, Poland; Michał DROŻDŻEK (*michal_drozdzek@sggw.pl*), Department of Wood Science and Wood Protection, Warsaw University of Life Sciences, Warsaw, Poland; Jacek NIEDŹWIECKI (*jacekn@iung.pulawy.pl*), Institute of Soil Science and Plant Cultivation, Department of Soil Science Erosion and Land Conservation, Puławy, Poland; Justyna Anna NOWAKOWSKA (*j.nowakowska@uksw.edu.pl*), Cardinal Stefan Wyszynski University in Warsaw, Faculty of Biology and Environmental Sciences, Warsaw, Poland; Rafał SOLECKI (*rafal.bruno.solecki@gmail.com*), Cardinal Stefan Wyszynski University in Warsaw, Institute of Archaeology, Warsaw, Poland; Anna TEREBA (*a.tereba@ibles.waw.pl*), Forest Research Institute, Department of Forest Ecology, Sękocin Stary, Poland; Piotr WITOMSKI (*piotr_witomski@sggw.pl*), Department of Wood Science and Wood Protection, Warsaw University of Life Sciences, Warsaw, Poland

Introduction

The early medieval site of Czermno is located in eastern Poland in the administrative district of Lublin Voivodeship, Tomaszów Lubelski County, in the Community of Tyszowce. Relicts of a gord (Slavic burgwall) and other constructions presented at the site (fig. 1) are alleged to be the capital city of the Cherven Cities [Poznański 2011; Piotrowski and Wołoszyn 2012], a territory which was an area of conflict in the 10th century between the tribe of Lendians and the Kievan Rus', and later in the 11th century between Poland and the Kievan Rus'. These confrontations are mentioned in the chronicle called *The Russian Primary Chronicle/The Tale of Bygone Years* which is the primary source describing the history of the Eastern Slavs and the Kievan Rus' [Cross and Sherbowitz-Wetzor 1953; Wołoszyn et al. 2015].

Fig. 1. The archaeological site of Czermno, visible relicts of the gord (Slavic burgwall) and other constructions, M. Poznański

The Huczwa river flows in its nearest proximity to the archaeological site of Czermno. Two meliorations of the river carried out in the 20th century [Documentation in the Archive of WZMiUW Lublin] and almost certainly climate change, resulted in the subsidence of the peatbog. This in turn, led to several dozen piles (constructional elements) to become visible in the turf (fig. 2). The subsidence of the peatbog may have a fundamental effect on the

in-situ preservation of the archaeological waterlogged wood in the future at this early medieval site.

Fig. 2. One of the oak (*Quercus sp*.) piles visible in the turf, M. Aniszewski

Since 2012, archaeological surveys have been conducted in the flood--meadows situated to the north of the gord, on plot number 23/1 in the local land record [Solecki et al. 2012, 2013, 2014]. One of the main purposes of this interdisciplinary research project, which combines archaeology, analyses and conservation of archaeological wood and analyses of the environment, is to determine the state of preservation and to identify the destructive factors posing a threat to the relicts of an early medieval wooden structure which is a part of the communication system of the Czermno settlement complex. The function of this structure – a pier, a bridge or a causeway – will be determined during the course of the research. So far, there hasn't been any excavations of this construction and its chronology is unknown. The analyzed structure leads from the gord's gate in a north-easterly direction and its track is clearly visible on the LIDAR documentation made for this area (fig. 3).

The state of the preservation of wooden relics depends more on the environmental conditions in which they were kept until the moment of their discovery, than on the duration of their deposition in archaeological layers. Of considerable significance to the speed and scale of decay of wood tissue, is the type of environment and its oxygenation, moisture, pH value, temperature, the

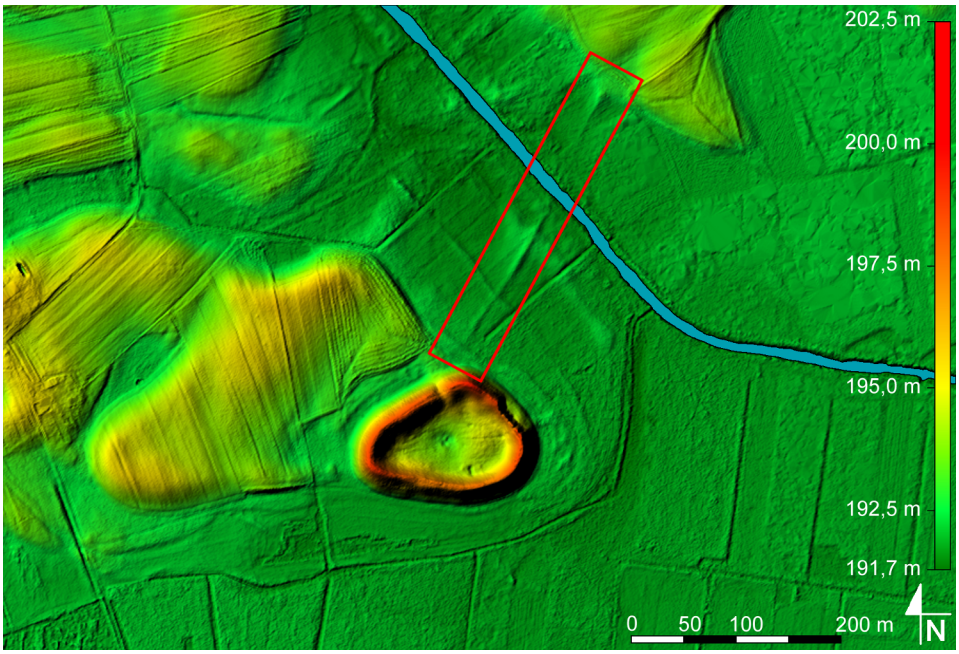

Fig. 3. The analyzed structure (area marked in red) visible on Digital Terrain Model, R. Solecki

presence of microorganisms, and sometimes, seasonal changes in conditions. The process of wood degradation proceeds more slowly under anaerobic conditions, occurring in swamps and peatbogs [Kim and Singh 2000; Aniszewski and Witomski 2013; Hoffman 2013; Broda et al. 2015]. In order to characterize the types of soils in which the historical construction (a part of the communication system of the Czermno settlement complex) is deposited, a pedological survey was carried out parallel to the archeological works. The level of degradation of the archaeological wood is often evaluated based on a fairly sophisticated physical, chemical and instrumental analysis – such as the maximum moisture content, the basic density, the porosity, the wet and dry volume densities and the content and proportion of structural substances [Florian 1990; Jensen and Gregory 2006; Babiński et al. 2014; Tamburini et al. 2015].

Due to the good state of preservation of the archaeological wood tissue – class III in De Jong's classification [McConnachie et al. 2008] – deposited in near anaerobic layers at the site of Czermno, an attempt was made to obtain a short fragment of DNA sequence, from samples acquired from the historical construction under examination. This is because its parameters provide the possibility for the best preservation of organic relicts and protect them from the most destructive degradation agents.

Plant remains carry molecular information, the analysis of which can provide new and substantial information. Molecular methods developed in forensic

sciences can be very helpful in increasing the effectiveness of work with DNA from ancient wood samples. However, the degradation process of nucleic acids over time is inevitable and double strand breaks rapidly reduces the length of the PCR product [Gilbert et al. 2007]. Jiao et al. [2015] indicates that it is possible to extract a DNA fragment ranging from 100 bp to 800 bp from samples of about 80 years old, however, it was not possible in archaeological samples (dating from 3600 year-old wood) using the same protocol. Nevertheless, a short DNA fragment of 100 bp was successfully amplified from oak wood of approximately 400 years old [Speirs et al. 2009]. An even more spectacular case was described by Tani et al. [2003], where a DNA fragment of 600 bp was obtained from a piece of wood from the excavations of a dam. The statistical data show that the ancient DNA is characterized by a much higher number of transitions than might be attributed to enzymatic errors [Hansen et al. 2001]. The combining of more efficient methods of recovery of aDNA helps in understanding the biochemical basis of damage occurring in aDNA, but this area of research still needs further exploration and development. The second problem in working with aDNA is contamination and appropriate controls must also be implemented [Yang and Watt 2005]. It seems that the best preserved DNA should be found in seeds and pollen tissue, because of the long-term ability of the survival of these organs but most of these plant remains, come down from the sediments flotation in water. The impact of water on the level of DNA preservation is not clear so far, however, waterlogged wood remains, prove to be a good reservoir of aDNA [Pollmann et al. 2005]. Furthermore, deciduous oaks are the most commonly found tree taxa group in Europe, archaeological remains can provide an important source of information on genetic diversity in the past [Deguilloux et al. 2006].

Materials and methods

The archaeological works and soils examination

During the archaeological excavations, in the four trial pits, 38 piles arranged in two parallel rows were discovered. To determine the present state of preservation and to identify the destructive factors, examinations were carried out on wood samples taken from the trial pits (W1/2012, W2/2012, W3/2013, W4/2014) to the north of the gord in various spots on the flood-meadows on plot number 23/1 (fig. 4).

According to the stratigraphy determined in the trial pits, wood samples for analysis were obtained from seven archaeological layers, the characteristics of which vary because of differences in the soil type. Soil samples for analysis were collected from seven morphological layers, which the wooden piles passed through vertically (fig. 5).

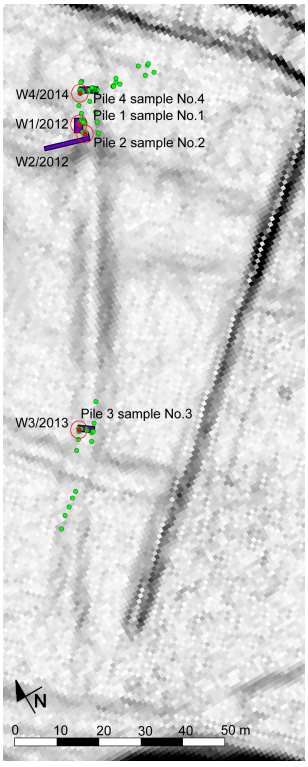

Fig. 4. Wood samples for examination were acquired from four trial pits located in various distances to the Huczwa river (Sample No 1 – 27 m, No 2 – 29,5 m, No 3 – 97 m, No 4 – 20 m), R. Solecki

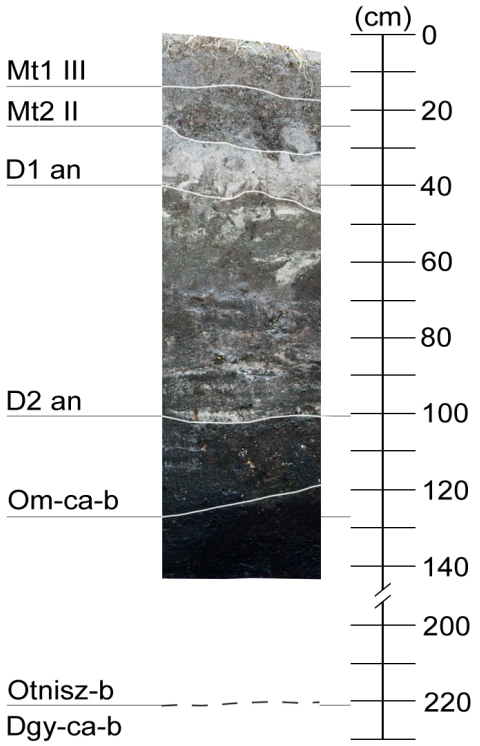

Fig. 5. Archaeological section in one of the trial pits with seven morphological layers visible, R. Solecki

The particle size distribution of the mineral layers was derived from tests using a laser diffraction particle size analyzer – Mastersizer 2000. The total *nitrogen* content was analyzed using a vario MACRO cube CN apparatus. To indicate the percentage of humus content in the morphological layers, the Tiurin method was used. The total carbonate ($CaCO_3$) was determined using the Scheibler calcimeter method. The potentiometric titration in aqueous solution technique was used for measuring the soil pH value in a ratio of 10 g of soil to 25 g solution.

Molecular methods

To verify the possibility of amplified DNA in samples from the conducted excavations, a pilot molecular study was performed, both in samples taken from

the ancient wood and from the modern one (as a control). Two kinds of DNA markers, i.e. the nuclear microsatellites and chloroplast DNA (cpDNA) were analyzed in four early medieval oak heartwood samples from the archaeological site of Czermno. Despite of higher polymorphism retrieved from nuclear sequences of DNA, one chloroplast (cpDNA) locus was investigated. In fact, the small molecules of plastid DNA are present in many copies in wood; and they are better preserved from the nuclease activities than long chains of nuclear DNA [Liepelt et al. 2006]. Total DNA from archaeological wood was isolated with NucleoSpin® Soil (Macherey-Nagel) with SL2 lysis buffer and without Enhancer SX. Each analysis of the sample was separate in time and space, carried out with proper blind controls (sample extraction without DNA added). To minimize the risk of contamination before each extraction procedure, the room was sterilized with a UV lamp and the set of pipettes were autoclaved before the extraction and PCR procedures. In addition, each procedure (extraction DNA, PCR, sequencing) was carried out in separate rooms. The amplification of cpDNA marker, located in polymorphic locus trnD-trnT, was carried out using a primers pair D2T2 [AAC TCC ATT TCT TCC ACT T and GAT CCA CAT CAT AAC GAA AT] described by Dumolin-Lapègue et al. [1999] with a total sequence length of 290 bp. The final reaction volumes were 20 µl, containing 10 pmol of each primer, 10 µl Multiplex PCR Kit (Qiagen®) supplemented with a sensitive hot-start polymerase, 4 µl of PCR water and 5-15 ng of extracted DNA. The PCR thermal profile was: 95°C for 15 mins followed by 40 cycles of 30 secs at 94°C for denaturation, 90 secs at an annealing temperature – 50°C, 90 secs at 72°C for elongation. The cycles ended with a final elongation of 30 mins at 60°C. In all the tests PCR reaction standard blind controls (PCR reaction without DNA added) were performed in thermocycler PTC-200™ Programmable Thermal Controller (MJ Research, Inc.), including controls from the extraction step. The PCR products were cleaned up with the Clean-Up Kit (A&A Biotechnology) and sequenced from both primers on ABI 3500 Genetic Analyzer (Applied Biosystems) with a standard manual. Sequences were proofread and checked in BioEdit software ver. 7.2.5 [Hall 1999]. For all four samples, all the steps including extraction, amplification and sequencing were performed twice.

For ancient wood samples, a PCR for eight microsatellite loci, dedicated to the oak species was also performed [Dow et al. 1995; Steinkellner et al. 1997; Kampfler et al. 1998; Muir and Schlötterer 2005]. All loci were amplified in one PCR multiplex reaction in a total volume 10 µl, containing 10 pmol of each primer, 5 µl Multiplex PCR Kit (Qiagen®) and 5-15 ng of DNA in the following thermal profile: 95°C for 15 mins followed by 35 cycles of 30 secs at 94°C for denaturation, 90 secs at a 50°C, 90 secs at 72°C for elongation. Samples were genotyped on an ABI 3500 Genetic Analyzer (Applied Biosystems) and read in Genemapper® ver. 5 (Applied Biosystems, USA).

Additionally, two modern oak samples (*Quercus robur* L., labelled A and B) from the same area, were analyzed for the analogous DNA fragments, i.e. chloroplast locus trnD-trnT and eight nuclear microsatellites. Extraction of total DNA from these samples was performed using the NucleoSpin® Plant II kit (Macherey-Nagel), following the manufacturer's protocol except for some small modifications. The volume of the three buffers was changed: 600 µl 266 of PL2, 150 µl of PL3 and 900 µl of the PC buffer were used. The conditions for sequencing a trnD-trnT fragment and genotyping microsatellites were the same as those for the four ancient wood samples, except the reduced number of PCR cycles (for 35) in the case of the trnD-trnT marker.

Results and discussion

Soils classification

According to the agricultural soil map developed for this region by the Institute of Soil Science and Plant Cultivation, flood-meadows situated to the north of the gord are located within the outline representing muck-peat soils [Agricultural soil map, Archive of ISSaPC Puławy]. In the course of *in situ* and laboratory analyses, the following soils, according to Polish Society of Soil Science classification [Polskie Towarzystwo Gleboznawcze 1989], have been indicated in their stratigraphic order – high mucked layer of peat muck horizon (*Mt1 III*), medium mucked layer of peat muck horizon (*Mt2 II*), anthropogenic mineral layer of loose sand texture classes (*D1an.*), an anthropogenic mineral layer of loamy sand texture classes (*D2an.*), fossil organic mud layer which contains small snails and mussel shells (*Om-ca-b*), a fossil layer of weakly decomposed reed peat of the fibrous structure (*Otnisz-bR1*) and a fossil layer of calcareous gyttja (*Ogy-ca-b*). The table below presents the laboratory results of the pedological survey carried out on the flood-meadows (tab. 1).

Historical wood examination

The microscopic images unambiguously determined that the samples extracted from the construction elements were waterlogged oak wood (*Quercus* sp., fig. 6).

Its colour, ranging from dark brown to almost black is the result of tannins contained in the wood reacting with water soluble iron compounds derived from the environment of deposition [Krutul et al. 2010; Mańkowski et al. 2013]. In the microscopic images (under an Olympus BX41) of the transverse cross sections, a ring-porous structure with an arrangement of large vessels in the earlywood and small ones in the latewood zones are visible. The most characteristic trait of this species is the presence of very wide rays which are clearly visible in the analyzed samples, prepared from the heartwood zone

Table 1. The results of the pedological survey carried out in a trial pit located on plot number 23/1

Morpho-logical layer	Approx. depth [cm]	Soil classiffication [PTG 1989]	Sand [%] 2.0- -0.05 mm	Silt [%] 0.05- -0.002 mm	Clay [%] < 0.002 mm	Total N	Organic carbon	$CaCO_3$	pH H_2O
						%			
Mt1 III	0-20	high mucked layer of peat muck horizon	n.o.	n.o.	n.o.	1.08	20.36	n.o.	7.0
Mt2 II	20-30	medium mucked layer of peat muck horizon	n.o.	n.o.	n.o.	1.92	33.22	n.o.	6.5
D1an	30-50	anthropogenic mineral layer	95.4	4.6	0	0.21	2.54	n.o.	7.0
D2an	50-100	anthropogenic mineral layer	76.1	22.8	1.1	0.26	4.69	n.o.	7.5
Om-ca-b	100-130	fossil organic mud layer	n.o.	n.o.	n.o.	1.80	41.67	33.44	7.5
Otnisz-b	130-220	fossil layer of weakly decomposed reed peat	n.o.	n.o.	n.o.	3.23	75.61	n.o.	7.0
Dgy-ca-b	< 220	calcareous gyttja	n.o.	n.o.	n.o.	0.77	14.48	41.80	8.0

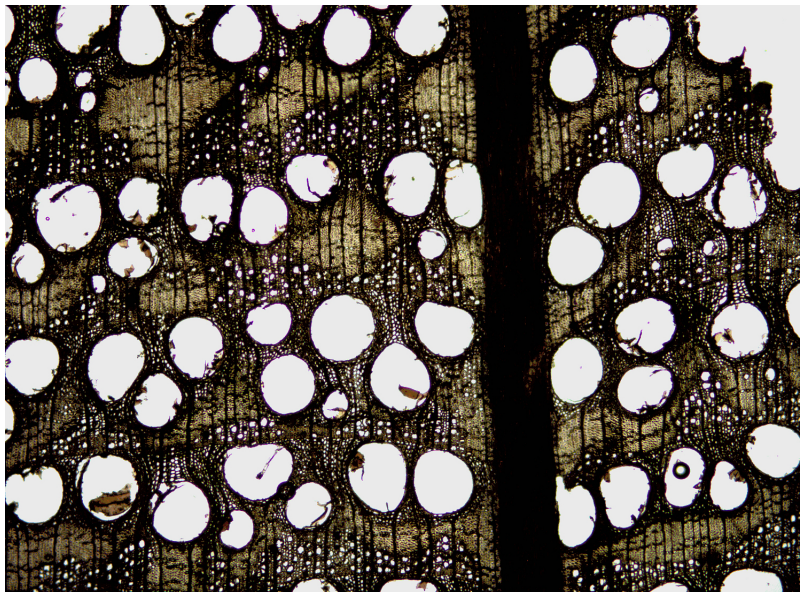

Fig. 6. The microscopic image of one of the piles with clearly visible oak wood (*Quercus sp.*) characteristics, A. Jankowska

[Wagenführ and Scheiber 1974]. The results of the microscopic identification of wood genus were also confirmed during the DNA analyses. Absolute dating, which can be provided by dendrochronology, was unobtainable due to the small cross sections of the piles (samples from trial pits – W1/2012, W2/2012, W3/2013) and a deficiency of the proper amount of tree rings (fig. 7), making it impossible to match the ring sequences with a dendrochronological local scale and obtaining certain results [Aniszewski et al. 2015]. A readable sequence of growth rings of suitable length (at least 30 rings) is required for dating wood with the dendrochronological method [Zielski and Krąpiec 2009]. The sequence of 101 growth rings which was obtained from the pile located in trial pit W4/2014 was impossible to match with a dendrochronological local scale.

Fig. 7. Cross section of one of the piles obtained from the remains of the historical construction (trial pit W1/2012), P. Kobek

Therefore, selected fragments collected from the piles have been dated using the radiocarbon method [Documentation in the Archive of LDB]. The table below presents the achieved results (tab. 2).

Table 2. List of calibrated radiocarbon dates obtained from oak piles

Trial pit number	Sample number	Wood species	Construction element	Period
W1/2012	MKL-1416	oak	pile	975-1155 calAD probability 95.4%
W2/2012	MKL-1629	oak	pile	1021-1155 calAD probability 95.4%
W3/2013	MKL-1841	oak	pile	1038-1213 calAD probability 95.4%
W4/2014	MKL-2373	oak	pile	776-982 calAD probability 95.4%

The oldest radiocarbon date was obtained from sample MKL-2373 which was taken in trial pit W4/2014. The indicated period between 776-982 calAD confirms the chronology of the archaeological site of Czermno from *The Russian Primary Chronicle*. The subsequent dates suggest that the construction was systematically repaired and lasted until the beginning of the13th century.

The obtained chemical and physical test results of historical wooden piles acquired from the northern flood-meadows indicate, that the state of preservation of the wood depends on the localization of trial pits in the area of archaeological recognition and varies depending on the conditions occurring in the individual stratigraphic layers. The most degraded sections of the construction elements were derived from the turf as well as from the high and medium (*Mt1 III, Mt2 II*) mucked layers of the peat muck horizon. Nonetheless, various physical and chemical parameters of the archeological oak wood under examination, taken from near-anaerobic layers of reed peat (*Otnisz-bR1*) and calcareous gyttja (*Ogy-ca-b*) did not diverge substantially from the values determined for modern material of this species, available in written sources [Fengel and Wegener 1989; Hoffman and Jones 1990]. This resulted from the deposition of the wood in an environment which preserved it from the most destructive degradation agents. In the near-anaerobic morphological soil layers, such as peat and gyttja, the wood tissue becomes degraded mainly through erosion, tunneling and due to the interference of cavitation bacteria. This process of deterioration proceeds very slowly and results in the gradual decline of cellulose and hemicelluloses content [Björdal et al. 1999; Blanchette 2000; Kim and Singh 2000; Helms et al. 2004].

DNA sequencing and genotyping

The analysis of trnD-trnT locus resulted in one sequence of 267 bp length of chloroplast DNA, obtained in one (originating from trial pit W4/2014) of four historical samples (fig. 8).

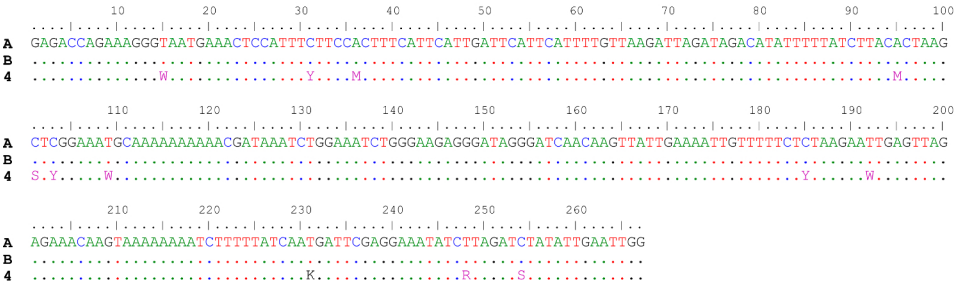

Fig. 8. Alignment of trnD-trnT sequences in two modern (A and B) and one archaeological sample (No 4) of oak wood (*Quercus* sp.), A. Tereba

The three remaining samples of historical wood failed in both sequencing and genotyping procedure. One sequence from historical wood sample was successfully aligned in BioEdit software, and the sequence was compared in the NCBI Blast database, indicating the *Quercus* genus. In this sequence, the 12 (from 267) nucleotide positions were unreadable, which may be related to PCR or sequencing errors.

In the case of microsatellite markers amplified in the wood sample No 4, only five out of eight tested loci were detected (tab. 3). This indicates the possibility of obtaining molecular data for short nuclear microsatellite fragments (up to 229 bp) in ancient wood. For aDNA studies, most DNA targets don't exceed 300 bp [Schlumbaum et al. 2008]. Similar studies based on nuclear markers and chloroplast trnL-trnF markers were useful in identification to the genus level of waterlogged *Prunus* fruits stones [Polleman et al. 2005]. Aberrations in the DNA molecules occurring in historical wood samples are the main obstacle to achieving a good DNA profiling in the laboratory. During aging the DNA molecules are prone to the degradation mainly caused by UV irradiation and reactive oxygen molecules [Watson and Riha 2011]. In our study, we can presume that the above factors are relatively absent in oak samples stored under water. However, the principal source of age-dependent DNA damage can be due to the hydrolytic activity of the cell-intrinsic nucleases maintaining their enzymatic activity at the very low level in anaerobic conditions.

Many findings confirm the usefulness of the phylogenetically meaningful sequences for the archaeobotanical study of the wooden species. In the present report, the analysis of two samples of modern oak wood resulted in two full sequences of chloroplast trnD-trnT fragments (fig. 8) and the genetic profile based on the studied nuclear loci (tab. 3). None of five microsatellites alleles identified in ancient sample No 4, did not match the alleles found in two modern samples A or B.

Analyzing the DNA of oak from ancient and modern wood, Dumolin--Lapègue et al. [1999] indicated an occurrence of indels and substitutions in the trnD-trnT chloroplast marker in France. In our study, no indels between the

ancient wood sample and the two modern ones (A and B) occurred. Moreover, the sequence of trnD-trnT chloroplast DNA in the ancient sample No 4 contained a specific motif of ten $(A)_{10}$ nucleotides, previously described by Deguilloux et al. [2003]. According to these authors, such a motif is characteristic for following a, b, f and i haplotype variants, mainly present in oaks from the central Europe. Taking into account that all three sequences (obtained in samples No 4, A and B) from the Czermno were identical (except 12 unreadable nucleotides) we can presume that the wood used to build the medieval wooden structure in the Czermno is originated from the central region of Europe.

Table 3. Genetic profile of the analyzed oak (*Quercus* sp.) sample based on 8 nuclear DNA markers alleles length expressed in base pairs

	Material type	Marker (ZAG7)	Marker (ZAG9)	Marker (36)	Marker (MSQ13)	Marker (ZAG20)	Marker (MSQ4)	Marker (102)	Marker (ZAG110)
4	historical DNA	141;141	n.a.	70,86	n.a.	161,161	n.a.	100,144	229;229
A	modern DNA	127;133	184;190	64;80	216;216	165;167	204;210	112;118	203;205
B	modern DNA	133;133	190;194	74;74	216;222	167;175	200;212	110;118	201;201

Based on the presented data, we can sum up that at least one ancient sample of oak originating from trial pit W4/2014 was successfully identified to the genus level. This wood sample was the most predisposed to the molecular investigation probably because of the smallest content of PCR inhibitors and the less degraded DNA structure. The success of microsatellite marker amplification in this one historical sample is promising from the perspective of population analysis in future research. However, further conclusions and recommendations are likely to arise after the analyses of the greater numbers of samples obtained from the Czermno early medieval wooden construction.

Conclusions

Soil samples for analysis, according to the stratigraphy determined in the trial pits, were collected from seven morphological layers, which the wooden piles of historical construction passed through. Parameters of the archeological oak wood under examination, taken from near-anaerobic layers (reed peat and calcareous gyttja) did not diverge substantially from the values determined for the modern material of this species, from written sources [Fengel and Wegener 1989; Hoffman and Jones 1990]. Nevertheless, despite the good state of preservation (class III in De Jong's classification made for archaeological oak) it

was possible to obtain well preserved DNA sequences for only one historical sample (No 4), which was collected from the trial pit located closest to the Huczwa River (W4/2014), radiocarbon dated to 776-982 calAD. The proximity to the riverbed during the deposition in the soil/archaeological layers provided the most stable environmental conditions and probably hindered the penetration and accumulation of chemical substances in the historical wood. The analysis of DNA from a wood sample as old as 1000 years shows that wood remains, could be a good reservoir of molecular information [Liepelt et al. 2006]. However, amplification of nuclear markers in a historical sample could be more problematic as in cpDNA. Additionally, targets are more susceptible to error due to polymerase slippage or advanced DNA degradation processes.

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