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Pisum & *Ervilia Tetovac* - Made in Early Iron Age Leskovac. Part Two. Extraction of the Ancient DNA from Charred Seeds from the site of Hissar in South Serbia

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Summary: The extracts were prepared from the samples of 3,200-year-old charred pea and bitter vetch seeds from the site of Hissar near Leskovac, South Serbia, using two different DNA extraction procedures. We used CTAB method with some modification and obtained low quantity of ancient DNA in comparison with the second method used - commercial available kit. After the extraction, a whole genome amplification using Phi29 DNA polymerase was performed. The amplified DNAs were used for PCR reaction using primers for 26S rDNA gene, which is located on the nuclear genome. The single band corresponding to 26S rDNA fragment from modern relatives was obtained. We conclude that DNA from charred pea and vetch seed can be extracted and used for further archaeobotanical analysis at the molecular level.
Key words: ancient DNA, charred pea seed, charred bitter vetch seed, PCR

Introduction

After death DNA is degraded by various biotic and abiotic processes, resulting in fragmentation and modification of original sequence information (Schlumbaum et al. 2008). It has emerged that cold, dry and low oxygen environments are beneficial for DNA survival; for this reason, freshly excavated material is best stored cold or frozen (Burger et al. 1999, Smith et al. 2001, Pruvost et al. 2007). However, these DNA fragments still contain information which may help us to understand agricultural or vegetation history.

Ancient DNA (aDNA) has received much attention since the mid-1980s, where the first sequence of an extinct animal species was recovered from a museum specimen. Since then, the majority of ancient DNA studies have focused predominantly on animal species, while studies in plant paleogenetics have been rather limited, with the notable exception of cultivated species found in archaeological sites (Gugerli et al. 2005).

The possibility that ancient DNA is preserved in some charred plant remains was first raised by Goloubinoff et al. (1993), who obtained polymerase chain reaction (PCR) products from extracts of 600-year-old maize cobs. There are many data considering general problems and doubts surrounding ancient DNA research (Cooper & Poinar 2000). Some of them are the proof of authenticity, contamination with other ancient and/or modern DNA and reproducibility of obtained results (Schlumbaum et al. 2008).

The procedure for aDNA analysis in plants involves excavation, morphological description, external cleaning if possible, powdering, DNA extraction, amplification of chosen target region within the plant's genome(s) by PCR, gel electrophoresis to establish presence of correct product size, cloning and sequencing PCR product and verification.

Nuclear ribosomal DNA (rDNA) genes are

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of interest for aDNA research, as they contain hundreds of unit of two variable spacer regions between the 18S, the 5.8S and the 26S genes, the so-called internal transcribed spacer regions 1 and 2, thus increasing the chance of DNA survival. These loci have been employed for phylogenetic analysis at the genus level in modern plants (Knaak et al. 1990, Soltis et al. 1992, Alvarez & Wendel 2003).

So far, there has been no preferred extraction method for ancient plant material, nor any comprehensive comparison protocols, such as exist for animals (Rohland & Hofreiter 2007). Generally, CTAB/DTAB methods, silica-based methods or DNA extraction kits are employed. All these methods were developed taking into account that plant parts are rich in secondary metabolites and other potential inhibitors of PCR.

Here we described the methods for aDNA isolation from pea (*Pisum sativum*), one of the first crops cultivated by man, and from bitter vetch (*Vicia ervilia*), two charred pulse crop storaged of the fortified hill settlement Hissar.

Our preliminary results show that even commercial kits for DNA extraction could be suitable for aDNA extraction from these species, but using some modification.

Materials and Methods

Charred pea (*Pisum sativum* L.) seeds were found in a single archaeobotanical sample from the hill fort settlement Hissar near Leskovac and represent a unique sample in Bronze/Iron Age research in the South East Europe. Bitter vetch (*Vicia ervilia* (L.) Willd.) was from the same site and period (Brnjica cultural group, beginning of the Iron Age in the Morava valley). Both pulse storages were almost pure with small amount of admixture from other crops, mainly cereals, other pulses, such as lentil *(Lens culinaris* Medik.) and faba bean (*Vicia faba* L.).

The DNA extraction procedure was performed using two different methods - commercial kit for DNA extraction (QIAGEN DNAesy kit) and modified version of the cetyltrimethylammonium bromide (CTAB) methods of Rogers & Bendich (1985). To eliminate possible contamination with DNA from other plants, we used UV treatment of laboratory over night, prior to DNA extraction. Also, all tubes and other equipment used for DNA extraction and PCR reaction were UV treated. Seeds of pea and vetch (500 mg for CTAB methods and 100 mg for DNAeasy) were ground in liquid nitrogen to a powder form with mortar and pestle, with adding of insoluble PVPP (polyvinylpolypirrolidone-SIGMA-ALDRICH, MO, USA, #856487), 2:1 (m/m). For CTAB methods the resulting powder was added to 1 ml CTAB extraction buffer (3% CTAB, 100 mM Tris-HCl pH8.0, 20 mM EDTA, 1.4 M NaCl, preincubated at 60^0C), mixed thoroughly and incubated at 60^0C for 4 h and at 37^0C over night. The mixture was centrifuged in a microfuge for 10 min and the supernatant extracted two times with 24 : 1 (v/v) chloroform : isoamyl alcohol. Two volumes of CTAB precipitation solution (1% CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA) was added to aqueous layer and mixed well. The mixture was incubated at room temperature for 1 h, and then centrifuged in a microfuge for 10 min. The supernatant was discarded and the pellet resuspended in 350 µl 1.4 M NaCl and 350 µl of chloroform. The mixture was centrifuged in a microfuge for 10 min, the aqueous layer was mixed with 0.6 vol of cold isopropanol, centrifuged for 10 min, and the pellet was washed 3 times with 70% ethanol. The pellet was dried in speed-vac and resuspended in 50 µl of water. The second method for DNA isolation (DNAesy kit) was performed according manufacturer's instruction.

The concentration of DNA was measured using NanoVue spectrophotometer (GE Heltcare).

For genome amplification, we used Illustra GenomiPhi HY DNA Amplification Kit (Amersham, GE Healthcare, UK) according to manufacturer's instruction. Whole genome amplification (WGA) is based on multiple displacement amplification. This is an isothermal (30°C), linear amplification using random hexamer primers and phi29 DNA polymerase. Primers attach randomly to the template adding complementary base pairs to one strand, while displacing the other DNA strand. Primary products initiate secondary priming on the displaced DNA strand and continue displacing to create multiple branches. If the initial sample of genomic DNA is high quality, it can be amplified one million-fold while maintaining accurate loci and allele representation. Phi29 DNA polymerase was inactivated at 60 °C. The product of reaction was diluted 50 times, and used for PCR reaction.

PCR reaction was performed using primers for 26S rRNA:

26Sf: 5'-ttcccaaacaacccgactc-3'; 26Sr: 5'-gccgtccgaattgtagtctg-3'.

Primers used for PCR reaction were developed

in Laboratory for Plant Molecular Biology at the Institute of Molecular Genetics and Genetic Engineering, Belgrade. They are not specific for legumes, but primers were designed according to conserved region of 26S rDNA gene present in all plants, and these primers were tested on different plant species (Arabidopsis, tobacco, buckwheat, *Ramonda nathaliae* and *R. serbica*, pea, vetch) and after PCR reaction one single band was obtained.

The reaction product was used as a template for PCR reaction. The reaction mixture (25 µl): 2 µl DNA from genome amplification reaction, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM primers, 1X Gold buffer (Applied Biosystems) and 1U Gold Taq polymerase (Applied Biosystems). PCR reaction was performed in a Biometra Thermocycler, using the following profile:

cycle: 95°C for 5 min (initial denaturation)

30 cycles: 95°C for 30 sec (denaturating), 60°C 30 sec (annealing), 72°C 1 min (extension), with final extension for 10 min at 72°C.

For positive and negative control reaction genomic DNA from *Pisum sativum* and *Vicia ervilia*, extracted using DNAeasy kit, and water were used, respectively.

The PCR products were separated on 2% agarose gel and visualised by UV/EtBr staining.

Results and Discussion

To perform isolation of DNA from ancient 3,200-year-old pea and vetch seeds, we tested two different methods for conventional DNA isolation, with some modifications. In the first method, we used conventional CTAB method, as described in the previous section. In this procedure we changed CTAB concentration in buffer, from 2% to 3%, in order to increase osmolarity of extraction buffer and obtain better disruption of the seeds. Apart of this modification, we used insoluble polyvinylpolipyrrolidone (PVPP). It is exceptionally good at absorbing polyphenols during DNA purification. Polyphenols are common in many plant tissues and can deactivate proteins if not removed and therefore inhibit many downstream reactions like PCR. Without the addition of PVPP, the precipitated DNA was brown, indicating the presence of oxidized phenols. This brown colour was lost on supplementation with PVPP in the extraction process (Angels et al. 2005). Regarding the fact that dry seeds possess large amounts of polyphenols and other components that may inhibit PCR reaction, adding of PVPP is first step in improving chance for isolation of ancient DNA. Using described methods, we observed that ancient pea seeds contain more fats than vetch seeds, and more extensive removing of these compounds was necessary. In addition, after removing of non-polar compounds, it was evident that vetch seed contained different pigments than pea seeds, according to colour of solution during DNA isolation. However, CTAB method yielded low-quality and low quantities of DNA, even though a detectable (about 2 ng μl^{-1}) amount of DNA were present.

Using different methods for DNA isolation-commercially available DNAeasy kit (QIAGEN), we obtained 7.2 ng μl^{-1} of DNA from vetch seeds, and 0.8 ng μl^{-1} from pea seeds. However, using these DNA isolates, as well as CTAB obtained isolates for PCR reaction, we did not observe any amplification products, nor after re-amplification of PCR products. Lower DNA amount in pea seeds could be explained by the fact that distinctive types of combustion are likely to have occurred with different archaeological remains, varying from the largely anoxic or low-oxygen events occurring in an archaeological place (Threadgold & Brown 2002). Another explanation is that not all seeds contain ancient DNA, suggesting that the critical factors include some morphological feature of the seed itself (Brown et al. 1998). Regarding this fact, we decided to perform whole genome amplification (WGA), using Illustra GenomiPhi HY DNA Amplification Kit. The concept of whole genome amplification is something that has arisen in the past few years as the polymerase chain reaction (PCR) has been adapted to replicate regions of genomes that are of biological interest. The applications are many - forensic science, embryonic disease diagnosis, bioterrorism genome detection, "immortalization" of clinical samples, microbial diversity, and genotyping. Whole-genome amplification (WGA) can replenish dwindling stocks of genomic DNA or bolster low yields, which limit the number of downstream assays that can be completed. PCR-based WGA methods can introduce amplification bias and generate products of insufficient length for some applications, such as RFLP analysis. The kit features Phi29 DNA polymerase, a bacteriophage enzyme normally involved in rolling-circle replication, which has the unique property of strand displacement. This is non-PCR method of WGA.

Using reaction product as a template for PCR

with primers for 26S rDNA, we observed single band, approximately 120 bp, corresponding to PCR product obtained on modern pea and vetch DNA (Figure 1).

Our goal is further optimization of methods in order to obtain PCR product which can be used for sequencing and archeaobotanical investigation of ancient plants and their modern relatives. The sequencing of PCR products should be imperative, in order to eliminate possible contamination with modern DNA.

Conclusions

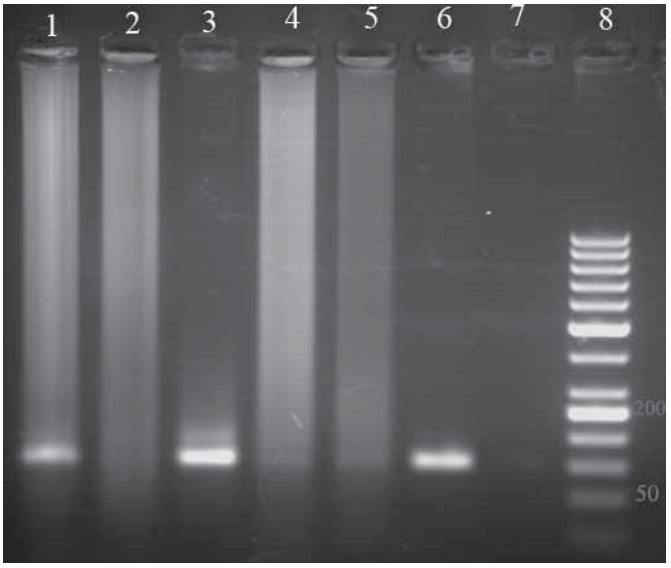

Figure 1. PCR products obtained after amplification of ancient and modern DNA from pea and bitter vetch with 26S primers. Lanes: 1 - ancient pea DNA extracted using kit, 2 - ancient pea DNA extracted using CTAB method, 3 - modern pea DNA, 4 - ancient bitter vetch DNA extracted using kit, 5 - ancient bitter vetch DNA extracted using CTAB method, 6 - modern bitter vetch DNA, 7 - negative control, 8 - DNA size marker (50bp Fermentas).

Slika 1. Produkti PCR reakcije dobijeni nakon amplifikacije drevne i moderne DNK graška i urova upotrebom 26S prajmera. Kolone: 1 - drevna DNK graška ekstrahovana upotrebom kita, 2 - drevna DNK graška ekstahovana primenom CTAB metode, 3 - DNK savremenog graška, 4 - drevna DNK urova ekstahovana upotrebom kita, 5 - drevna DNK urova ekstrahovana primenom CTAB metode, 6 - DNK savremenog urova, 7 - negativna kontrola, 8 - marker veličine DNK.

Although there are data about aDNA isolation from ancient plants, this is the first time that aDNA from ancient pea and bitter vetch seed was isolated. In addition, more interesting fact is that these seeds were from a local Serbian archaeobotanical site which could be very important for further investigation. Moreover, we showed that aDNA from charred pea and bitter vetch seeds could relatively easily be isolated, using commercial kit for DNA extraction. Although some limitations for use of aDNA for PCR still exist, we showed that fragment of nuclear ribosomal DNA gene-26S rDNA can be amplified by PCR and further applied for investigating and answering archaeobotanically and archaeologically relevant questions. These include questions such as species identification, origin and spread of cultivated plants or monitoring the state of domestication; these are the questions that cannot be answered by morphological archaeobotanical studies alone.

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Grašak i urov *Tetovac* - made in ranogvozdenodobni Leskovac. Deo drugi. Ekstrakcija drevne DNK iz ugljenisanih semena sa nalazišta Hisar u južnoj Srbiji

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Izvod: Primenom dve različite procedure ekstrahovana je drevna DNK iz ugljenisanih semena graška i urova starih 3200 godina sa lokaliteta Hisar kod Leskovca. Korišćena je modifikovana CTAB metoda i dobijena je relativno mala količina drevne DNK u poređenju sa drugom primenjenom metodom – komercijalno dostupnim kitom za izolaciju DNK. Nakon ekstrakcije primenjeno je umnožavanje celog genoma pomoću Phi29 DNK polimeraze. Umnožena DNK je korišćena za PCR reakciju primenom prajmera za 26S rDNK gen, koji je lociran u jedarnom genomu. Dobijen je fragment iste veličine kao i PCR fragment 26S rDNK savremenih srodnika graška i urova. Može se zaključiti da je moguće ekstrahovati drevnu DNK iz ugljenisanih semena graška i urova i koristiti je za dalje arheobotaničke analize na molekularnom nivou.
Ključne reči: drevna DNK, PCR, ugljenisano seme graška, ugljenisano seme urova