An archaeobotanical and molecular fairy tale about the early Iron Age Balkan princess and the charred pea

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Pea *(Pisum sativum* L.) is one of the most important crops in Serbia and other Balkan countries. It is used for human consumption, animal feeding and for various non-food uses such as green manure (1). Apart from the cultivated pea (P. *sativum* susbsp. *sativum),* the flora of the southeast regions of Serbia is rich in 'tall pea' *(Pisumsativum* subsp. *elatius* (Steven ex M. Bieb.) Asch. & Graebn.), most likely being one of the northernmost borders of its distribution in southeast Europe (2).

Pea is one of the most ancient and the most persistent grain legume crops in the archaeological findings of the Balkans, where the 'agricultural revolution' of Europe began about 9000 years ago. In Serbia, especially in its northern parts, pea is found in tells belonging to Neolithic times, such as the site Gomolava, Bronze Age, with Zidovar and Feudvar, Early Iron Age, with Gomolava, Feudvar and Gradina-upon-Bosut, up to La Tene, represented by the tell of Gomolava (3). Together with lentil *(Lens culinaris* Medik.), chickpea *(Cicer arietinum* L.), bitter vetch *(Vicia ervilia* (L.) Willd.) and some other grain legume and cereal crops, pea travelled up the Danube valley into the continental interior and quickly reached its farthest regions (4).

Charred plant remains are possibly the most unexpected type of preserved biological material to have yielded ancient DNA (aDNA), and even though they are fragmented, they still contain information which may help us to understand agricultural or vegetation history (5, 6). Ancient DNA has received much attention since the mid-1980s when the first sequence of an extinct animal species was recovered from a museum specimen (5, 6). Since then, the majority of ancient DNA studies have focused predominantly on animal species, but the investigation of plant aDNA was limited, with the exception of cultivated species found in archaeological sites. There have been studies on charred archaeological samples of other species such as maize and wheat (7). However, there are still problems with charred plant ancient DNA - primarily the enhancement of DNA degradation upon charring. Despite these problems, investigation of ancient plant DNA may provide opportunities for palaeogenetic studies in plants for phylogenetic analysis and species identification (8).

Materials and Methods

The fortified hill settlement of Hissar in Leskovac is a multilevel settlement belonging to the Brnjica cultural group in the Morava Valley and dates back to 1350-1000 B.C. in the Iron Age I (9). At an altitude of 341 masl the Hissar hill has a strategic position over the confluence of Jablanica and Veternica rivers in South Morava and over the greates part of the Valley of Leskovac. It is fifty kilometers long and forty-five kilometers wide.

Figure 1. Charred pea seeds from the Iron Age fort Hissar, southern Serbia

The archaeological excavations at Hissar began in 1999, and in 2005 two rich grain legume samples were collected from the deposits belonging to Brnjica II a level from the 12th century B.C. Flotation of 7 liters of earth substrate yielded 2572 charred seeds of pea (Fig. 1). In one of the previous archaeobotanical reports (10) only one uncertain

record of pea from the deposits of Brnjica cultural group was made. In addition to pea, a collection of bitter vetch with about 3000 seeds and supplements of faba bean and lentil was found.

The 50 g of charred pea seeds were ground to as close a powder form as possible in liquid nitrogen with a mortar and pestle. During grinding insoluble polyvinylpolypirrolidone (PVPP) was added. The resulting powder was added to 1 ml CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, preincubated at 60 °C), mixed thoroughly and incubated at 60 °C overnight. The mixture was centrifuged in a microfuge for 10 min at 14,000 rpm, and the supernatant extracted twice with 24 : 1 (v/v) chloroform : isoamlyl alcohol. Two volumes of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA) were added to the aqueous layer and mixed well. The mixture was incubated at room temperature for 1h and then centrifuged for 10 min at 14,000 rpm. The supernatant was discarded and the pellet was suspended in 50 ul sterile distilled water. The concentration of DNA was measured using a NanoVue spectrophotometer (GE Heltcare, Little Shalfont, UK). For genomic amplification, the GenomiPhi DNA Amplification Kit (GE Heltcare, Little Shalfont, UK) was used according to manufacturer's instructions. The PCR reaction was performed using primers for 26S rRNA: 26Sf: 5'-attcccaaacaacccgactc-3'; 26Sr: 5'-gccgtccgaattgtagtctg-3'. The reaction mixture (25 ul): 1 ul DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4uM primers, 1X Taq buffer with KCl (Fermentas) and 1U Taq polymerase (Fermentas). The PCR reaction was performed in a Biometra Thermalcycler, using the following profile: 95 °C for 5 min (initial denaturation); 30 cycles - 95 °C for 30 s (denaturating); 60 °C 30 s (annealing); 72 °C 1 min (extension); with a final extension for 10 min at 72 °C. For positive and negative control reactions genomic DNA from pea and water were used, respectively. The PCR products were separated on 2% agarose gel.

Results and Discussion

It is almost impossible to precisely determine a charred pea seed solely by its morphology if it misses its specific coffee-bean-shaped hilum (Fig. 2). The seed shape, regularly spherical with few flattenings depending on their position and space within a pod, and the seed size are not sufficient to determine if one deals exactly with pea. The reason for this lies in the fact that pea seeds can be as small as some *Vicia* seeds and, if the hilum is lost, they cannot be differentiated from each other.

In the Hissar pea sample, only few seeds maintained an almos intact testa with hilum. Fortunately, among the pea seeds, 32 seeds of lentil, bitter vetch and faba bean were observed and their identity was quite clearly determined (Table 1). The seeds of lentil and bitter vetch differed from pea in shape and from faba bean in size. By applying the exclusion principle, it is almost certain that the remaining seed were pea.

Figure 2. A charredpea seed from Hissar with preserved important morphological features (drawing by A. Medovic)

Table 1. The specification of the Hissar pea sample.

Remain type	Quantity (number)	Quantity (%)	Mass (mg)	Mass (%)
Pulses				
Pisum sativum	2572	85.68	62768	97.82
Lens culinaris	21	+	113	r
Vicia faba	8	r	472	+
Vicia ervilia	3	r	21	r
Cereals				
Hordeum vulgare vulgare	10	r	126	r
cf. *Triticum aestivum* s.l.	1	r	6	r
T. aestivum s.l., rachis internodes	1	r	3	r
Triticum dicoccon	1	r	11	r
Triticum monococcum	1	r	2	r
Cerealia indeterminata	1	r	8	r
Millets				
Panicum miliaceum	320	10.66	553	+
Oil and fibre plants				
Linum usitatissimum	21	+	20	r
cf. *Lallemantia iberica*	1	r	2	r
Dye plants				
cf. *Carthamus tinctorius*	1	r	2	r
Fruits				r
Sambucus	1	r	0	r
Weeds and ruderals				
Convolvulus arvensis	16	+	40	r
Echinochloa crus-galli	8	r	2	r
Polygonum convolvulus	4	r	5	r
Chenopodium album	3	r	0	r
Setaria viridis	2	r	0	r
Digitaria sanguinalis	1	r	0	r
Galium aparine	1	r	2	r
Lolium temulentum	1	r	3	r
Medicago	1	r	2	r
Plant families				
Fabaceae	1	r	3	r
Poaceae	1	r	0	r
Total	3002	100	64164	100
Other finds				
Insecta, larva	1		0	

r < 0.5%; 0.5% < + < 1.0%

Since this work was also aimed at investigating the possibility of applying molecular methods to analyze archaeological pea remains, the DNA from ancient pea seeds was isolated and fragments of 26S rDNA were amplified by PCR. The standard DNA extraction procedure without genome amplification did not provide adequate quantities of DNA material for the PCR reaction. The DNA concentration in the isolate was 4.5 ng ul^{-1} and total yield of DNA was about 200 ng from 200 mg of seeds. However, using the GenomiPhi DNA Amplification Kit for random amplification of genomic DNA and using the product of this reaction in PCR we observed a single band (150 bp) corresponding to the gene for 26S rRNA (Fig.3).

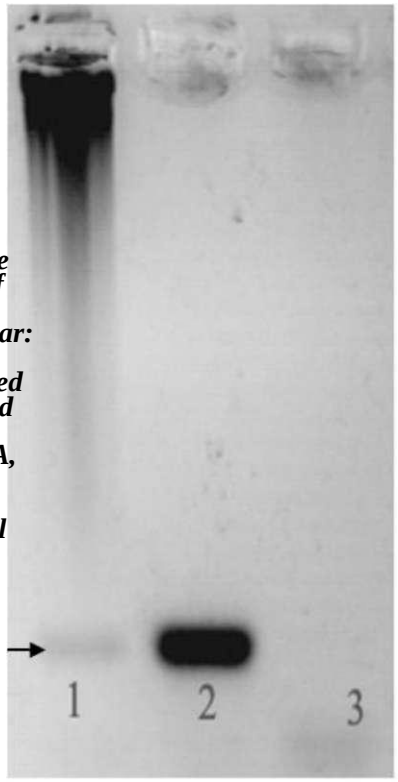

Figure 3. Electrophoretic separation of the PCR products of the charred pea seeds from Hissar: (1) the PCR product indicated by the arrow and obtained with ancientpea DNA, (2) positive control and (3) negative control

Conclusions

Despite the fact that pea was one of the first cultivated crops, discovery of 2572 pea seeds in a single sample at Hissar represents a unique example in the archaeobotany of southeast Europe. The pea seeds are often poorly represented in the archaeobotanical records, not only due to rather small chances of being carbonized, but also because of the difficulties related to their proper determination. Pea at Hissar was a crop of its own and was stored separately from other crops. It also seems that pea was cultivated on small, patch-like fields where intense crop care was implemented.

These results encourage future research to use standard DNA extraction procedures, with the addition of insoluble PVPP and an extended period of incubation, accompanied by random genome amplification to obtain appropriate material for molecular analyses of archaeological plant material. The charred pea seeds contained aDNA, which could be used for further molecular analyses.

Acknowledgements

This work was financially supported by the Ministry of Science and Technological Development, Republic of Serbia, Grants No. 143017, 20090 and TR31024.

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