Extracting Genomic DNA of Fossilised Pollens from Volcanic Soil Sediment of Liyangan Site-Central Java

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Abstract. Liyangan buries some artifacts in volcanic sediment, including pollens. Reconstructing ancient plant diversity requires good quality in an adequate amount of genomic DNA from fossilized pollen. The current study was aimed to separate pollens from their sediment and extract the pollens. The separation was done according to different protocols (BALAR’s and Geology’s), DNAs were extracted by applying modified-CTAB and mini Genomic DNA Mini Kit. Current data showed the two separation and extraction protocols were able to separate fossilized pollens and so to extract them. The BALAR method combined with CTAB resulted in 314µg/ml to 887µg/ml while a lower amount of 382µg/ml to 852µg/ml were obtained when extracted by DNA Mini Kit. The Geology protocol combined with CTAB resulted in even lower concentrations (43µg/ml to 230µg/ml); and 22 µg/ml to 216 µg/ml by DNA Mini Kit. The BALAR’s protocol performed a better result in separating fossilized pollens from other materials. Both extraction methods resulted in an adequate amount of genomic DNA, although in quite low quality.

1. Introduction

Pollens, male reproductive organs of Angiospermae and Gymnospermae, are produced by microsporocyte following meiotic [1]. Pollen might undergo fossilization due to its outer layer, which is composed of sporopollenin [2, 3, 4]. Fossilized pollens are important material to trace the ancient plant diversity [5].

It is predicted that prehistoric people relied more on the availability of collective foods and raw material sources from the surrounding environment [6]. Reconstructing plant diversity and their abundance during the prehistoric age becomes an essential step in studying the lifestyle of the people. The Liyangan buries some artifacts of hunting tools made of stones and bones, ceramics, and fossilized-burned woods, rice, and pollens, as well as Hinduism temple and artifacts [7, 8].

The identification of fossilized pollens based on their morphological characters could not be able to identify their species or even genera [9]. Molecular analyses might then be applied [10], and that molecular analysis performs better than morphological characters, more stable and has no environmental effect [11, 12] though it requires good quality DNA at adequate concentration [13]. Extracting fossilized pollens needs special treatment before extraction [14]; however, the success of extracting fossilized pollens is quite low as it is strongly related to the treatment; thus, very limited data were available so far [15]. Extraction might be done either by CTAB [16] or DNA extraction Kit [17, 18] following preparation methods of BALAR’s and Geology’s protocols.
2. Methods

2.1. Research Design

The research used a survey method employing a purposive sampling design. The sampling was performed in 5 different sites within my field, an area where ancient people did their activities. The sediment was taken from the soil, as far as 20 cm from topsoil. Twenty grams soil sediment was then taken and split into two parts, and put in the plastic clip which covered by aluminum foil before be brought to laboratories for separation (BALAR and Geology Department). The Liyangan site was divided into five spots (Fig. 1).

![Figure 1](image)

**Figure 1.** Soil sediment sampling sites within the Liyangan

Remark:
Spot 1: Area E: 07°15' 07,4” SL and 110°01'39,3” EL and 1184 masl.
Spot 2: Area C: 07°15'07,1” SL, 110°01'38,1” EL and 1186 masl.
Spot 3: Area M: 07°15'07,7” SL, 110°5” EL and 1173 masl.
Spot 4: Area A: 07°15'07,4” SL, 110°01'36,8” EL and 1169 masl.
Spot 5: Area O: 0°15'07,9” SL, 110°01'36,9” EL and 1152 masl.

Soil sediment was divided into two parts before separation from the fossilized pollens according to BALAR and GEOLOGY protocols. The parameter observed from these two protocols was the morphological characters of fossilized pollen.

2.2. Research protocols

2.2.1 The BALAR'sprotocol

Seven grams of sediment powder was put into the reaction tube and added with 5 ml 30%HCl, then left for 10 minutes and centrifuged at 2,500 rpm for 10 minutes. The pellet was added with 5 ml H$_2$O, for triplicates. Natant was added with 70% HF, incubated in the darkroom for 24 hours, re-centrifuged, and cleaned with H$_2$O four times. The natant was added with 5 ml 30% HCl, heated for 15’ and re-centrifuged, cleansed three times with H$_2$O. Natant was added with 5 ml 30% KOH and heated for 20’ and re-centrifuged, add pellet with 5 ml 30% KOH three times, and re-centrifuged. Natant was added with 50% HNO$_3$ and 30% HCl (100:5), heated for 10’ and re-centrifuged. Natant was then added with H$_2$O and re-centrifuged in triplicates.
2.2.2 The Geology protocol

Seven grams of sediment powder was placed into a beaker glass and added with 50% HCl, homogenized and incubated for 3 hours. The natant was neutralized with H₂O to pH 7, sank in 40% HF, homogenized and incubated for 24 hours. The natant was reneutralized with H₂O, then added with 50% HCl, and heated for 2 hours, neutralized with H₂O, then sieved sequentially with 150µ and 5µ sieves. The natant was transferred to a tube, then 50% HNO₃ was added, and heated for 10 minutes, neutralized with 10% KOH, and H₂O.

The obtained pollens fossils were extracted using modified CTAB and genomic DNA extraction kit PureLink® then quantified.

2.2.3 Modified CTAB [16].

0.5-gram of fossilized pollens were ground, added with 1,500 ml warmed-CTAB buffer and 15 µl β mercaptoethanols. The samples were transferred into a 1.5 ml microcentrifuge tube, warmed in a water bath at 65°C for 60 minutes and homogenized, centrifuged at 11,000 rpm for 10 minutes. The supernatant was transferred into a new tube, added with 800 µl of cold CIAA 24:1 (chloroform isoamyl alcohol), vortexed for 5 minutes, and centrifuged at 11,000 rpm for 20 minutes, transferred to a new tube, added with ammonium acetic 1/10 of the supernatant volume, and 2/3 absolute cold ethanol, homogenized and incubated in -20°C overnight. The mixture was recentrifuged at 11,000 rpm, 4°C for 20 minutes. The pellet was added with 750 µl 70% Ethanol, centrifuged for 5 minutes at 11,000 rpm, and placed the tube upside down on a tissue paper.

2.2.4 Genomic DNA extraction Kit

0.5-gram fossilized pollens were placed into a sterile Eppendorf tube and added with 180 µl PureLink® Genomic Digestion Buffer plus 20 µl K Proteinase then were homogenized. The mixture was incubated in a water bath at 55°C for 2 hours and cooled down before centrifuged at 6,000 rpm for 3 minutes. The supernatant was transferred into a new tube, then added with 20µl RNase A, vortexed for 10’ and left for 2’ at RT. The mixture was added with 200 µl PureLink® Genomic Lysis/Binding Buffer, vortexed, added with 200µl absolute ethanol, and re-vortexed. It was transferred into the PureLink® Spin Column tube, added with 500 µl Wash Buffer 1, centrifuged at 10,000 rpm for 1’, transferred into a new column tube. The mixture was added with 500 µl Wash Buffer 2, centrifuged for 3 minutes at 10,000 rpm. The spin-column was transferred into a new tube, added with 50 µl PureLink® Genomic Elution, left for 1 minute, centrifuged at 10,000 rpm for 1 minute, added with 50 µl PureLink® Genomic Elution before recentrifuged at the same speed and time. The tube containing DNA was kept at -20°C.

Parameter observed from this step was DNA extract, which was quantified and scored, and interpreted for their quality [26] at absorbance of A260/230nm and A260/230nm as pure, contaminated by carbohydrate, protein, or RNA.

3. Results

Both preparation methods succeeded in separating fossilized pollens from their volcanic material and gases and visible under a microscope (Fig. 2).
Figure 2. Visualization of fossilized pollens prepared with (I) the BALAR method, (II) Geologi method (400x), and documented using an opti Lab camera.

Both extraction methods (CTAB and mini Kit) also succeeded in extracting the DNA in various amounts and purity (Fig. 3). CTAB, preceded by BALAR protocol, resulted in 314µg/ml to 887µg/ml DNA, which was slightly higher than that obtained using another protocol, i.e., 382µg/ml to 852µg/ml. On the other hand, CTAB following Geology protocol obtained a much lower amount (43µg/ml to 230µg/ml; and 22 µg/ml to 216 µg/ml).

Figure 3. The DNA concentration of fossilized pollens applying CTAB and KIT extraction methods following sediment preparation by BALAR and GEOLOGY protocols.

The DNA extracted from both methods, however, were contaminated by either carbohydrate, RNA, protein, or humic acid (HA) as reflected in its absorbance either values of <1.8 or >1.8 (Fig. 4-6).
Remarks:  
B. = CTAB and KIT, separated according to BALAR and extracted by the CTAB and DNA extraction kit
G. = CTAB and KIT separated according to Geology and extracted by the CTAB and DNA extraction kit

**Figure 4.** The Purity of DNA of fossilized pollens at the ratio of 260/280Å

The extract DNA’s obtained from that separation and extraction were then visualized in agar plate following the PCR.

**Figure 5.** Visualization of Extracted DNA of fossilized pollens (A) BALAR and CTAB (I), DNA Mini Kit (II); (B) GEOLOGY and CTAB (I), DNA mini Kit (II).

**4. Discussion**

Discrepancies in the visualization of morphologic fossilized pollens (Fig. 2) might probably due to different concentrations of HCl applied during separation. The clear vision in Geology protocol was obtained from the use of 50% HCl, in contrast to 30% in BALAR’s. HCl is a strong monoprotic. Thus, the higher concentration would result in the faster release of sediment from pollens [19], but not the gasses like humic acid (HA).

It is reported volcanic ashes or sediment containing the HA abundantly as a polyelectrolyte-organic macromolecule and might interact with metal to form metal HA complex or involving in oxidizing processes to form quinone to covalently ties to the DNA [20, 21]. In attempts to minimize it, it is suggested the use of sodium carbonate to release HA [22]; meanwhile, other report states differently [23]. The extraction of the DNA initially obtained from volcanic sediment material might not clean up the HA thoroughly, thus affecting the quality and quantity of the DNA obtained. For this purpose, it is suggested the use of PVP in the lysis buffer to lyse the hard outer part of the pollen shell [24].

The current study, unfortunately, did not apply the last two suggestions and so caused the poor quality and quantity of DNA obtained. Alternatively, the low concentration of extracted DNA obtained might be caused by the volume of KOH, incubation time, and homogenization of the
sediment during separation. The addition of KOH will automatically increase the pH of the solution to 11 and forces to dissolve HA [25]. However, both concentrations of 10% KOH in Geology and 30% in BALAR did not perform well in dissolving it entirely. Another possibility was centrifugation, where the BALAR protocol includes this step in its protocol while Geology’s does not.

Apart from that, it is reported that the very tiny size of fossilized pollen, as well as types, and numbers led to low concentrations of extracted DNA in this study [9]. The pollens of Hibiscus rosa-sinensis varied from 89.66 µm to 112.92 µm, thus requires specific precautions and treatment on extracting them fully. The purer the fossilized-pollens, the better the result of DNA extracts obtained [26]. Current data were classified as contaminated by carbohydrates, where the absorbent was <2 [27]. In most of the cases, the DNA of volcanic sediment was mainly contaminated by HA [28].

Apart from the HA, RNA or protein might also contaminate the DNA [28] as noted here, the absorbant score was either <1.8 or >18 at a ratio of A260/280 (Fig 4.). The slight smear on DNA bands indicated that such things contaminated the current extract DNA, but still possible to be used as material for further analyses [29].

5. Conclusion

Current data concluded that both separation protocols (BALAR and GEOLOGY) could separate fossilized pollens from their volcanic sediment. Both extraction methods are also performed well in extracting the DNA of fossilized-pollens. However, some suggestions might also be written to overcome such problems in the separation of fossilized-pollens from their volcanic sediment and gasses and extracting the fossilized-pollens to obtained good quantity and quality DNA for amplification.

Acknowledgment

We would like to pass our gratitude to the BALAR (ARCHAEOLOGICAL AGENCY) for allowing us entering the excavation site and bringing some soil sediment from the site. We would also thank the Geology Faculty of the Universitas Jenderal Soedirman-Purwokerto-Indonesia, for allowing us to compare the sediment preparation protocol. The last but not the least, we thank the Molecular and Genetic Laboratory of Faculty of Biology Universitas Jenderal Soedirman-Purwokerto-Indonesia for allowing us extracting and analyzing the fossilized pollen.

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