Comparison of Three Methods for Extracting Genomic DNA From Alcohol-Preserved Samples of Freshwater Sponges

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Comparison of Three Methods for Extracting Genomic DNA From Alcohol-Preserved Samples of Freshwater Sponges

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Natural Sciences at Carroll College, Helena, Montana

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April 9, 2001
This thesis for honors recognition has been approved for the Department of Natural Science.

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Date: 4/9/01
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Abstract

With greater emphasis being placed on molecular approaches to systematics, it is becoming increasingly important to be able to extract DNA from alcohol-preserved specimens, such as those maintained in museum collections. As a preliminary step to DNA sequence analyses of alcohol-preserved freshwater sponge specimens, this study compared the purity and yield of genomic DNA isolated from alcohol-preserved specimens by three different DNA extraction methods. One method (standard method) made use of a proteinase K digestion in the presence of detergent and EDTA, followed by extraction with phenol-chloroform. A second method (CTAB method) involved proteinase K digestion in the presence of cetyltrimethylammonium bromide (CTAB), followed by extraction in chloroform. A third method employed a cell lysis solution containing guanidium thiocyanate, followed by extraction in phenol-chloroform. Analysis of isolates indicated (1) no difference in purity or yield between DNA extracted from alcohol-preserved and frozen samples and (2) no difference in purity among methods, although yield was highest using the CTAB method. Preliminary evidence also suggested that all DNA preparations contained appreciable amounts of RNA.
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Introduction

Traditional freshwater sponge phylogeny relies heavily on the presence of gemmules, gemmoscleres, and microscleres, which are not present in all sponge species (Boury-Esnault and Volkmer-Ribeiro, 1992). Due to sponges without these features, sponge phylogeny is currently being re-examined with DNA sequence analysis (Chombard et al., 1997, 1998). These sequence analyses can be performed on traditionally identified sponges found in museum collections, or on newly collected specimens. Both of these specimen types are often preserved in ethanol.

As the preliminary step to sequencing DNA from alcohol-preserved specimens, this study attempts (1) to determine the feasibility of isolating DNA from alcohol-preserved specimens by comparing the purity and yield of DNA from specimens stored at -70°C to specimens stored in ethyl alcohol, and (2) to compare the purity and yield of DNA isolated by the following three methods: a standard method utilizing proteinase K catalyzed digestion followed by phenol-chloroform extraction (Sambrook et al., 1989); a method utilizing proteinase K and cetyltrimethylammonium bromide (CTAB) (Winnepennickx et al., 1993); and finally a method using guanidium thiocyanate in place of proteinase K digestion (Fernandez-Busquets, 2000).

Extraction of DNA from tissues generally occurs in the following manner: (1) cells are lysed, and DNA degradation by nucleases is prevented by proteinase K and ethylenediaminetriacetic acid (EDTA). Detergents are often added to ensure cell lysis. In this study, all cells were additionally lysed by freezing with dry ice (-78.5°C) and grinding in a mortar and pestle. (2) Proteins are removed by the addition of
phenol:chloroform. Upon mixing the DNA is separated from the proteins by centrifugation.

The guanidium thiocyanate method was selected due to the inhibitory action of guanidium thiocyanate on nucleases (Lippke et al.; 1987, Pitcher et al., 1989). High nuclease activity may be a complication in sponge DNA purification since at least some nucleases are not inhibited by EDTA. Nucleases not inhibited by EDTA have been described in the genus Geodia (Dawes et al., 1983). In this method the detergent sarkosyl was used to ensure cell lysis. In addition to guanidium thiocyanate, proteinase K and EDTA were used to inhibit nuclease activity in this method.

The CTAB method was selected because CTAB removes carbohydrates. In this method, β-mercaptoethanol was added to break disulfide bonds. The breaking of disulfide bonds should denature proteins, including nucleases. This method uses chloroform:isoamyl alcohol instead of phenol:chloroform:isoamyl alcohol.

The standard method was chosen as a control method. It utilizes sodium dodecyl sulfate (SDS) to lyse cells. Proteinase K and EDTA are then used to inhibit nucleases. Proteins and DNA were separated by phenol:chloroform:isoamyl alcohol.
Methods

Samples

The sponge species used were *Eunapius fragilus*, *Spongilla lacustris*, and *Ephydatia muelleri*. Samples were preserved either in ethanol or by freezing. Samples preserved in ethanol were collected and placed into individual containers filled with 70% ethanol. Samples preserved by freezing were placed on dry ice (-78.5°C) in the field, and then stored at -70°C in the laboratory. Immediately before use all sponges were placed in a pre-chilled mortar (-78.5 °C) and crushed with a pestle.

Prior to crushing, ethanol was removed from ethanol-preserved specimens and replaced by Tris and EDTA buffer (10 mm Tris-HCl, pH 8.0; and 1mm EDTA, pH 8.0) (TE). The replacement was performed over a 2 h period by pouring off 1/2 of the ethanol and replacing with TE every 30 min. After 2 h all liquid was poured off, and the specimen was frozen in a pre-chilled (-78.5 °C) mortar and ground with a pestle.

In the experiments that compared the three DNA isolation methods, samples from the same individual were used. This was accomplished by grinding a specimen then placing equal masses in separate centrifuge tubes.

Standard Method (modified from Sambrook, 1989)

In this method, 10 volumes by weight of extraction buffer (10mm Tris-Cl, pH 8.0; 0.1 M EDTA, pH 8.0; and 0.5% SDS) was added to the crushed tissue. This was mixed by inversion, and then proteinase K was added to a final concentration of 100 μg/mL. The sample was mixed and incubated at 50 °C for 3 h with periodic swirling.
The sample was then removed and cooled to room temperature. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The tube was mixed thoroughly by inversion and then centrifuged at 12,000x g for 15 s. The aqueous layer was transferred to a new tube, and an equal volume of chloroform:isoamyl alcohol (24:1) was added. The tube was thoroughly mixed before being centrifuged (12,000x g, 15 s). The aqueous layer was transferred to a new tube where it was precipitated with 2/3 volume isopropanol and left overnight. The next day the sample was centrifuged at 13,000x g for 1 min, washed with 70% ethanol, air-dried, and finally re-suspended in an appropriate volume of TE buffer.

CTAB Method (modified from Winnepeninckx, 1993)

In this method, one volume of pre-heated (60 °C) CTAB buffer [2% (w/v) CTAB; 1.4 M NaCl; 20mM EDTA; and 100mM Tris-HCl, pH 8.0] was added to the specimen in a ratio of 100μL / 100mg tissue. Proteinase K was then added to a concentration of 0.1 mg/mL, and β-mercaptoethanol was added to 0.2% (v/v). This was incubated for 30 min at 60 °C. After the incubation, an equal volume of chloroform:isoamyl alcohol (24:1) was added. The tube was centrifuged at 7700x g for 10 min, and the aqueous phase was transferred to a new tube.

The DNA was precipitated by adding 2/3 volume of isopropanol and allowing to stand overnight. The next day the DNA was centrifuged at 7700x g for 10 min. The isopropanol was poured off, and the pellet was washed in 10 volumes of 70% ethanol. The DNA was centrifuged at 7700x g for 10 min. The ethanol was poured off, and the
DNA was left to air-dry. Finally, the DNA was re-suspended in an appropriate volume of TE buffer.

Guanidium Thiocyanate Method (modified from Fernandez-Busquets, 2000)

In this method GES was prepared by combining the following: 60g guanidium thiocyanate, 25 mL of 400mm EDTA (pH 8.0), 5mL 10% Sarkosyl, and filling to 100 mL with distilled H$_2$O. The GES was then added to ground tissue. Various ratios of GES to tissue were tried, from 2-10 volumes GES, depending on the volume of sample and available tube-space.

The solution was vortexed, incubated 10 min at room temperature, mixed by inversion, incubated at 37 °C for 30 min, and then chilled on ice. Once chilled, ammonium acetate (10 M) was added to a final concentration of 2.5 M. The sample was inverted, and a volume of phenol:chloroform:isoamyl alcohol (25:24:1) equal to the volume of GES was added. The tube was then mixed by inversion and centrifuged at 2900x g for 10 min. The aqueous phase was removed to a new tube, where an equal volume of chloroform was added, mixed by inversion, and centrifuged 5 min at 2900x g. The aqueous layer was transferred to a new tube and precipitated with 0.54 volumes of isopropanol. This tube was centrifuged at 13,000x g for 20 s. The isopropanol was poured off, and the pellet was washed in 70% ethanol. The ethanol was poured off, and the pellet was allowed to dry overnight before being re-suspended in an appropriate volume of TE.
Analysis

DNA purity was assessed by determining $A_{260}/A_{280}$ ratios. DNA (2 µL) was placed in de-ionized water (498 µL), mixed by inversion, and read in a spectrophotometer. DNA yields were determined by the formula $A_{260}/0.020$.

RNase Treatment

In one set of experiments RNase was included in the lysis buffer of the standard method, as in Sambrook (1989). In other experiments, DNA isolated using the guanidium thiocyanate and CTAB procedures was treated with RNase after the isolation. A volume equal to the original tissue volume of each of the following was added: 4x 10mM Tris, 4x 0.1 M EDTA, and 4x 20µg/mL RNase. This solution was incubated 1 h at 37°C, extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and centrifuged 7700x g for 10 min. The aqueous layer was transferred to a new tube. Chloroform:isoamyl alcohol (24:1) (1 volume) was added and centrifuged at 7700x g for 10 min, and then the aqueous layer was removed. DNA was precipitated by adding 0.54 volumes of isopropanol and centrifuging at 13,000x g for 20 s. The isopropanol was poured off, and the pellet was then washed in 70% ethanol. The ethanol was poured off, and the pellet was allowed to dry overnight and then re-suspended in TE.
Results

One of the objectives of this study was to determine the feasibility of isolating DNA from ethanol preserved specimens. Table 1 shows a comparison of DNAs isolated from ethanol preserved specimens and specimens preserved by freezing at -70°C. The A$_{260}$/A$_{280}$ ratio is an indication of the purity of the DNA isolate. Pure DNA gives a ratio of between 1.8 and 1.9. Pure RNA gives a ratio of 1.9 to 2.0. With the exception of the alcohol-preserved guanidium-thiocyanate isolate (2.44), all A$_{260}$/A$_{280}$ ratios were close to 1.9. Furthermore, while Table 1 shows that A$_{260}$/A$_{280}$ ratios are similar it also shows that the yield of DNA from CTAB extractions (3.51 and 2.54 μg DNA/g of tissue) is higher than the other two methods. Table 1 also lists A$_{230}$ readings, which indicate some contamination by aromatic moieties. In proportion to the A$_{260}$ values, A$_{230}$ values are higher with the standard method (0.646 for the standard method, 0.473 for the CTAB method, and 0.227 for the guanidium thiocyanate method for frozen specimens) (Table 1).
Table 1. Comparison of DNA isolated from frozen and alcohol-preserved specimens.

All samples from *E. fragilis*.

<table>
<thead>
<tr>
<th>Isolation Method</th>
<th>Method</th>
<th>A&lt;sub&gt;260&lt;/sub&gt;</th>
<th>A&lt;sub&gt;280&lt;/sub&gt;</th>
<th>A&lt;sub&gt;260&lt;/sub&gt;/A&lt;sub&gt;280&lt;/sub&gt; Ratio</th>
<th>A&lt;sub&gt;230&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A&lt;sub&gt;325&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Yield&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen Specimens</td>
<td>Standard</td>
<td>0.096</td>
<td>0.051</td>
<td>1.88</td>
<td>0.062</td>
<td>0.003</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>CTAB</td>
<td>0.478</td>
<td>0.251</td>
<td>1.90</td>
<td>0.226</td>
<td>0.011</td>
<td>3.51</td>
</tr>
<tr>
<td></td>
<td>GTC</td>
<td>0.066</td>
<td>0.035</td>
<td>1.89</td>
<td>0.015</td>
<td>0.000</td>
<td>0.91</td>
</tr>
<tr>
<td>Alcohol-Preserved Specimens</td>
<td>Standard</td>
<td>0.038</td>
<td>0.021</td>
<td>1.81</td>
<td>0.027</td>
<td>0.002</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>CTAB</td>
<td>0.458</td>
<td>0.239</td>
<td>1.92</td>
<td>0.212</td>
<td>0.008</td>
<td>2.54</td>
</tr>
<tr>
<td></td>
<td>GTC</td>
<td>0.039</td>
<td>0.016</td>
<td>2.44</td>
<td>0.005</td>
<td>0.000</td>
<td>0.24</td>
</tr>
</tbody>
</table>

<sup>a</sup> detects contaminants containing aromatic moieties, such as phenol

<sup>b</sup> detects particulate contaminants

<sup>c</sup> µg DNA / g tissue

<sup>d</sup> DNA isolated by the standard method

<sup>e</sup> DNA isolated by the CTAB method

<sup>f</sup> DNA isolated by the guanidium thiocyanate method

A second objective of this study was to determine which DNA extraction method yields the highest purity DNA. Table 2 shows the results of all three extraction methods from all three sponge species. It should be noted that in this experiment RNase was incorporated into the extraction buffer of the standard method.

The standard method, which used RNase, has the lowest A<sub>260</sub>/A<sub>280</sub> ratios (0.955 to 1.410). The guanidium thiocyanate and CTAB methods have similar A<sub>260</sub>/A<sub>280</sub> ratios of 1.630 to 1.855 and 1.620 to 1.950, respectively. The species of sponge used did not appear to affect the A<sub>260</sub>/A<sub>280</sub> ratio.
Table 2. Comparison of DNA isolated from three alcohol-preserved specimens of three sponge species.

<table>
<thead>
<tr>
<th>Extraction Method</th>
<th>$A_{260}/A_{280}$ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard*</td>
<td></td>
</tr>
<tr>
<td>$E.\text{ muelleri}$</td>
<td>0.955</td>
</tr>
<tr>
<td>$E.\text{ fragilis}$</td>
<td>1.250</td>
</tr>
<tr>
<td>$S.\text{ lacustris}$</td>
<td>1.410</td>
</tr>
<tr>
<td>CTAB</td>
<td></td>
</tr>
<tr>
<td>$E.\text{ muelleri}$</td>
<td>1.855</td>
</tr>
<tr>
<td>$E.\text{ fragilis}$</td>
<td>1.730</td>
</tr>
<tr>
<td>$S.\text{ lacustris}$</td>
<td>1.630</td>
</tr>
<tr>
<td>GTC</td>
<td></td>
</tr>
<tr>
<td>$E.\text{ muelleri}$</td>
<td>1.950</td>
</tr>
<tr>
<td>$E.\text{ fragilis}$</td>
<td>1.800</td>
</tr>
<tr>
<td>$S.\text{ lacustris}$</td>
<td>1.620</td>
</tr>
</tbody>
</table>

* standard samples treated with RNase

It was observed that samples treated with RNase had the lowest $A_{260}/A_{280}$ ratios.

In a follow-up experiment, DNA isolated by the guanidium thiocyanate and CTAB procedures was treated with RNase. Table 3 shows $A_{260}/A_{280}$ ratios before and after RNase treatment.
Table 3. Comparison of guanidium thiocyanate and CTAB methods before and after RNase treatment

<table>
<thead>
<tr>
<th></th>
<th>A$<em>{260}$/A$</em>{280}$ Ratio</th>
<th>Before RNase</th>
<th>After RNase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GTC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. muelleri</em></td>
<td></td>
<td>2.00</td>
<td>1.17</td>
</tr>
<tr>
<td><em>S. lacustris</em></td>
<td></td>
<td>1.61</td>
<td>1.14</td>
</tr>
<tr>
<td><em>E. fragilis</em></td>
<td></td>
<td>1.80</td>
<td>1.33</td>
</tr>
<tr>
<td><strong>CTAB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. muelleri</em></td>
<td></td>
<td>1.81</td>
<td>1.27</td>
</tr>
<tr>
<td><em>S. lacustris</em></td>
<td></td>
<td>1.63</td>
<td>1.08</td>
</tr>
<tr>
<td><em>E. fragilis</em></td>
<td></td>
<td>1.73</td>
<td>1.00</td>
</tr>
</tbody>
</table>

A$_{260}$/A$_{280}$ ratios dropped in all isolates treated with RNase. RNase-treated isolates had A$_{260}$/A$_{280}$ ratios of between 1.00 and 1.33.
Discussion

This study’s first objective was to determine the feasibility of extracting DNA from ethanol-preserved specimens. This study did not show any notable differences in the purity of DNA isolated from sponges preserved in ethanol or by freezing (Table 1). Therefore, this study suggests that sponge DNA can be extracted effectively from ethanol-preserved specimens. Consequently, ethanol is recommended for sponge preservation in situations where dry-ice or long-term freezing facilities are not available. Ethanol additionally has the advantage of being easier to handle in the field than dry ice.

The experiment comparing DNA extracted from frozen and ethanol preserved specimens (Table 1) suggests that nucleic acids were isolated because the $A_{260}/A_{280}$ ratios are between 1.80 and 1.90, which suggests DNA. The $A_{260}/A_{280}$ ratio of the guanidium thiocyanate-extracted, alcohol-preserved specimen is high (2.44). This is probably due to either a contaminant that absorbs at 260 nm, or the fact that it has low $A_{260}$ and $A_{280}$ values. Low absorption values may create a high $A_{260}/A_{280}$ ratio if they are near the spectrophotometer’s lower detection limit and consequently misreported by the instrument.

The second objective of this study, to compare the purity and yield of DNA isolated by the different methods, did not show that any one of the DNA extraction methods used was preferable to the others in terms of purity (Tables 2 and 3). This study did, however, suggest that the CTAB method is preferable to the other two methods due to its higher yield of DNA/g of tissue.
Finally, this study found that digestion with RNase after the initial DNA isolation lowered the $A_{260}/A_{280}$ ratio (Table 3), suggesting that RNA is present after DNA isolation in all three methods. Furthermore, Table 3 suggests that after RNase treatment the standard method’s $A_{260}/A_{280}$ ratio is similar to the other two methods.

In an effort to determine the molecular weight of DNA isolated in these experiments, the DNA isolates were electrophoresed on agarose gels and stained with ethidium bromide. The isolates that were untreated with RNase exhibited long smears of low molecular weight nucleotides (result not shown). Redman (1999) reported a similar smear and observed that it was no longer present after RNase treatment. An attempt to confirm that the low molecular weight smear was due to RNA contamination by treating the DNA sample with RNase and then running the sample on an agarose gel was unsuccessful.

In conclusion, the CTAB method yields the most DNA per gram of sponge (Table 1). Further advantages to this method are that it has the fewest steps, is the easiest to perform, and does not involve phenol. The standard method has the second highest yield, but due to a long incubation in a water bath it is the most time-consuming method. The advantage of the standard method is that RNase can be used in the initial isolation, whereas RNase is not used in the other two methods and must be added as a final series of steps. DNA purity from all three isolation methods is similar. DNA isolated with any of the three methods described here should be treated afterwards with RNase. Future work could focus on reducing the amount of RNA present in the final isolate.
References


