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Article

Sepia bandensis ink inhibits polymerase chain reactions

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SUMMARY

While cephalopods serve critical roles in ecosystems and are of significant interest in scientific studies of the nervous system, medicinal toxins, and evolutionary diversification. The absence of a genomic library and the lack of comprehensive gene analysis present challenges to conducting efficient and thorough research. One difficulty in advancing cephalopod genomics is the presence of inhibitors (such as ink) that impede the amplification of DNA samples with PCR. We tested the hypothesis that *Sepia bandensis* (dwarf cuttlefish) ink inhibits PCR by running PCR reactions with and without the back addition of ink to *Turbo fluctuosus* (marine sea snail) DNA with the inclusion of the appropriate positive and negative controls. The experimental results show that ink added to *T. fluctuosus* DNA extracted using two kit-based extraction methods or phenol chloroform extraction prevents the amplification of the cytochrome c oxidase subunit I (COI) mitochondrial gene. Also, while modern extraction methods like quaternary amine resin and silica column isolation failed to produce genomic products viable for PCR from *S. bandensis*, phenol chloroform extraction eliminated the inhibitors and resulted in successful amplification. The results of this investigation could further cephalopod genomic studies and serve as a model for experiments aiming to determine the cause of PCR inhibition.

INTRODUCTION

Mollusks occupy a key role in ecosystems around the world: they purify water by passing it through their internal biological systems, recycle nutrients in the ocean, and serve as integral parts of many food webs (1). However, due to climate change and habitat destruction, their populations may become threatened (2). This fact poses threats to the stability of many ecosystems and to the survival of several species in the mollusk phylum, including those in the Cephalopoda class.

Although cephalopods play significant roles in natural ecosystems and in medical research, there is currently no assembled cephalopod genome, as challenges in accessing samples and keeping cephalopods (specifically oceanic and deep-sea species) in a lab setting have prevented the large scale analysis of their molecular and morphological features (3-5). Thus, there is limited information on mollusk transcriptomes and genomic regulation, but advancements could be made through genetic studies and DNA barcoding. Many sequencing efforts are underway due to the popularization and increased ease of molecular sequencing, yet difficulties, such as repeated regions and gene duplication events, have prevented the compilation of a full genome (1, 4, 6).

Biocurious's cuttlefish project team chose *Sepia bandensis* (the dwarf cuttlefish) as a potential model organism for mollusks due to its small size and diagnostic features, like aligned suckers, chromatophores, and dorsal and ventral protective membranes as shown in **Figure 1** (7, 8).

The initial goal of the project was to sequence the *S. bandensis* genome and study the cephalopod's gene expression, but difficulties arose while attempting to prepare PCR products for genomic sequencing. Originally, the isolation of cuttlefish DNA by silica column and quaternary amine resin failed to produce genomic DNA products that were viable for PCR amplification of a segment of the cytochrome c oxidase subunit one COI mitochondrial gene (unpublished findings). We performed gel electrophoresis and saw no band of the expected 710 base pairs. Since COI is found in all eukaryotic organisms, including the closely related cuttlefish *S. officinalis* (the common cuttlefish), it is highly improbable that *S. bandensis* lacks this gene. We hypothesized that the failure was due to interference from inhibitors or inferior enzymes, as those are well-documented causes of PCR failure.

Repeatedly, inhibitors have been shown to increase error, reduce assay resolution, and produce inaccurate results in both quantitative and qualitative PCR assays (9, 10). Common mechanisms of PCR inhibition include the binding of an inhibitor to the polymerase, erroneous polymerase interaction with DNA, and inhibitor interaction with the polymerase during primer extension. Inhibitor sources may include reagents used during sample preparation, contamination,

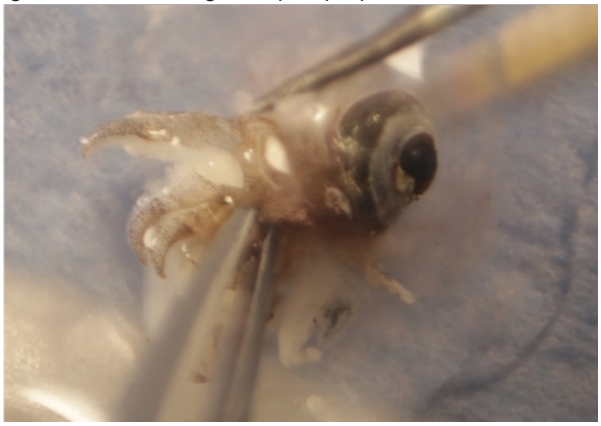

Figure 1. Dissecting *S. bandensis* (Photo credits: Eric Aker).

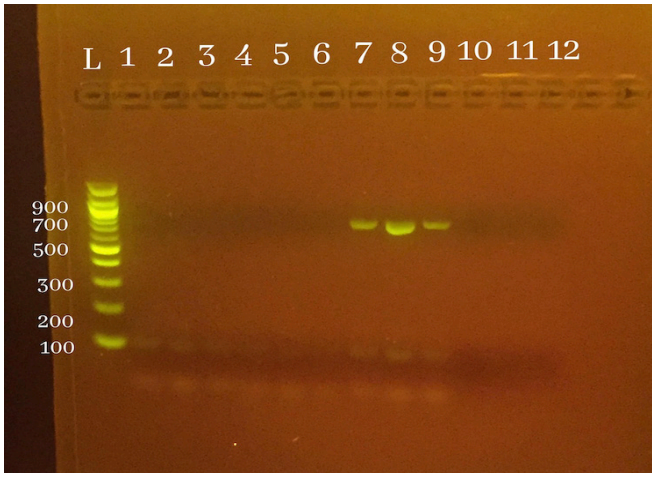

Figure 2. PCR amplification using the three DNA extraction methods with or without ink. Gel electrophoresis shows a clear band of ~710 bp for each of the three extraction methods where no ink was added (#7-10). **L**: 10 µl 100bp ladder, **1**: Phenol chloroform + 0.5 µl ink, **2**: MagListo + 0.5 µl ink, **3**: AccuPrep + 0.5 µl ink, **4**: Phenol chloroform + 1.5 µl ink, **5**: MagListo + 1.5 µl ink, **6**: AccuPrep + 1.5 µl ink, **7**: Phenol chloroform + no ink, **8**: MagListo +no ink, **9**: AccuPrep + no ink, **10**: Phenol chloroform + no primers, **11**: MagListo+ no primers, **12**: AccuPrep + no primers**.** Each PCR product lane has 2 µl 6X loading dye and 7 µl PCR products.

or the sample itself. Some common examples of inhibitors are organic compounds (such as urea, phenol, ethanol, and polysaccharides) and proteins (such as melanin and collagen) (10-12). Differences in relative amplification efficiency and changes in the slope of the exponential amplification curve compared to a non-inhibited control sample may be used to reveal decreased PCR efficiency due to the presence of such inhibitors (13). Furthermore, the concentration of the inhibitor tends to be correlated with the degree of inhibition (11).

S. bandensis ink is negatively charged and composed of heavily glycosylated proteins (specifically monomeric units of dihydroxyphenylalanine (DOPA) and/or cysteinyl-DOPA). It also includes other proteins (such as melanin), peptidoglycan, dissolved amino acids, and metals (14). The ink may inhibit PCR reactions by interacting with the DNA template or by binding to enzymes in the PCR mix. In our case, the S. bandensis DNA isolated with the DNA extraction had a purple tint. This strange coloration led the team to guess that ink was the probable inhibitor.

We guessed that the ink blocked the PCR reaction and that greater concentrations of ink would have stronger inhibitory effects. In order to test the hypothesis that *S. bandensis* ink is a PCR inhibitor, we designed a series of experiments based around the PCR amplification of the COI gene with and without the presence of ink. Appropriate negative and positive controls were included.

In addition, we hypothesized that the mucus may also have inhibitory effects due to its composition of protein-polysaccharide complexes, inorganic salts, and water. Glycoproteins help determine the mucus's properties, while glycosaminoglycans include numerous linear carbohydrate chains and do not notably contribute to the mucus's characteristics; still, more research is required to confirm these observations (15, 16).

We chose to amplify the cytochrome c oxidase subunit I (COI) gene in this study due to its effectiveness in barcoding species. COI is expressed in all cells of all eukaryotic organisms under normal conditions, and mRNA and protein are usually found at stable levels. Importantly, it is heavily conserved among members of the same species but differs significantly by several percentages among different species (17). COI is considered to be a housekeeping gene, as it maintains cellular function due to its key role in the electron transport chain. Furthermore, it plays a role in maintaining homeostasis by converting arachidonic acid to prostaglandin H2, a precursor to other biologically significant prostaglandins (18). Thus, it is very common and well-studied.

Phenol chloroform is an extraction method that has been used when PCR inhibitors are suspected or when the DNA quantity in samples is low (e.g. in forensic analysis). We predicted that this method would separate the glycosylated proteins and lipids as well as the ink and mucous from the DNA through phase separation. Typically, phenol chloroform extraction uses the Proteinase K enzyme and sodium dodecyl sulfate (a detergent) to lyse and digest cells. The addition of phenol prompts phase separation based on differences in solubility. Chloroform helps increase the efficiency of phenol, aids in the denaturation of lipids, and helps prevent phase inversion. When performed effectively, this process yields high-quality DNA in significant quantities (19). However, there are more opportunities for contamination with this method than with modern kit-extraction methods due to the greater procedure complexity and hands-on effort. One additional difficulty includes separating the interphase layer containing DNA from the aqueous and organic layers without mixing (19, 20). Despite these challenges, a modified version of phenol chloroform extraction performed on extracted cuttlefish organs in our study successfully produced viable PCR templates while modern kit-based protocols did not.

Here, we show the inhibitory properties of ink through results that depict PCR failure after back-additions of ink to DNA extracted with two kit-based extractions and phenol chloroform extraction. We also describe a detailed protocol of phenol chloroform extraction optimized for mollusk samples and explain its effectiveness in the presence of inhibitors. The article concludes by exploring this study's implications for mollusk research.

RESULTS

Ink inhibits amplification of COI from *T. fluctuosus*

To test whether ink inhibits COI amplification, we added 0 µl, 0.5 µl, or 1.5 µl of *S. bandensis* ink to extracted *T. fluctuosus* samples. We found that the addition of 0.5 µl and 1.5 µl of ink prevented amplification of COI: all three extraction methods (phenol chloroform, MagListo, and AccuPrep) failed to produce a template that showed a clear band after PCR.

Comparing #1-3 (addition of 0.5 µl ink) to #4-6 (addition of 1.5 µl ink)

	Phenol Chloroform	MagListo	AccuPrep
A260/A230 #1-3 had a lower A260/A230 ratio for MagListo and higher ratios for Phenol-chloroform and AccuPrep	#1 Average: 1.2967 #4 Average: 1.24	#2 Average: 1.0167 #5 Average: 1.4167	#3 Average: 1.0933 #6 Average: 0.78
p value	0.0607	<0.0001	<0.000138
significant?	not significant	highly significant	highly significant
A260/A280 #4-6 had lower A260/A280 ratios than #1-3 across the three extraction methods	#1 Average: 1.75 #4 Average: 1.74	#2 Average: 1.6667 #5 Average: 1.77	#3 Average: 1.7333 #6 Average: 1.6267
p value	0.1587	0.002068	0.023168
significant?	not significant	significant	significant

Comparing #1-3 (addition of 0.5 µl ink) to #7-9 (no addition of ink)

	Phenol Chloroform	MagListo	AccuPrep
A260/A230 #1-3 had lower A260/A230 ratios than #7-9 across the three extraction methods	#1 Average: 1.2967 #7 Average: 1.88	#2 Average: 1.0167 #8 Average: 1.67	#3 Average: 1.0933 #9 Average: 1.5533
p value	0.010658	<0.0001	<0.0001
significant?	significant	highly significant	highly significant
A260/A280 #1-3 had lower A260/A280 ratios than #7-9 across the three extraction methods	#1 Average: 1.75 #7 Average: 1.8167	#2 Average: 1.6667 #8 Average: 1.7667	#3 Average: 1.7333 #9 Average: 1.8
p value	0.013054	0.001892	0.075168
significant?	significant	significant	not significant

Comparing #4-6 (addition of 1.5 µl ink) to #7-9 (no addition of ink)

	Phenol Chloroform	MagListo	AccuPrep
A260/A230 #4-6 had lower A260/A230 ratios than #7-9 across the 3 extraction methods	#4 Average: 1.24 #7 Average: 1.88	#5 Average: 1.4167 #8 Average: 1.67	#6 Average: 0.78 #9 Average: 1.5533
p value	0.008044	0.000408	<0.0001
significant?	significant	highly significant	highly significant
A260/A280 #4-6 had lower A260/A280 ratios than #7-9 for phenol-chloroform and AccuPrep and a higher ratio for MagListo	#4 Average: 1.74 #7 Average: 1.8167	#5 Average: 1.77 #8 Average: 1.7667	#6 Average: 1.6267 #9 Average: 1.8
p value	0.006170	0.643330	0.283891
significant?	significant	not significant	not significant

Table 1. Analysis of spectrophotometer data. A two-tailed t-test was performed using VassarStats to determine the level of significance of the variance in purification ratios. *P*-values <0.0005 are considered statistically highly significant, *p*-values <0.025 are considered statistically significant, and *p*-values >0.025 are considered not significant. Bonferroni's correction was applied to correct for multiple comparisons by dividing the alpha value (0.05 as statistically significant) by 2 (the number of comparisons).

The negative controls (where no primers were added) did not produce a band. In contrast, samples where no ink was added produced distinct bands (**Figure 2**).

Analysis with a spectrophotometer disclosed the effects of the addition of ink to DNA-purity ratios. A ratio of about 1.8 is seen as pure for dsDNA, which generally absorbs at 260 nm. A low A260/230 ratio is often an indication of a contaminant that has an absorbance of 230 nm or less while a low A260/280 ratio is often an indication of a contaminant that absorbs at around 280 nm or less. For reference, proteins absorb at around 280 nm.

A260/A230

Samples with low A260/A230 ratios can indicate the presence of contaminants, such as organic compounds or chaotropic salts. The standard recommended ratio should exceed 1.5, with the ideal being close to 1.8. The average A260/A230 ratios for our PCR products after the addition of 0.5 µl of ink were -0.503, -0.783, and -0.707 away from the recommended 1.8 ratio, and the ratios for the addition of 1.5 µl of ink were -0.560, -0.383, and -1.02 away from the ideal for phenol chloroform, MagListo, and AccuPrep respectively. The average A260/A230 ratios where ink was not added were +0.080, -0.130, and -0.267 away from 1.8 for the three methods (**Table 1 and Figure 3**). When comparing the addition of 0.5 µl of ink to no addition of ink, we observed a significant difference in the A260/A230 ratios as indicated by *p*-values of 0.010658, <0.0001, and <0.0001 for phenol chloroform, MagListo, and AccuPrep respectively. Similar significant differences were seen when comparing the addition of 1.5 µl of ink to no addition of ink (*p*-values 0.008044, 0,000408, and <0.0001).

These results indicate that the reactions where ink was not added had purer DNA with fewer contaminants that absorb at 230 nm (the ratios were closer to 1.8). Except for MagListo, the addition of greater volumes of ink (1.5 µl instead of 0.5 µl) was correlated with lower A260/A230 ratios.

A260/A280

The A260/A280 ratio is often used to assess protein contamination of DNA, for proteins absorb at 280 nm due to their aromatic ring structures. Pure DNA samples have A260/A280 ratios greater than or equal to 1.8. The average A260/

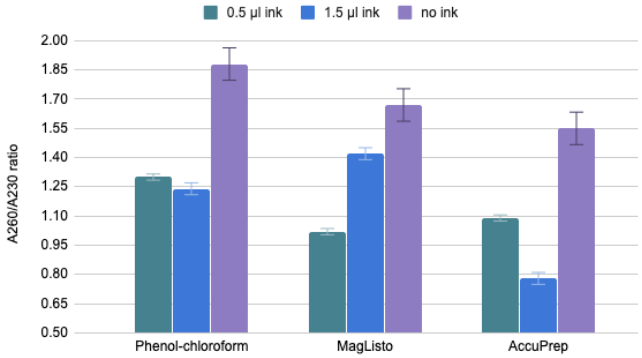

Figure 3. Addition of no ink produced better A260/A230 ratios than the addition of 0.5 µl, and 1.5 µl of ink. Analysis of the samples after PCR amplification focusing on the A260/A230 ratios shows significant contrasts between reactions where ink was added (0.5 µl or 1.5 µl) and reactions where ink was not added. The error was calculated by finding the ± standard deviation: 0.01588 for 0.5 µl ink, 0.0305 for 1.5 µl ink, and 0.083312 for no ink. The values shown are the average of three measurements with the DeNovix spectrophotometer; n=3.

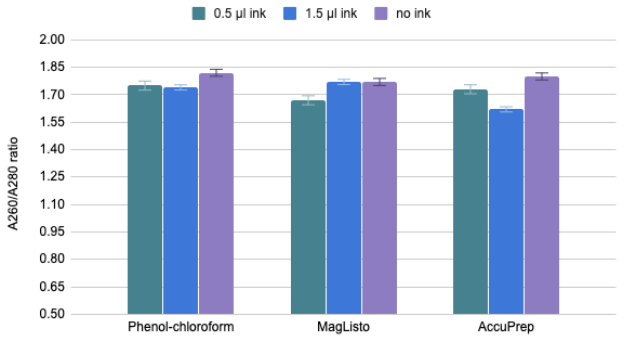

Figure 4. Addition of no ink produced better A260/A280 ratios than the addition of 0.5 µl, and 1.5 µl of ink. Analysis of the samples after PCR replication focusing on the A260/A280 ratios shows significant contrasts between reactions where ink was added (0.5 µl or 1.5 µl) and reactions where ink was not added. The error was calculated by finding the ± standard deviation: 0.0245 for 0.5 µl ink, 0.014048 for 1.5 µl ink, and 0.01913 for no ink. The values shown are the average of three measurements with the DeNovix spectrophotometer; n=3.

A280 ratios after the addition of 0.5 µl of ink were -0.500, -1.33, and -0.067 away from the recommended 1.8 limit, and the ratios for the addition of 1.5 µl of ink were -0.060, -0.030, and -0.173 away from the recommended limit (for phenol chloroform, MagListo, and AccuPrep respectively). The average A260/A230 ratios where ink was not added were +0.0167, -0.0333, and 0 away from 1.8 for the three extraction methods (**Table 1 and Figure 4**).

When comparing the addition of 0.5 µl of ink to no addition of ink, we observed a significant difference in the A260/A280 ratios for phenol chloroform (*p*-value 0.013054) and MagListo (*p*-value 0.001892) but not for AccuPrep (*p*-value 0.075168). When comparing the addition of 1.5 µl of ink to no addition of ink, a significant difference was seen in the phenol chloroform extraction (*p*-value 0.006170).

While the differences in the A260/A280 ratios among the various extraction methods were not as extreme as those of the A230/A260 ratios, the fact that the ratios for PCR samples with no ink were closer to the recommended benchmark of 1.8 indicates higher quality DNA samples.

Mucus

While the mucus may have had some inhibitory effects, they were not significant because PCR amplification was successful even with the MagListo and AccuPrep kits, which did not remove the mucus. Its composition of protein-polysaccharide complexes, inorganic salts, and water did not seem to inhibit the PCR reactions (15, 16). Further studies need to be conducted to confirm this hypothesis.

Successful amplification of COI from *S. bandensis* after extracting DNA with phenol chloroform

Phenol chloroform DNA extraction of *S. bandensis* produced samples viable for PCR. Analysis with gel electrophoresis showed clear amplification of the COI gene from the buccal and tentacle samples, with tentacle DNA having better amplification as indicated by a brighter band. In contrast, DNA templates from the eye, ovary, and ink sac produced very faint bands or no bands at all, perhaps due to the limited amount of COI in those tissues (**Figure 5**). Sanger sequencing showed notable sequence alignment of our sample with the *S. officinalis* genome from NCBI (ranging from 93.80% to 95.08 %).

Analysis with a spectrophotometer produced a value of 1.70 for the A260/230 ratio and 1.76 for the A260/A280 ratio (n=3), as well as a distinct peak in the 260nm wavelength region. The A260 value was 21.95, and there were 1098 ng of DNA per µL of the solution.

In previous runs, a slight peak at 270 nm indicated the presence of residual phenol. The addition of more chloroform counteracted this issue by dissolving the phenol while allowing theDNA to remain in the aqueous layer. While phenol has been shown to denature DNA polymerases, no such properties were noted in this experiment, as the COI was amplified and produced a clear band on a gel as shown in **Figure 2** and **Figure 5** (22).

DISCUSSION

This study shows the effectiveness of phenol chloroform in removing inhibitors from cephalopod samples to produce a viable template for PCR. This can be seen from the distinct bands produced on the gel (**Figure 5**). The brightness of the tentacle band is likely the result of the presence of many copies of the COI gene in tentacle cells due to the high energy metabolism of muscle.

Figure 5. COI gene amplified from the buccal and tentacle samples. The 1.5% agarose gel was loaded with 2 µl 6X loading dye mixed with 8 µl of PCR product. **L**: 10 µl 100bp ladder, **1**: unknown sample, **2**: eyeball, **3**: buccal mass, **4**: tentacle, **5**: ovary.

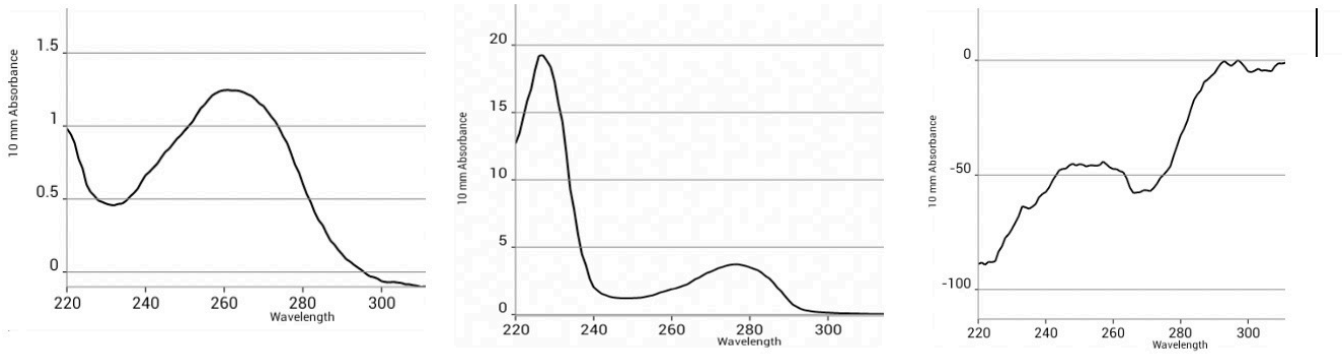

Figure 6. Ink affects spectrophotometer graphs showing wavelength versus 10 nm absorbance. (left to right): Graph generated with *S. bandensis* DNA extracted using the phenol chloroform method, graph generated with *S. bandensis* DNA extracted using the Accuprep kit, and graph generated from a pure ink sample from an isolated *S. bandensis* ink sac.

Because phenol chloroform is immiscible with water, mixing it with the sample permitted the formation of distinct phases due to differences in density. The heavily glycosylated ink entered the dense organic layer, while the nucleic acids entered the less dense aqueous layer. This phase-separation was critical in isolating the DNA and preventing ink contamination of the sample. Other extraction methods—specifically kit-based ones—proved effective in isolating *T. fluctuosus* DNA (no ink), but not *S. bandensis* when ink was present. Analysis of isolated *S. bandensis* samples with a spectrophotometer showed that phenol chloroform extraction produced a clear peak at 260nm, while the MagListo extraction had a high peak at approximately 230nm, a lower peak at 280nm, and an upward-sloping trend between 230nm and 280nm. The Accuprep extraction produced a low peak at about 250nm and a much higher peak at about 290 nm; the plotted data was also irregular, as it did not create smooth curves (**Figure 6**). When using kit-based methods, the ink may have prevented the nucleic acids from effectively binding to the silica gel membrane or flowing through the spin column. More data is needed to support these speculations.

Also, adding ink to *T. fluctuosus* illustrates that ink likely causes PCR inhibition. While all three extraction methods—phenol chloroform, MagListo, and Accuprep—proved effective in isolating samples for PCR, the addition of ink following purification impeded PCR amplification for all three methods (**Figure 2**). By forming a reversible complex with the DNA polymerase, the melanin in the ink may have altered the DNA polymerase, preventing it from binding to the template and elongating the strand (21). Other glycoproteins in the ink may have also compromised the effectiveness of PCR by binding to cofactors or by blocking reaction components (10, 13).

When isolating *T. fluctuosus* with phenol chloroform, the purity of the sample may have been further away from the ideal than samples isolated with MagListo and Accuprep due to residual components left in the product, such as phenol, chloroform, or salts. Contamination may be minimized by taking more care in pipetting the supernatant, centrifuging at higher speeds, or performing additional purification procedures. Other solutions could be performing additional washes or one more round of chloroform extraction; however, this could result in a decrease in the quantity of DNA. The data suggests that phenol chloroform may not be the best extraction method for isolating DNA from tissues without PCR-inhibiting components; nevertheless, it could be superior when inhibitors are present.

These results are notable because an effective procedure to produce PCR templates in the presence of inhibitors is crucial to galvanizing more studies of cephalopods and other organisms whose samples may include interfering compounds. Advances in cephalopod genomics could notably improve the efficiency and effectiveness of cephalopod research by providing scientists with a comprehensive library of the species' genes, identifying critical protein identities, and improving the effectiveness of gene analysis (1). This information would be of significant value to those seeking to understand cephalopod gene expression and mutations as well as to those studying comparative development of metazoans. Future research into cephalopod genomics could also reveal new molecular regulatory mechanisms and protein expression patterns (1).

Analysis on a molecular level of the impact of changing oceanic conditions—such as ocean acidification, warming of waters, and lower oxygen tension in oxygen minimum layers—on mollusks can help predict their responses to new selective pressures (4, 23, 24). Mollusks could also be used to study the broader implications of a changing climate, as their intricate evolutionary history and prevalence in oceanic habitats around the world could offer insights into the adaptations and diversification of traits (3, 4).Furthermore, cephalopods are of special interest to many researchers due to their unique adaptations (such as chromatophores, differentiated brains, and specialized sensory organs) and intricate structures, including vascular systems and neurological mechanisms (4). Studying these features could provide insights into homologous structures and similar regulatory mechanisms in other organisms.

Moreover, members of the Mollusca phylum, especially cephalopods, are used in medical research for developing biologically active substances, in toxicology studies of pollutants and for commercial uses in dietary supplements and cosmetics. Biological compounds isolated from the tissues and ink of cephalopods have been shown to exhibit antioxidant properties; besides that, they may increase lipid metabolism and inhibit angiotensin-converting enzymes involved in high blood pressure and impaired homeostasis (5). Improved procedures to isolate cephalopod DNA would prove useful for these objectives.

Ultimately, the ability to remove inhibitors without compromising DNA quality is crucial to developing isolates that can be amplified. The control experiments performed in this study could serve as a model for experiments aiming to determine the cause of PCR inhibition.

While the control experiments showed that ink was the probable inhibitor, future studies are needed to further analyze the specific components of ink that may be causing this inhibition. Other future studies could examine the effectiveness of phenol chloroform extraction in removing inhibitors from other mollusk samples.

METHODS

Ink Inhibition Experiment

A multi-part control protocol was designed to establish the cause of PCR inhibition by determining whether ink caused the failed amplification. The same PCR conditions and primers as were used for the *S. bandensis* PCR amplification were used to amplify COI from Turbo fluctuosus, which has no ink. Three different extraction methods—phenol chloroform extraction, genomic DNA extraction with Bioneer's MagListo kit, and genomic DNA extraction with Bioneer's AccuPrep kit—were used.

The MagListo kit involves lysing the sample with Proteinase K and RNase and then using chaotropic agents to facilitate the absorption of DNA onto magnetic nano beads. A magnetic plate and a series of washes are used to isolate the DNA. The Accuprep kit involves a series of centrifugations following additions of a tissue lysis buffer, Proteinase K, RNase A, and a binding buffer. After adding absolute ethanol, the sample is washed to remove residual components. Phenol chloroform extraction uses proteinase K to digest tissue and then phenol and chloroform to facilitate phase separation (see the introduction for a thorough explanation of the phenol-chloroform method).

The independent variable for each extraction methods was the amount of ink added (0.5 µl or 1.5 µl). The positive controls were the extracted DNA samples with no addition of ink. The negative controls were the PCR reactions performed without the addition of primers.

First, the sea snail was anesthetized by covering it entirely with 20mM MgCl2 in a beaker for 10 minutes at room temperature (25). The tissue was cut away and put into a tube, where scissors were used to cut it into even smaller pieces. For each extraction method, about 30-60 mg of tissue was used. The ink was obtained from an isolated S. bandensis ink sac.

Before use in PCR, 40 µl of extracted DNA from each of the three methods was cleaned using the AccuPrep PCR Purification Kit from Bioneer according to the manufacturer's protocol. After purifying the template DNA, the three following PCR reactions were prepared using *T. fluctuosus* tissue samples: Phenol chloroform extracted DNA, MagListo extracted DNA, and AcuuPrep extracted DNA. These reactions were done with either 0.5 µl ink, 1.5 µl ink, no ink, and a negative control containing no primers or ink.

Each reaction contained 26 µl of nuclease-free water (30 µl for the negative controls), 4 µl of 10x buffer, 2 µl dNTPs,2 µl of MgCl2, 2 µl of the forward COI primer (omitted for the negative controls), 2 µl of the reverse COI primer (omitted for the negative controls), 1 µl of hot start Taq polymerase, 1 µl of the extracted DNA template combined for a 40 µl total volume in each PCR tube. The PCR products were then analyzed using gel electrophoresis and a spectrophotometer.

Phenol chloroform extraction

All reagents were purchased from Thermo Fisher Scientific. Phenol chloroform extraction was performed to extract DNA samples from cuttlefish organs including the tentacles, muscle from ovaries, eye, stomach, buccal parts, and ink sac tissue. The PCR was performed on a variety of organs to determine which ones resulted in the highest PCR efficiency based on the amount and quality of product. It was predicted that tentacle and buccal samples would be best due to the high metabolic activity of those organs and the high density of muscle cells. The procedure was revised chiefly by Johan Sosa and Eric Espinosa, Ph.D. and optimized using marine snail samples.

After isolating the extracted organs into 25-50 mg tissue samples in separate microcentrifuge tubes, 500 µl of TRIzol (which is composed of phenol, guanidine isothiocyanate, and red dye) was added to each sample to break up the proteins and cell membranes. This reagent was chosen due to its ability to dissolve organic compounds in a solution and to sequentially isolate DNA, RNA, and protein without compromising the structure of the nucleic acids (26). The phenol in TRIzol forms droplets throughout a solution when mixed and due to its lower polarity than water, causes proteins in the water phase to denature and enter the phenol droplets, thus separating nucleic acids from protein.

After the addition of TRIzol, the sample was homogenized. Following incubation for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes, 400 µl of 12.4 M chloroform was added, and the samples were kept at room temperature for another 2-3 minutes. The homogenate was allowed to separate into three distinct layers: a bottom organic layer containing proteins and some DNA, an interphase containing DNA, and a clear upper aqueous layer containing RNA. After centrifugation for 10 minutes at 12,000

rpm, the layers became more distinct. The upper RNA layer was transferred to a new tube by pipetting and discarded. Afterward, 300ul of a solution of 20mM Tris (pH 8), 1% SDS, and 6M urea was added to the remaining sample before centrifuging again for 5 minutes at 12,000 rpm to separate the DNA and organic layers. The DNA was isolated by pipetting into a separate tube and recovered through mixing with 200ul 17.1 M ethanol. The organic layer remained in the original tube and was not used. The separated DNA was purified using the AccuPrep PCR Purification Kit from Bioneer.

The PCR reactions to amplify COI were performed using 1 µl of each primer, 13 µl of nuclease-free water, 2 µl of 10X buffer, 0.5 µl of hot start Taq polymerase, 0.5 µl of the extracted DNA template, 1 µl of MgCl2, and 1 µl of dNTPs (for a total volume of 20 µl). The primers used were the universal cytochrome c oxidase subunit I gene (COI) primers LCO1490: 5'-ggtcaacaaatcataaagatattgg-3' and HC02198: 5'-taaacttcagggtgaccaaaaaatca-3 (27). PCR reaction conditions were as follows: 98°C for 1 min, 98°C for 5 seconds, 48°C for 15 seconds, 68°C for 1 min, repeat steps 2-4 29 more times, bring the temperature up to 72°C for 5 min, 10°C until the end.The Zymo Research DNA Clean and Concentrator kit was used to clean the PCR product before sending it to Elim Biopharmaceuticals for Sanger sequencing.

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