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Comparative isolation and amplification of *Cytochrome oxidase 1* DNA from *Oncorhynchus mykiss* (Rainbow Trout) of Azad Jammu & Kashmir

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Abstract
DNA extraction plays a pivotal role in the polymerase chain reaction (PCR) based molecular and genetic characterization methods. In order to find out the exact genetic lineage of the trout fish found in AJK, the fish DNA was extracted and analyzed further. Due to complicatedness regarding the fish DNA extraction, three different extraction methods were employed. We extracted genomic DNA from Rainbow trout by using standard phenol Chloroform method, a modified phenol Chloroform method and DNAzol DNA extraction method. The yield and quality of DNA extracted through different methods was compared. It was found that the extraction made by treating the samples with sucrose during initial steps of DNA extraction (Sucrose, Tris HCl (pH 7.5), $MgCl_2$, 1%V/V Triton, SDS and proteinase K) showed the best results while DNAzol method was the least productive. Likewise the modified method was also found to be the most productive in terms of PCR yield of *cytochrome oxidase 1* gene.

Keywords: Fish DNA isolation, phenol-chloroform methods, choloform-Isoamyl alcohol extraction, comparative quality analysis, PCR

1. Introduction
[DNA-based molecular study of vulnerable and endangered species has been one of the foremost routine matters to identify the problems in the population diversification, conservation and genetic scrutiny [1-3]. For molecular analysis, the effective, reliable and speedy extraction of DNA is required. Therefore various extraction techniques of mitochondrial and genomic DNA has been progressively practiced [4-6]. DNA is extracted from the muscles and other tissues using a choice of protocols like Trizol extraction method [7]. Cetyl Trimethyl Ammonium Bromide (CTAB) method [8] and Chelex-100 method as formerly described by Walsh *et al*. [9] and later modified by Santrude *et al*. [10] and Manuja *et al*. [5]. Similarly, DNA is extracted from tissues/muscles by standard Phenol-Chloroform protocol as described by Birren*et al*. [11] and Sambrook & Russell [12] with minor adjustments (500 µL lysis buffer; 50 mMTris, 50 mM EDTA, 200 mMNaCl, 1% SDS) with 25 µL proteinase K and 25 µL DTT was added and mixture was incubated at 56 ºC for 3 hrs followed by centrifugation.
Onset techniques of DNA analysis by PCR have heightened the concern in animal systematic. DNA sequencing data is used to discern the affiliation among species and integrate these obtaining statistics with available morphological records. However, for factual DNA sequences, it is imperative to isolate qualitative DNA, free from any impurities [13]. Thus pure DNA derivation is a basic step in molecular investigation which performs a vital role in the settlement of issues in gel-based PCR systems. The aim of this study is to extract qualitative DNA through minor modification of existing protocols for outstanding PCR amplification.

2 Materials and Methods
2.1 Collection of Samples
The required number of fresh Rainbow trout specimens was procured from government trout hatcheries Pattika (34º26.769 N and 73º32.986E) Muzaffarabad, Azad Jammu and Kashmir. Afterward the fish was brought to fisheries laboratory, Department of Zoology for further processing.

Each fish sample was thoroughly washed with sterilized water, cut into pieces (muscles tissues), placed in labeled zip bags and stored at -20 °C. DNA can be extracted within 6 months of the storage [14].

2.2 DNA Isolation

Protocol 1a: (Phenol-Chloroform-Isoamyl alcohol method with or without sucrose). The genomic DNA was extracted from tissue samples following Sambrook & Russell [12].

2.3 Reagents and Solutions of Protocol 1a

Solution A was made by adding 0.32 M Sucrose, 10 Mm Tris HCl, 5 Mm $MgCl_2$, in 400 ml of d.H_2O, (pH 7.5), then autoclaved, cooled and mixed with 1%V/V Triton X-100 (5 ml).

Solution B was made with 10 Mm Tris HCl (pH 7.5), 400 Mm NaCl, 2 Mm EDTA (pH 8.0) in the volume of 500 ml.

Solution C represented 400 µl phenol only (pH 8.0).

Solution D is expressed by Chloroform, Iso-amyl alcohol mixture in 24: 1 proportion.

Procedure: 100 mg muscles were taken in eppendorf tube and add 750 µl of Sol A. Mixed it and kept it at room temperature for 5-10 min. Centrifuged at 13000 rpm/ min discarded the supernatant and mixed up the residues gain in 600 µl of Sol A. For a second time repeat the step 1 and again mixed the pellet in 600 µl of Sol B. Then, added 50 µl of 10% SDS and 20 µl of Proteinase-K (20 mg/ml), after incubation for 3 hrs at 65 °C, added equal volume (500 µl) of Sol C and D, mixed well and centrifuged it for 10 min at 13000 rpm. Then, collected aqueous phase in new tubes and added Sol D (500 µl) and centrifuged yet again at 13000 rpm for 10 min. Once more, shifted aqueous layer in the new tube, added 55 µl of sodium acetate (3M, pH 6.0) and 500 µl iso-propanol, mixed softly and centrifuged with same rpm. Then supernatant was carefully removed without disturbing DNA pellet. Now in the DNA pellet, added 70% ethanol (350 µl) and then again centrifuged with same above mentioned rpm. Removed ethanol and dried the pellet in vacuum dryer (15-25 min.) or at room temperature (12 hrs). In the last, the DNA was dissolved in PCR water (200-250 µl) or TE buffer.

2.4 Protocol 1b: (Chloroform Isoamyl alcohol Method)

The genomic DNA was extracted from tissue samples through chloroform-Isoamyl alcohol (without phenol).

2.4.1 Reagents and solutions

Lysis buffer (TNES buffer) was made by dissolving 10 Mm Tris HCl (pH 7.5), 400 Mm NaCl, 2 Mm EDTA (pH 8.0) SDS in d.H_2O to made volume of buffer 50 ml. With the same above mentioned procedure.

2.4.2 (DNAzole Protocol 2): is an inclusively ready solution to use; followed by Chomczynski *et al*., [15]. Contain guanidine thiocyanate and detergent:

2.4.3 Reagents and solutions: DNAzol isopropanol, 70% ethanol, TE buffer.

Procedure: 250 µl blood serum/100 mg minced muscle was taken in eppendorf tube and added 500 µl DNAzol and kept it for 5 min at room temperature. Then added 250 µl isopropanol in and kept it for 5 min. Centrifuged it at 7,000 rpm for 5 min. Discarded the supernatant, again added 250 µl DNAzol after thoroughly mixing it and centrifuged at 7,000 rpm for 5 min. Discarded supernatant and added 70% ethanol (500-700 µl) in the pellet and again centrifuged it at 7,000 rpm for 5 min. After Removing ethanol dried the pellet and finally dissolved dried pellet in TE buffer (50 µl).

2.5 DNA Confirmation

Presence of DNA was confirmed by

2.5.1 Gel electrophoresis

The extracted genomic DNA run on 1.0% agarose gel, staining with ethidium bromide and the gel was visualized in the UV Tran illuminator and photographed by Canon 205.

2.5.2 Confirmation of Quantity of DNA

Quantity of extracted DNA was checked through Spectrophotometer (UV-1601, UV visible spectrophotometer, SHIMADZU) by using formula

DNA concentration (µg/µL) = [(A (260) × DF × 50)] / 10,000 [16].

Where: A (260) = absorbance at 260 nm and DF = dilution factor.

2.6 DNA Amplification

Polymerase chain reaction (PCR) was carried out in thermo cycler (SimpliAmp) in final mixture volume of 25 µl. **(**4 µl DNA, 2.5 µl of 10X PCR buffer, 2.5 µl of $MgCl_2$, 0.5 µl of dNTPs (10 mM; Fermentas), 1 µl of forward and reverse primers, 0.5 µl of Taq DNA polymerase and 13 µl of PCR water). Conditions were Optimized as 3 minutes at 95 ºC for the initial denaturation of DNA template followed by 39 cycles of amplification, 1 min at 95 ºC for DNA denaturation into single strand; 1 min 55-63 ºC for primers to bind to their complementary DNA template, and one minute at 72ºC for elongation of complementary DNA. Finally last ten minutes at 72 ºC for Taq polymerase to amplify the remaining DNA strands and a finally held out at 4 ºC and amplified PCR products were also visualized by UV Trans aluminator and images were obtained by digital camera.

3 Results and Discussion

The removal of qualitative DNA for genetic analysis had become apprehension for DNA based techniques. In the present study, two protocols were tried for better DNA extraction. 1) Phenol-chloroform isoamyl alcohol with minor amendment that is one case involving only addition of 40 µL of proteinase K during overnight lysis of samples [17] and in other case samples were treated with sucrose during 3 hours lysis by Tris HCl (pH 7.5), $MgCl_2$, 1% V/V Triton, SDS and proteinase K. Obtained results demonstrated the success of Phenol-Chloroform-Isoamyl alcohol method with sucrose treatment. It yielded good quality of DNA. Some DNA samples visualized in Figures 1&2.

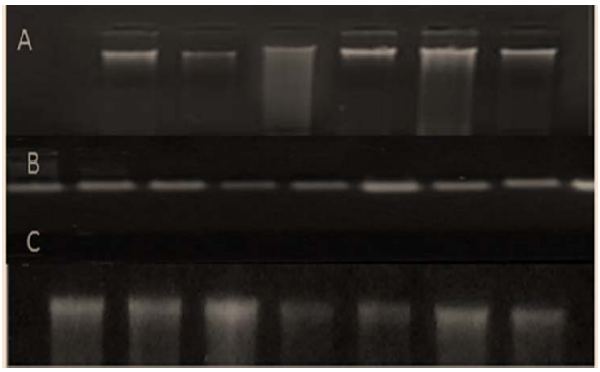

Fig 1: The agarose (1 %) gel profile of DNA isolation from fish samples by using (A) phenol-chloroform-Isoamyl alcohol, (B)the phenol-chloroform-Isoamyl alcohol treated with sucrose, (C) chloroform-Isoamyl alcohol without phenol.

The absence of phenol also yielded better quality of DNA (protocol 1b). Omission of phenol showed not any negative effects on DNA extraction (Figure 1 & 2). The present results demonstrated that organic solutions with or without phenol was equally useful. It was also noted that addition of sucrose during extraction qualifies the DNA extraction and bestowed with better result (band of CO1 gene) in PCR than other procedures (Figure 1b & 2b).

However, the extraction of DNA with DNAzol (Protocol 2) showed completely negative results in our laboratory conditions and did not extract DNA from fish muscles or its serum.

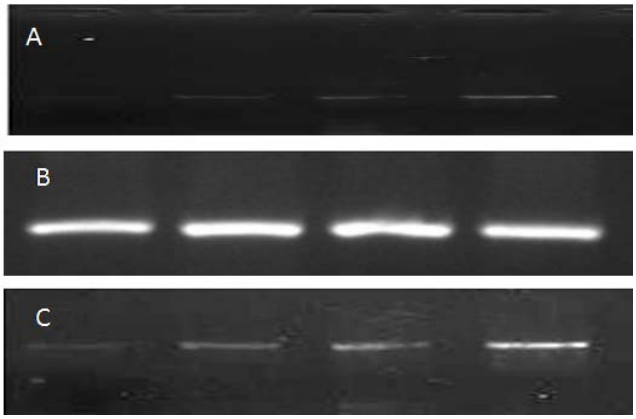

Fig 2: PCR products amplified by 2.0% agarose gel electrophoresis, using Cytochrome Oxidase 1 (CO1) primers with the DNA extracted from: (A) Phenol-chloroform-Isoamyl alcohol, (B) phenol-chloroform-Isoamyl alcohol with addition of sucrose (C) chloroform-Isoamyl alcohol.

3.1 Quantification of DNA

The quantification of DNA measured by spectrophotometer at 260 nm a showed the better result (Table 1). Quantity of DNA extracted by Phenol: Chloroform: Isoamyl alcohol (sucrose) was remaining 0.895 and 0.870 at 260 nm and, 0.805 and 0.782 at 280 nm and the final purity of DNA was 1.12 and 1.1 which is followed by the DNA extracted by without Phenol with 0.893 and 0.856 at 260 nm while, 0.892 and 0.832 at 280 nm which gave the purity 1.0 (Table 1).

Table 1: Concentration (ng/µL) and purity (A260/A280) of fish genomic DNA according to each method tested

Methods		Absorbance		
		260 nm	280nm	Purity
Protocol #1				
Phenol:chloroform:isoamyl	Sample 1	0.895	0.805	1.12
with Sucrose				
	Sample 2	0.870	0.782	1.1
Protocol #2	Sample 3	0.893	0.892	1.0
Phenol:chloroform:isoamyl				
Without Phenol				
	Sample 4	0.856	0.832	1.0

The phenol-chloroform-Isoamyl alcohol method effectively extracted pure DNA from fins, scales and muscles of fish (Table 1). DNA samples with absorbance ratios of 1.0 -2.0 were used for PCR amplification. Crude extractions could result in a DNA contaminated with proteins that may not be stable for long-term storage [18].

DNAzole Protocol (2); was tried to extract DNA from muscles and from blood serum [15] but the present protocol did not have any positive response in case of fish's DNA isolation. Although DNAzol protocol, offers a fast and effective method in 20–30 minutes and isolated genomic DNA and DNA fragments from tissues down to 0.1 kb in length successfully [15] and also productively extracted DNA from blood serum in case Hepatitis C [19].

The 650bp fragment of the CO1 gene was amplified from the DNA samples isolated with the phenol-chloroform-Isoamyl alcohol with or without sucrose and chloroform-Isoamyl alcohol method without phenol (Figure. 2).

DNA extraction is the basic step used in the study of genetic analysis and molecular biology [20]. Hence, the methods which are used for extraction of DNA should be cheap, fast and free of contaminants that yield qualitative DNA. Only qualitative DNA can give better results in the procedure of polymerase chain reaction (PCR) [21, 22] with minimal fragmentation ([20].

On the other hand, many customary DNA extraction protocols do not have this uniqueness. The PCR frequently repressed the amplification and important amount of DNA can be lost [23], thus restraining their efficacy for constructing DNA templates in PCR (Manuja *et al.,* 2010) [5]. DNA extracted with phenol-chloroform, treated with sucrose gave excellent visible PCR products with standardized band formation going on 2.0% agarose gel electrophoresis.

It can be concluded that the phenol–chloroform protocol in addition with sucrose, gave quantitative and qualitative DNA. By practicing this protocol, we reached to obtain pure and clear throughput yield from a small amount of sample which makes current method applicable. Nevertheless, DNAzole did not extract the DNA either from fish muscle or from its serum.

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