Comparative analysis of genomic DNA extraction protocols: Maxi-preparation of quality DNA for genetic evaluation and phylogenetic studies

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ABSTRACT. Four different DNA extraction protocols were compared for ability to produce DNA from the various tissues (caudal fin, pectoral fin, pelvic fin, muscle and gills) of three different freshwater fish species (*Hypophthalmichthys molitrix*, *Labeo rohita* and *Sperata sarwari*). Study describes the comparison of Phenol-chloroform; salt extraction; TNES and Urea methods in terms of ability to extract high quality yield and amplifiable DNA from the various tissues of fish species available in Pakistan. Although notable variations were by mean of purity and yield of quality DNA were observed among study methods, all our methods produced DNA suitable for PCR amplification. The DNA obtained from all methods were quantified and tested by Polymerase Chain Reaction. Test results indicated superiority of one out of four protocols, Phenol chloroform method a least hazardous and simplest in order to perform, a significant higher DNA yields were found than other studied methods.

Keywords: Freshwater; Fish species; DNA isolation; Protocol comparison; DNA quantification

INTRODUCTION

DNA extraction remains as an important issue in molecular biology (Pandey RN et al., 1996). DNA extraction is basic step in many biological studies including identification, inference of genes, genetics and genomic as molecular base study; also have importance in medical examinations, clinical diagnosis and investigations. So,

multiple methods were shaped to isolate DNA for biological means (Milligan BG, 1998). Despite of that various methods have diverse effect on extraction of DNA (Waldschmidt AM et al., 1997; Chen M, 2008). Foremost step necessary for ideal DNA extraction protocol are: Optimal DNA extraction protocol, reduction in degradation of DNA, cost effective and useful in terms of labour and time (Chen H et al., 2010). Addition to that DNA extraction protocols should not use toxic chemicals or produce hazardous wastes (Dittrich-Schroder G et al., 2012). Even though purpose of DNA extraction is to purify DNA by binding or splitting it (Hajibabaei M et al., 2005), the simplified DNA extraction protocol, such as the protocol that uses a commercial group, is more cost-effective and allows hundreds of thousands of samples encoded in the same facility each year (Wong EHK, 2008).

MATERIALS AND METHODS

Fish samples

Samples of freshwater species of fish (*Hypophthalmichthys molitrix*, *Labeo rohita* and *Sperata sarwari*) were collected from Chenab River, Multan, Pakistan. All specimens were identified on morphological basis by using taxonomic key based on visible characteristics as length, weight, various fin ray counts and so. Samples were stored in laboratory for freezing at –20°C further analysis.

Tissue preparation

Tissues of various regions like Caudal, Pectoral, Pelvic, Gill and Muscle were used for DNA extraction.

DNA extraction

Four different extraction methods for Genomic DNA were used and conducted in triplicate analyses for each sample.

Method I: Modified phenol chloroform extraction protocol

50–100 mg of each tissue with 600-800 µl extraction buffers was used for grinding and immediately transferred into MCT. Add 12 µl Proteinase K, vortex it and were incubated at 37 and 55° C for 1 hour respectively, Centrifuged at 5000rpm for 10 minutes. Then supernatant was collected in new MCT, add equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) in it, mix it by repeated MCT inversion and centrifuge it at 12000rpm for 10 minutes. Supernatant was then collected and add equal volume of chloroform: isoamyl alcohol (24:1) in it, again repeat the process of inversion on centrifugation at 12000 rpm for 10 minutes, collect supernatant, add 0.1 volume of 3 Molar sodium acetate and 100% ethanol in equal volume. MCT were placed at -20°C for 1-2 hour and centrifuged again at 4000 rpm for 4 min. Wash DNA pellets with 70% ethanol, keep it for air dry and suspend adequate volume of triple distilled water (AquaPro Injection, Pakistan) and stored at -20°C until further processing.

Method II: High salt protocol

Tissue of ~0.5 cm^2 was used for analysis, sample were cut with a sterile blade. The samples was transferred to a labelled 1.5 ml MCT and add 600 µl of buffer (TNES) with 35 µl of Proteinase K. Mixing of samples were done by the repeating inversion of tubes and then incubated it for overnight at 50ºC. After incubation add 166.7 µl of 6 Molar Nacl, shaken it vigorously for few seconds and centrifuge it at 14,000rpm for 10 minutes. Supernatant was taken to newly labelled MCT and add equal volume of 100% cold ethanol, gently mixed by repeated inversion of tube.

Centrifugation was again done at 14,000 rpm for 15 minutes at 4ºC. Remove the Supernatant carefully due course for prevention in DNA pellet disruption. Pellet was washed in using 500 µl of pure and 70% ethanol simultaneously. After removing that sample were centrifuges to get the last of ethanol in bottom of tube; remove out the remaining ethanol. Sample was left to air dry for 30 minutes. As soon as the sample was just dry, re-the DNA was suspended in about 100 µl sterile distilled water.

Method III: TNES Extraction protocol

Tissue of sample were taken 20 mg, add 800 µl buffer solution and 10 µl of RNase. Homogenized it and kept it for incubation at 42°C for 1 hour. Also added 10 µl of Proteinase K and kept it at 42°C for overnight incubation. 800 µl of Phenol: Chloroform: Isoamyl Alcohol (25:24:1) was added to new MCT, mix it by repeated inversion. Sample was then centrifuged for 15 min at 10,000 rpm and then recovered top aqueous layer. DNA was precipitated in one volume of 1 Molar Nacl and two volumes of ethanol. The DNA becomes washed with ethanol 70%; kept it for air dry and resuspended it in 60 µl of doubled distilled water.

Method IV: Urea extraction protocol

Tissue become dry by using filter paper and placed it in 2 ml of lysis buffer in falcon tube. Added RNase with volume of 30 µl and incubated it at 42°C for 1 hour. After that 30 µl of Proteinase K was added, mixed it by repeated inversion and kept it at 42°C for 10 hours. Equal amount of Phenol: Chlorophorm: Isoamyl Alcohol (25:24:1) was added in MCT, mixed it by repeated inversion of tube, and become centrifuged for 15 minutes at 13,000 rpm and recovered the top aqueous layer. 1 M NaCl and 2-3 volumes of chilled ethanol were added in the tube. Sample was kept at -20°C for 2hours and centrifuged it at 13000 rpm for 15 minutes. DNA pallets were washed with 70% ethanol, become air dried and resuspended in appropriate volume of distilled water and stored at -20°C for further analysis.

Statistical analysis

For DNA concentrations were used for comparison from studied fish with the different DNA methods of DNA extraction. ANOVA was performed by using SPSS. ANOVA was also utilized in order to compare the DNA purities from three different fish species. Differences were considered statistically significant at $P<0.05$. Tukey's, fisher and Dannett's multiple comparison procedure was used when interactions were significant in order to interpret which interaction effects differed. Graphical representations were performed using R software.

RESULTS

DNA concentration and its purity obtained from the *Labeo rohita*, *Hypophthalmichthys molitrix* and *Sperata sarwari* using four different methods of extraction. Statistical study employ purity and yield may differ significantly by analysing significant interaction existed among various methods of DNA extraction. Various DNA extraction methods were performed accordingly; the Phenol chloroform method consistently extracted highest purity and yield of quality DNA as compare to other methods. It is also pin pointed that PC method also required more tissue (50-100 mg) of sample for extraction of DNA than other methods.

For the *Hypophthalmichthys molitrix*, *Labeo rohita* and *Sperata sarwari*, DNA yield and concentration obtained with the Phenol Chloroform extraction method found significantly higher than obtained with other method like High Salt, Urea and TNES respectively as in Figure 1A and Figure 1B. Same trend were found in all the fish species.

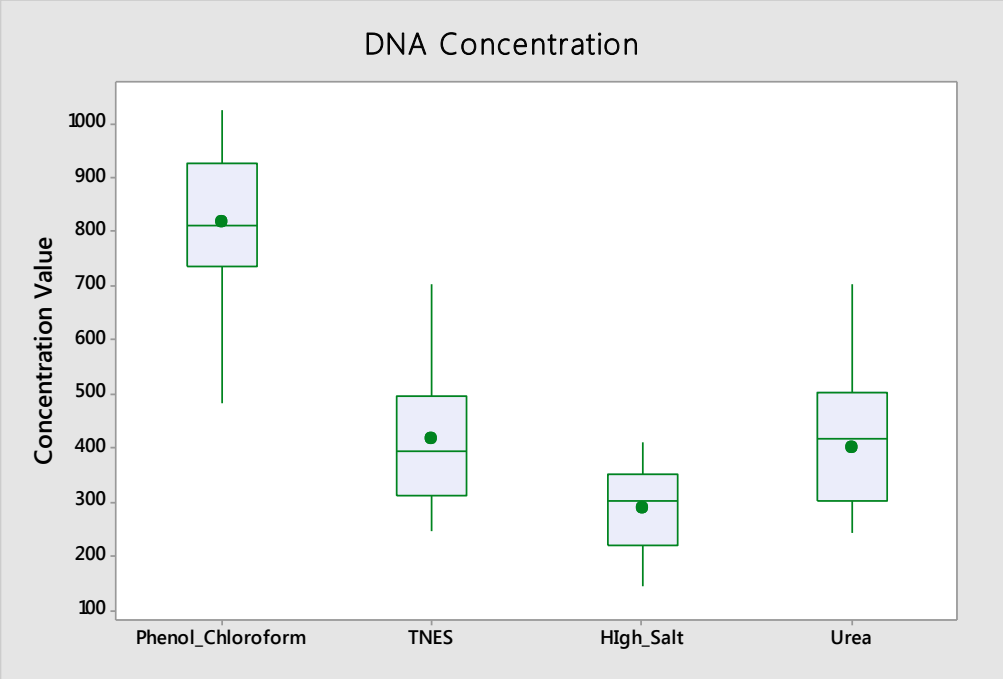

Figure 1A. Method wise DNA concentration

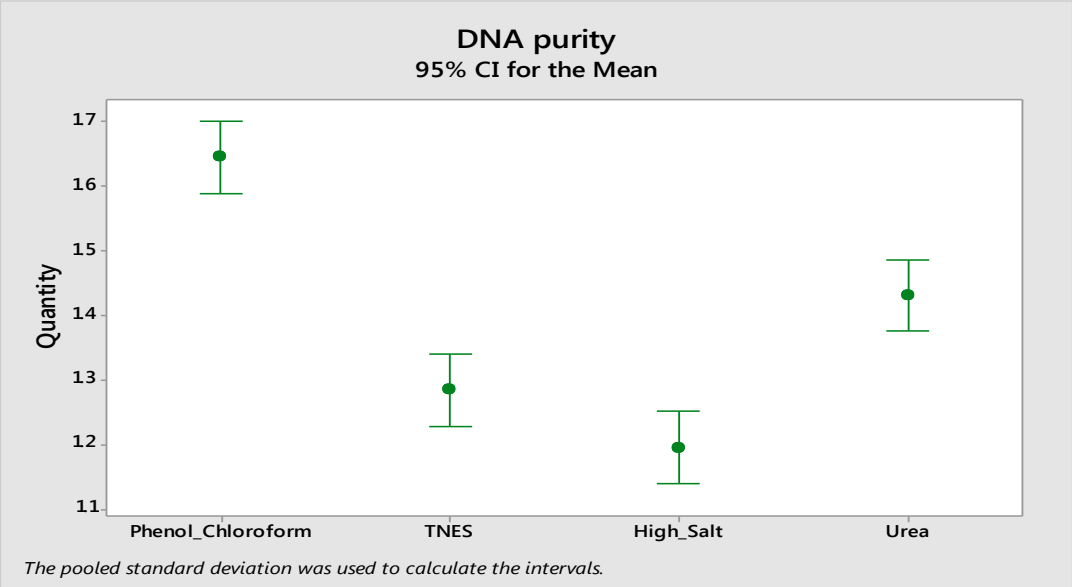

Figure 1B. Method wise DNA purity

Comparison of *Hypophthalmichthys molitrix*, *Labeo rohita* and *Sperata sarwari* as per method respectively as according to concentration and yield of DNA shown in Table 1 and Table 2 and Figure 1C and Figure 1D. DNA concentration were found significant correlation ($P<0.05$) comparatively in TNES-Phenolchloroform; High Salt - Phenol chloroform; Urea-Phenol chloroform High Salt-TNES and Urea-high salt. While shows a non-significant correlation ($P>0.05$) in Urea-TNES, meant that in case of Urea-TNES it doesn't matter who is been selected for extraction both shows same results (Figure 1C). Same trend were found in all fishes.

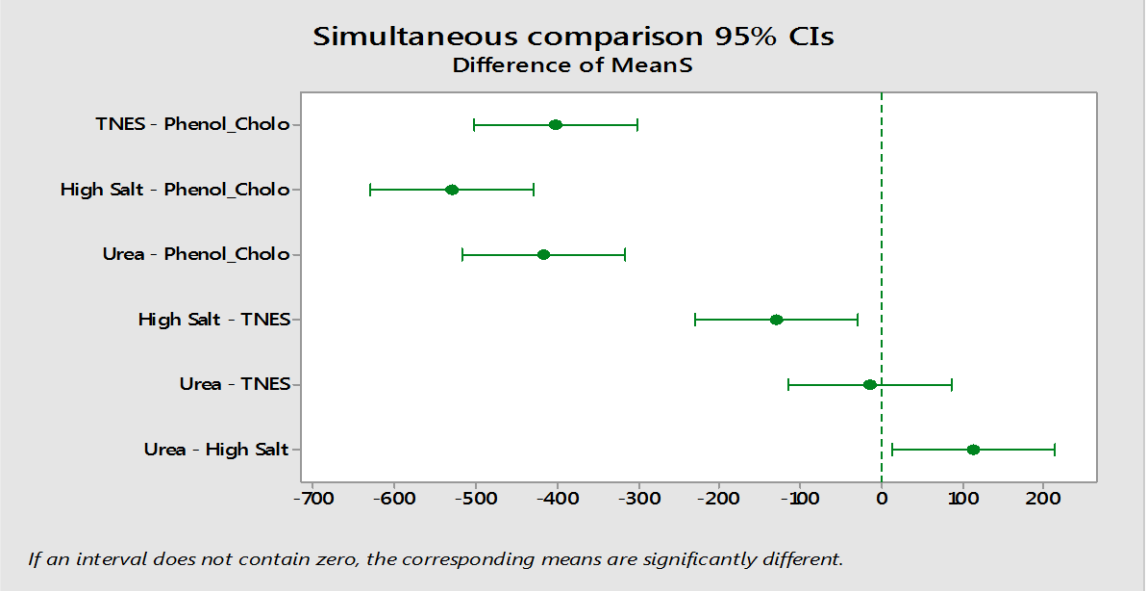

Figure 1C. Comparative analysis of various methods for DNA concentration.

Table 1. Analysis of Variance. Means and Grouping Information of DNA concentration

Analysis of Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	3078273	1026091	73.73	0.000
Error	72	1002057	13917		
Total	75	4080330			
Means					
Factor	N	Mean	StDev	95% CI	
Phenol_Choloform	19	819.1	130.2	(765.2, 873.1)	
TNES	19	417.3	124.8	(363.4, 471.3)	
High Salt	19	287.9	82.8	(234.0, 341.9)	
Urea	19	401.2	127.6	(347.2, 455.1)	
Grouping Information of DNA concentration					
Factor	N	Mean	Grouping		
Phenol_Choloform	19	819.1	A		
TNES	19	417.3	B		
Urea	19	401.2	B		
High Salt	19	287.9	C		

DNA concentration shows a significant correlation (P<0.05) as compare to TNES-phenol-chloroform; High salt-phenolchloroform; Urea-phenol chloroform; Urea-TNES and urea-high salt. While shows a non-significant correlation (P>0.05) in High salt-TNES, meant that in case of High Salt-TNES it doesn't matter who is been selected for extraction both shows same results (Figure 1C). Also draw three variables in Figure 1E.

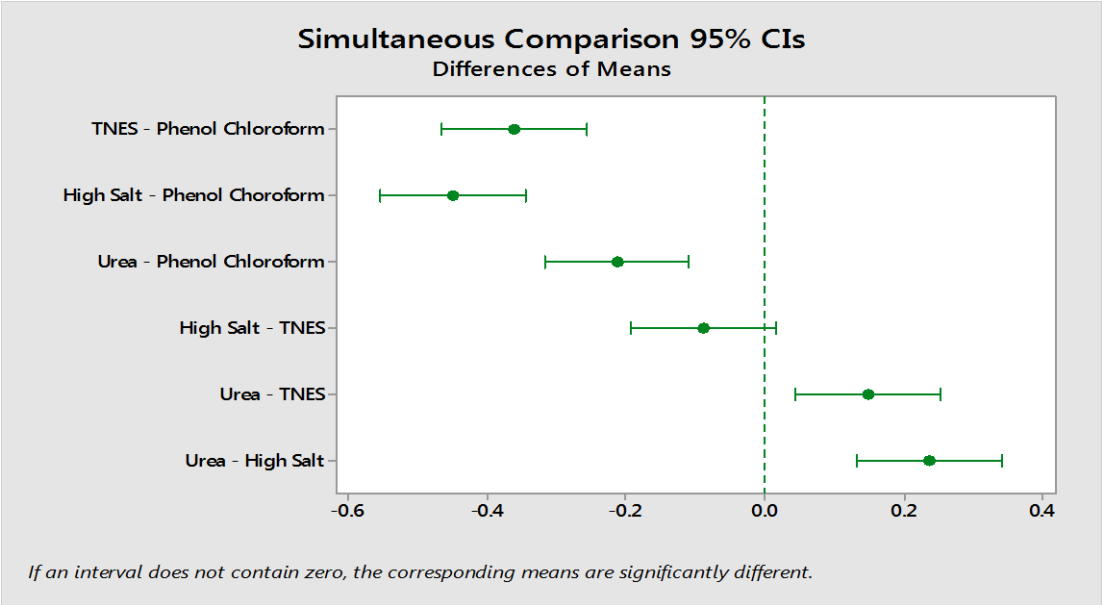

Figure 1D. Comparative analysis of various methods for DNA purity

Table 2. Analysis of variance. Means and grouping information of DNA purity

Analysis of variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	3	2.203	0.73438	48.98	0.000
Error	72	1.08	0.01499		
Total	75	3.283			

Means

Factor	N	Mean	StDev	95%CI
Phenol_chloroform	19	1.6453	0.1006	(1.5893, 1.7013)
TNES Method	19	1.2842	0.1366	(1.2282, 1.3402)
High Salt	19	1.1953	0.0911	(1.1393, 1.2513)
Urea Method	19	19	1.4311	(1.3751,1.4871)

Grouping information of DNA purity

Factor	N	Mean
Phenol_chloroform	19	1.6453 A
TNES Method	19	1.4311 B
High Salt	19	1.2842 C
Urea Method	19	1.1953 D

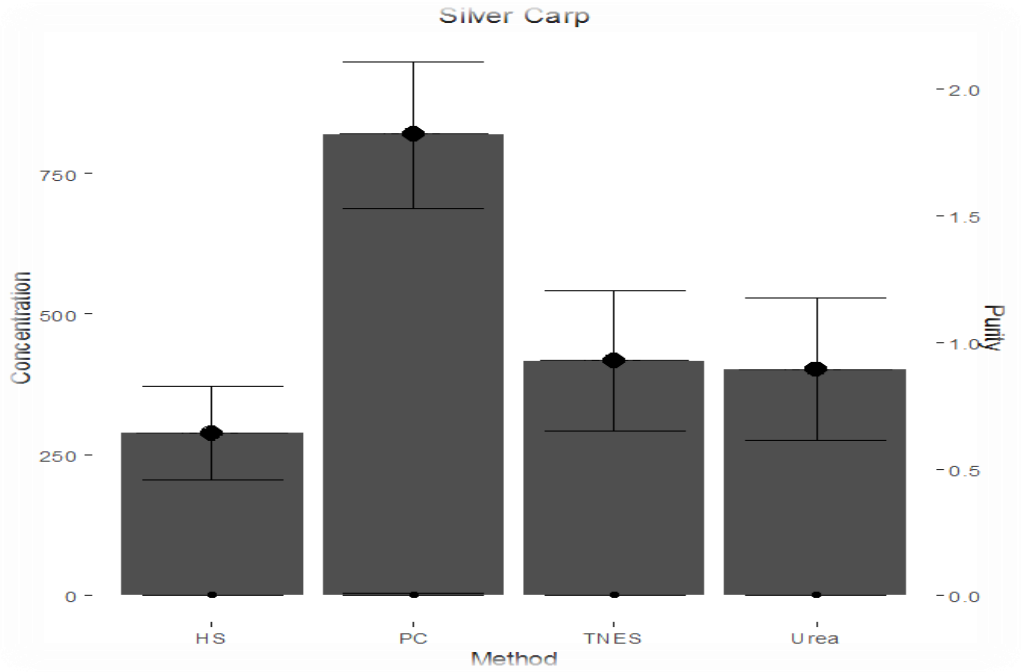

Figure 1E. Three variables in a figure simultaneously.

DISCUSSION

It is obvious that when DNA extraction methods were used, a significant difference in yield and concentration of DNA in species were found. Thus, it seems liable that the composition or nature of tissue in different species had an effect on ability of extracted DNA with some method were being more suitable than others. Studied reported earlier that the lower quantity of DNA extraction can be allied with samples with higher contents of fats (Saunders GC., et al. 2001), even though this was not apparent in present study.

Of three DNA extraction methods using 50 to 100 mg sample the phenol chloroform method found significant with higher yields than others while lowest yields were from tissues with High Salt method. Method suitability for the DNA extraction from muscle tissue would subsequently depends upon either the user entail consistently bring similar yield or method deliver higher DNA yield is mandatory. Mean yield of DNA attain from muscle tissue was well supported in the literature as compare to the other.

DNA purities from various fish species by using different extraction method were estimated by A260/A280 ratio. By Statistical analysis a significant differences among the various species by using various method of extraction were resolute. DNA was found satisfactory when A260/A280 ratio were remained from 1.6 to 2. (Rapley R, 1996; Aljanabi SM, 1997; Lopera-Barrero, 2008; Ferrara GB, 2006; Sambrook J, 2001). Reduction in value of A260/A280 were marked contaminated DNA with impurities in it. Remaining impurities from DNA extraction process were from chloroform, ethanol or phenol, were reported by absorption A260/A280 ratio (Mackie IM, 1998; Wilfinger WW et al., 2006). DNA extracted with Phenol Chloroform method was found considerably higher than others. Absorbance values can't be distinguished, an accurate quantification of DNA is thus dependent upon the DN with their high purity (Wasko AP, 2003).

Polymerase Chain Reaction was used to assess the suitability and quality of DNA. Despite of yield and purity ranges noted for the DNA extraction by using different method; all four methods quantify DNA successfully and also amplify through Polymerase Chain Reaction. However, when DNA extracted with these methods, the PCR was optimized using 3 ml of quality DNA in reaction mixture. Polymerase Chain Reaction are concentration dependent it may inhibited by high or low concentration (Nazar AZ, 2019). The concentration, purities and yields of extracted DNA from species standardised method were presented. Present results suggest that Phenol Chloroform protocol is able to delivering high DNA yields than others protocols.

CONCLUSION

To be the best of our knowledge, present study was used to compare the efficiency of various method of DNA extraction from fish tissues. Present result marked variability in yield, purity of different DNA extraction method for quality DNA. DNA of a high yield and purity is important for molecular techniques success, as Polymerase Chain Reaction and further sequencing of DNA. Present study marked that Phenol Chloroform extraction method as most appropriate extraction method for quality DNA, due to their high yield of DNA deliver relatively safe method, ease for use and applicable for high throughput for various specimens.

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