Comparison of different DNA extraction methods for variously processed fresh/frozen and fried nuggets of freshwater fish, *Labeo rohita*

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Abstract- Fish species identification after its processing leads to difficulty because of the loss of morphological characteristics. Good quality and quantity of DNA is the first step for molecular based fish identification. In present study, five out of nineteen DNA extraction methods were shortlisted after employing on non-marinated fried fish nuggets. For analyzing the effects of differently processed and fried fish on DNA extraction, raw and samples cooked at 170-180 ^{0}C for 5 minutes in different oils with a freezing (at -20 ^{0}C) interval were subjected to five DNA extracting techniques. Addition of marinating ingredients of fish products adversely affected both the DNA quality and quantity. Overall, the control (non-marinated and non-fried) nugget of *Labeo rohita* (rohu) yielded the highest DNA (14988 ng/µl) whereas NMMO (marinated and fried in mustard oil) yielded the lowest (9011 ng/µl) value, with a little difference in their purity ratio (260/280) being 1.75 highest for control and 1.62 for NMCO (nuggets marinated and cooked in cooking oil). A significant difference ($P<0.001$) in DNA yield was found for applying different marinades and oils for cooking while in purity significant difference was found only for different oils. A significant difference ($P<0.001$) in the DNA content was recorded before and after the freezing interval manifesting a decreasing trend for frozen samples. SNET extraction method proved to be best in terms of yield whereas the salt out method gave the highest purity with respective values of 16177 ng/µl and 1.77 respectively. These findings permit to design a powerful tool for thermally treated processed fish' forensics.

***Index Terms*:** DNA extraction from fish, DNA extraction from processed fish, DNA extraction from cooked fish, Fish Identification, DNA degradation, Species fraud

I. INTRODUCTION

Since prehistoric times, fishes are used worldwide as a vibrant source of high value protein [1]. In Pakistan, highly preferred freshwater fish is *Labeo rohita* (rohu) because of its taste [2]. Consumers are preferring fish and fish products due to low saturated and high unsaturated fatty acids in addition to the high-quality protein content. Fish oils and fats have also been reported to resist many diseases [3]. Furthermore, fish and its products are accepted in all cultures and religions. While considering all this importance, the increase in fish product demand is valid globally, which is obvious to surge in the coming future. Now a days, multiple challenges are being faced by the rapidly thriving fish products market. One of these, is the malpractices of few entrepreneurs, wishing excessive profit. These corrupt entrepreneurs mislabel the quality and nature of the food products to mislead customers [4]. Species substitutions have been reported in many fish products [5]. In such cases products commercial names mentioned, do not correspond to the actual product which is mostly an easily available or cheap product [6]. It is a reported practice worldwide to replace costly fish products with lower-valued ones [3].

Morphometric features are usually helpful for fish identification but they are lost while processing the fish products. For instance, frying changes the outlook of the product which makes it harder for speciation [7]. Many other identifying methods for species like tracing biomarkers through insight work, electrophoretic techniques [8], immunological approaches [9] and chromatographic methods [10] are also not valid for processed and/or cooked fish products. These kinds of tests are in some specific situations but are mostly unsuitable for commercially available fish products because the application of heat degrades the structure of proteins [11]. In addition, these analytical procedures are interfered with other ingredients like oil [12].

Conversely, very stable nature of nucleic acids allows them to withstand numerous food processing treatments *viz.*, pressure, mixing and heat exposure [13]. However, such processings might convert DNA into fragments which even then are capable of detection. Conclusively, DNA is known to be highly suitable molecular marker for species identification [14]. Being highly sensitive, DNA markers offer more reliability for identifying processed fish products than protein-based tests [15]. Presently, for testing and checking food authenticity, DNA-based testing is widely applied [12]. This method is becoming more famous globally as it is capable of applying on all over the animal kingdom in addition to its being economical, reliable and less time-demanding [16].

The first step for DNA-based forensics is the isolation of the good quality and quantity of DNA from a processed food sample. PCR is then employed for the amplification and sequencing of different genes to act as identification tools of fish products. Nowadays, several traditional DNA isolation methods and commercial kits are available [17]. Depending on the fish products processing and different isolation methods, the yield of the DNA varies accordingly [18]. In the presence of a long list of DNA extraction methods, it is very difficult to select

a suitable method for DNA extraction for specific fish products. Aim of the present study was to compare different DNA extraction methods from freshly processed, frozen nuggets cooked in different oils of freshwater fish, *Labeo rohita* quantitatively as well as qualitatively.

II. MATERIALS AND METHODS

A. *Purchase of fish specimens*

The specimens of freshwater fish, *Labeo rohita* (rohu) of comparable weights (1.5+0.1 Kg) were identified using a local identification key [19] and then purchased from the local market of Gujranwala (32°18′77″N 74°19′45″E), Punjab, Pakistan. All fish specimens were kept in ice box transported to the Institute of Zoology, University of the Punjab, Lahore within two hours for further study.

B. *Phase 1: Comparison of different DNA extraction methods using non-marinated fried fish nuggets*

Each fish specimen was washed, air dried after descaling and dissecting. Muscle pieces (fish nuggets) of about 50 g each were cut and used in the present study. Although, many methods have been established for successful DNA extraction from raw fish but less work has been done for the isolation of DNA from processed/ cooked fish manually. To identify economical manual method(s), total 19 methods were employed to extract DNA from fried fish nuggets in about 1.5-liter cooking oil at 170-180 ^{0}C for 5 minutes. Around 20 mg of a muscle sample was taken from fried fish nuggets to extract DNA for each of the DNA extraction method separately, including SNET, Urea-SDS, Rapid MT Method, TNES Method, salt out [20], CTAB [21], CTAB, HotSHOT [22], CTAB, Tris- EDTA Method, Alkaline Method, Urea Method [23], HotSHOT [24], Phenol-chloroform Extraction [25], Sambrook [26], Quinteiro [27], Desalle and Birstein [28], Koh (1998), Phenol-chloroform extraction [29]. In addition to these manual methods, Qiagen kit (Blood and Tissue kit; cat. No. 69504) was also employed for comparison. Out of the 19 different methods, those methods which yielded DNA successfully from fried fish nuggets were used further. Summary of the procedural steps is depicted as Fig.1.

C. *Phase 2: Comparison of different DNA extraction methods for differently processed fish nuggets*

To extract DNA from differently processed fish nuggets, fish specimens were purchased, dissected as described earlier. Almost equally weighed (50g) muscle pieces (fish nuggets) of *L. rohita* were divided into 6 groups (C; non-marinated and non-fried (control), NMR; marinated and non-fried, NWCO; non-marinated and fried in cooking oil, NWMO; non-marinated and fried in mustard oil, NMCO; marinated and fried in cooking oil and NMMO; marinated and fried in mustard oil.

Marination of fish nuggets

One Kg fish nugget pieces were taken in 2 glass containers each of about 2 L capacity. In first container non-marinated fish was mixed with only table salt (25.0g). Whereas, for preparing marinated products table salt (25.0 g), powdered black pepper (10.5 g) and pulverized red chili (5.91g) were added. After mixing properly the fish nuggets were kept at 4ºC for 1 hr. After marination, the fish nuggets were coated with all-purpose flour followed by immersing in two well-beaten hen eggs and then the fish pieces were coated with bread crumbs whereas the non-marinated pieces were not coated.

Frying of fish nuggets

Two batches of fish nuggets were fried separately in 1.5-liter of mustard and cooking oil at 170-180 ^{0}C for 5 minutes.

Collection of samples from fried fresh and long frozen fish nuggets

After frying of the fish nuggets of different categories, known-weight fish nuggets were taken as fried fresh sample for DNA extraction. For long frozen fish nuggets samples, each non-marinated, marinated fish samples with control were packed separately in polythene bags and stored in freezer at -18^{0}C for three months. After three months of freezing period, the samples were fried again following same separate frying procedure as described above after thawing at room temperature. These were further process for DNA extraction. Out of 19 different DNA extraction methods employed in phase 1, only those DNA extraction methods which yielded DNA from fried fish nuggets (phase 1) were used to extract DNA from fresh and frozen processed fish nuggets to compare their relative efficiency.

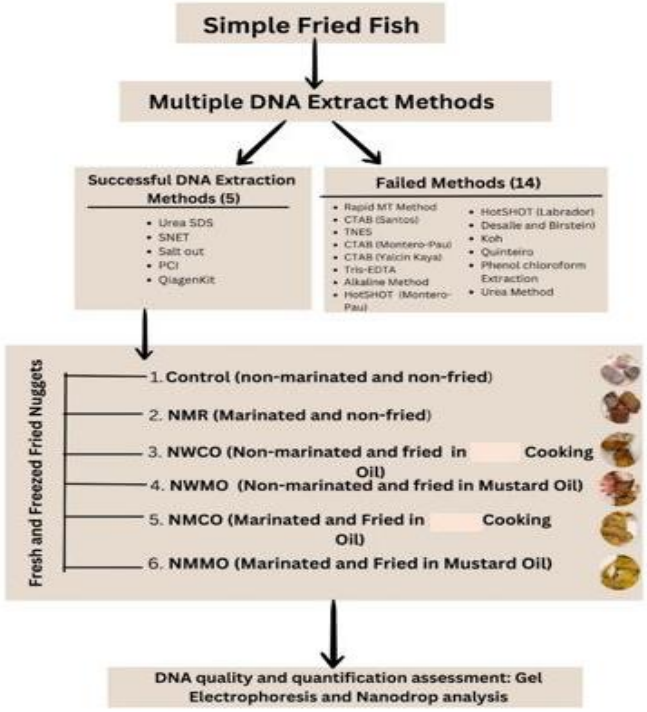

Fig. 1. Overview of processed/ fried fish samples for DNA extractions

D. Visualization and quantification of extracted DNA

The quality of DNA isolated from each category of the methods was evaluated by gel electrophoresis to identify most suitable method for each category of the fish product. To check DNA integrity, 10 µl of extracted DNA was mixed with 2 µl DNA loading dye and then loaded on 1% agarose gel stained by ethidium bromide. The DNA bands were then visualized by UV trans-illumination after running the gel for almost 45 min at 90V.

The extracted DNA was quantified by directly comparing the samples' DNA with 1Kb mix standard marker (Fermentas) using spectrophotometry (Nanodrop R ND-8000). DNA concentration (ng/ul) was assessed from the absorbance at nanophotometer. In addition, the quality/purity of extracted DNA was inferred by its A260/A280 ratio. The ratio from 1.7 to 2.0 usually denotes a sample with high-quality DNA [25].

E. Statistical analyses

General linear model using Tukey post-hoc test with 95.0% confidence level was executed by Minitab 16 for evaluation of significant differences from the outcomes of different DNA extraction methods, different oils and different processed fish products.

III. RESULTS AND DISCUSSION

Phase 1: In the present study, five out of nineteen DNA extraction methods namely; Urea-SDS, SNET, Salt out [20], Phenol-chloroform Extraction [29] and Qiagen kit (Blood and Tissue kit; cat. No. 69504) gave positive results for fried fish nuggets (Table 1; Fig. 2).

Table 1: Quantification of positive DNA extraction methods (Phase 1)

DNA extraction Methods	Extracted DNA	
	Yield (ng/ul)	Purity (260/280)
Urea-SDS	6033±579.51^{a}	1.4±0.04^{d}
SNET	2666±208.16^{b}	1.9±0.05^{a}
Salt-out	2383±236.29^{b}	1.6±0.13^{c}
PCI	5283±388.37^{a}	1.9±0.02^{ab}
Qiagen kit	1816±160.72^{b}	1.7±0.07^{bc}
Significance (P)	<**0.001**	<**0.001**

Values are Means ± SD of three replicates in column with different alphabet represent significant differences (P<0.05)

The positive results of DNA extraction might be associated with use of proteinase-k in these methods. Proteinase K increased the DNA isolation efficiency specifically for samples incubated up to 55 ºC [31]. Overnight incubation was used in all positive DNA extraction methods, except for Qiagen kit whose incubation time was of almost 3 hrs. [32]. Mezzomo *et al.* (2020) reported that reasonable incubation times for the lysis of the samples in buffer increases its efficiency spectrum. In addition, he reported that PCI/SDS protocols are helpful in DNA extraction yielding concentration from 106.3 to 183.3 ng in fresh as well as processed fish samples. In three out of the five positive DNA extraction methods, sodium chloride was used which might had assisted in extraction of good quality and quantity DNA. Lopera-Barrero *et al.* (2008) reported that sodium chloride play a role in removing the interfering compounds like polysaccharides during DNA extraction [33].

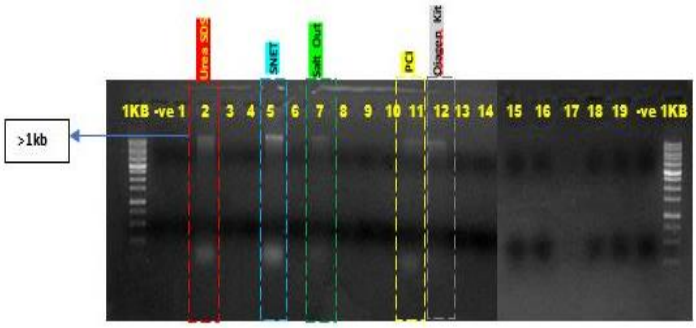

Fig. 2: DNA extraction from simple fried fish employing 19 different DNA extraction methods. Names of only successful methods have been earmarked

Abbreviations: From left to right, 1 Kb DNA ladder, -ve: negative control, 1: Rapid MT Method; 2: Urea-SDS, 3: CTAB (Santos), 4: TNES, 5: SNET, 6: CTAB (Montero-Pau), 7: Salt out, 8: CTAB (Yalçınkaya), 9: Tris- EDTA, 10: Alkaline Method, 11: Phenol-chloroform Extraction (Muhammed), 12: Qiagen kit, 13: HotSHOT (Montero-Pau), 14: HotSHOT (Labrador), 15: Desalle and Birstein, 16: Koh, 17: Quinteiro, 18: Phenol-chloroform extraction, 19: Urea Method

In present study, majority of unsuccessful DNA extraction methods *i.e.*, Rapid MT method, CTAB [21], TNES, CTAB, HotSHOT [22], CTAB, Tris- EDTA, Alkaline Method [23], HotSHOT [24], Desalle and Birstein [27], Koh [28], Quinteiro [26], Phenol-chloroform extraction [25] and Urea Method [23], were found failed to yield DNA. Similar results had earlier been reported by other researchers. Negative results of CTAB for cooked fish had been associated with un-optimized concentration of CTAB that compel DNA to form complexes with lipids. DNA condensation with CTAB occurs producing insoluble complexes when the ratio of CTAB to DNA is >1.0 [34].

Phase 2: DNA yields as well as purity values for differently processed samples differed significantly (P<0.001). Control sample yielded the highest value (Table 2). Likewise, Tumerkan (2021) reported at raw anchovy highest quantity of DNA [35]. Whereas, NMMO (marinated and fried in mustard oil) yielded the lowest value in the present study. The order from higher to lower for all the samples' yields was as Control<NMR< NWMO< NMCO< NWCO< NMMO. Tagliavia *et al.* (2016) have shown that degraded DNA yielded low quantity in processed samples as compared to the raw. For instance, frying causes significantly low yield of DNA [36]. Tumerkan (2021) mentioned that cause of low yield is not only thermal treatment but also marination reduces the amount of extracted DNA [35]. Spychaj *et al.* (2021) described that application of spices, salt

and acidic medium decreases DNA yields [37]. It is known that pressure and thermal treatments, presence of additives like acid or oil and enzymatic impurities inhibit the presence of DNA extractions [17], [38].

The highest purity ratio with 1.75 was found in case of control, whereas the lowest value 1.62 was found in NMCO (Table 2). The order from higher to lower in all samples' yields was as control<NMR<NWMO<NMMO<NWCO<NMCO. Tumerkan (2021) has also reported significant differences in raw and processed anchovy at domestic and industrial levels of processing. He recorded lowest DNA purity (1.58) for the sample marinated with pepper sauce and assumed the addition of pepper, acid and other ingredients might be responsible for reducing the purity [35]. The present study has also shown that, overall fried samples i.e., NWCO, NWMO, NMCO and NMMO gave low purity values of 1.63, 1.64, 1.62 and 1.64, respectively as compared to control and NMR samples that expressed purity values of 1.75 and 1.72 respectively. Likewise, Tumerkan (2021) also found that roasted samples produced low purity. Whereas, raw and marinated samples yielded purity values as 2.07 and 2.04 respectively [35].

A statistically significant difference ($P<0.001$) was found in DNA yields as well as purities in case of fresh and frozen samples. Highest DNA yield upto 12226 ng/ul was found before and low yield i.e., 10709 ng/ul after the freezing. The highest purity ratio 1.71 was found before freezing, whereas the lowest 1.63 after the freezing interval (Table 2). Storage of the samples by freezing for 3 months might had degraded some of the DNA. Storage conditions, presence of marinades and thermal treatment significantly degrade DNA of samples [3]. Guo *et al*. (2018) stated that degradation of DNA continues even during optimal storage conditions like buffers, low temperature, or sterile conditions [39]. In contrast to these studies, Oosting *et al*. (2020) found no significant effects of different storing conditions. He reported relative DNA stability after storing samples at 5°C in DESS and retention of >50% DNA content after about 3 months' storage at about -18^0 C [40].

Regarding efficiencies of different DNA extraction methods, SNET gave highest yield upto 16177ng/µl, whereas Qiagen kit method yield the lowest 7925 ng/µl value (Table 2). Similarly, Akkurt (2012) has shown significant difference ($P < 0.01$) between 6 different protocols including 3 traditional and 3 protocols of commercial kits and found the economical and better usage of traditional methods [30]. Considering yield of DNA different methods can be categorized as SNET>Urea-SDS>Salt out>PCI>Qiagen kit. Likewise, Chowdhury *et al*. (2016) rated SNET a superior method was the superior method in terms of the quantity of isolated DNA from fish tissue samples. They reported DNA concentration from *L. rohita* as 177.85ng/µl and 200.72 ng/µl from *T. ilisha*. These authors have attributed these higher yields of DNA by SNET method to the availability of higher NaCl (400 mM) and Proteinase K (400 µg/ml) concentrations [20]. In the present study, highest purity ratio 1.71 was found for the salt out method which is comparable to the results of Chowdhury *et. al.* (2016) who reported the purity value of 2.00 for the salt out method [20]. The salt-out method also proved to be the most efficient method in the present study, whereas the lowest value 1.62 was found in case of Urea-SDS. On the basis of purity of extracted DNA from all the samples, the different methods can be ranked as Salt out>SNET>PCI> Qiagen kit>Urea-SDS (Table 2). Contrary to the present results Chowdhury et. al. (2016) had reported results with Urea-SDS with purity levels of 1.77 and 1.74 for *L. rohita* and T. ilisha, respectively [20]. Lower DNA quality and quantity for different methods is attributed to various factors like variations in handling and differences in the chemicals used within the protocols. Piskata (2017) described impurities such as ethanol, phenol residues which influence the efficiency in different DNA extraction methods [41].

Table 2: Comparative DNA yield (ng/µl) and purity (260/280) with their standard error of means (SEM) and significance of differently processed fish nuggets' samples with control

Sample	Yield (ng/µl)	Purity (260/280)
Control	14988^{a}	1.75^{a}
NMR	12170^{b}	1.72^{ab}
NWCO	9990^{d}	1.63^{c}
NWMO	12043^{b}	1.64^{bc}
NMCO	10604^{c}	1.62^{c}
NMMO	9011^{e}	1.64^{bc}
SEM and Significance	**68.07*** **	**0.019*** **
Duration		
Fresh	12226^{a}	1.71^{a}
Frozen	10709^{b}	1.63^{b}
SEM and Significance	**39.30*** **	**0.011*** **
Method		
Urea SDS	14642^{b}	1.62^{b}
SNET	16177^{a}	1.68^{ab}
Salt out	9488^{c}	1.71^{a}
PCI	9106^{d}	1.67^{ab}
Qiagen kit	7925^{e}	1.65^{ab}
SEM and Significance	**62.14*** **	**0.017** **
Marination		
Non-marinated	12340^{a}	1.67^{a}
Marinated	10595^{b}	1.65^{a}
SEM and Significance	39.30***	0.010
Oil		
No oil	13579^{a}	1.73^{a}
CO	10297^{b}	1.63^{b}
MO	10527^{c}	1.63^{b}
SEM and Significance	**48.13*** **	**0.012*** **

A significant difference in DNA yield (ng/µl) was also found for the use of oils like no oil, cooking oil, mustard oil and different marination patterns i.e., without marination and marinated fish products. Similarly, Radstrom *et al*. (2004) found that products

prepared in different liquids like oil, brine, tomato and vinegar can cause variations in extracted DNA quality and quantity and also act as PCR inhibitors [42]. Whereas, purity value (260/280) was also found significantly different for consuming different oils while non-significant during application of spices for marination. Low DNA values can also be because of impurities. According to Varma *et al*. (2007) presence of abundant protein can lead to produce low DNA purity [43]. Piskata (2017) also ascribed the samples with low purity (<1.7) values to contaminations with different components like protein [41]. Whereas, Saunders and Rossi (2008) nominated this component to be higher fat [44]. Likewise, Armani *et al*. (2013) found no significant differences in 260/280 ratios within different marinades. This could be because of his different extraction methodology patterns in addition to the varying nature and the amount of additives used [38].

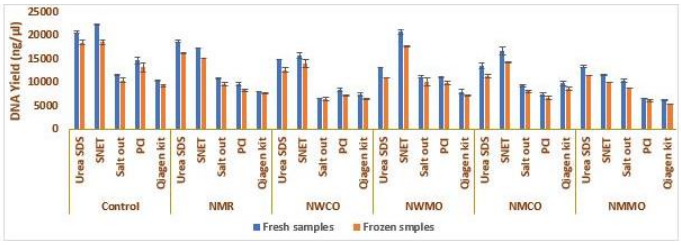

Fig. 3: Comparative DNA yield (ng/µl) of fresh as well as 3-months frozen fish samples cooked varyingly, by different successful DNA extraction methods

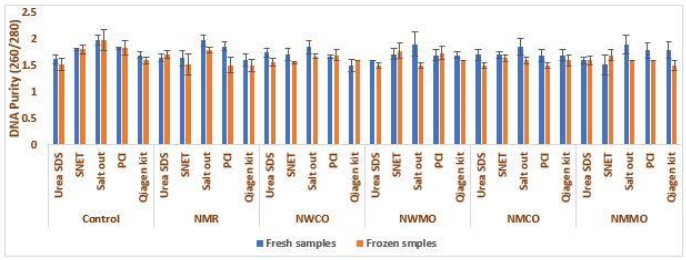

Fig. 4: Comparative DNA purity (260/280) of fresh as well as 3-months frozen fish samples, cooked varyingly, by different successful DNA extraction methods

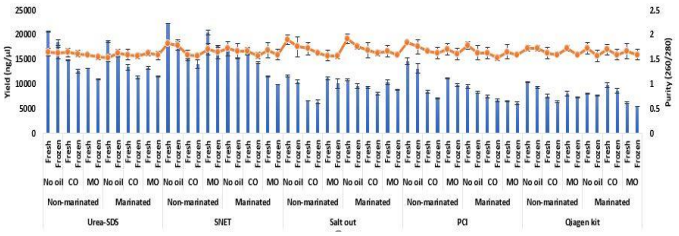

Fig. 5 Comparative DNA yield (ng/µl) and purity (260/280) of fresh as well as 3-months frozen fish nuggets cooked varyingly, by different successful DNA extraction methods, marinations

Considering overall efficiency of DNA extraction methods, control sample showed highest yield and purity as compared with the varyingly processed and cooked samples. DNA isolation protocols aimed to obtain pure DNA in high yield from processed (supposed to be degraded) Rohu (*Labeo rohita*) samples for practical application.

Five DNA isolation protocols including a commercial kit were compared in this study (Fig. 3-5). SNET and Salt out methods were found best. Whereas, kit method was found to work below average. Likewise, Di Bernardo *et al*. (2007) and Akkurt (2012) also found low yield in case of kits as compared to traditional methods [30]. Similarly, Buntjer *et al*. (1999) found lowest yield of DNA while employing Food DNA Isolation Kit (Norgen Biotek) but highest outcome by employing phenol—chloroform extraction [45]. An ideal DNA isolation technique, costs, labor, and removal of PCR inhibitors, it should produce high yield and purity levels and must lessen the DNA degradation [42].

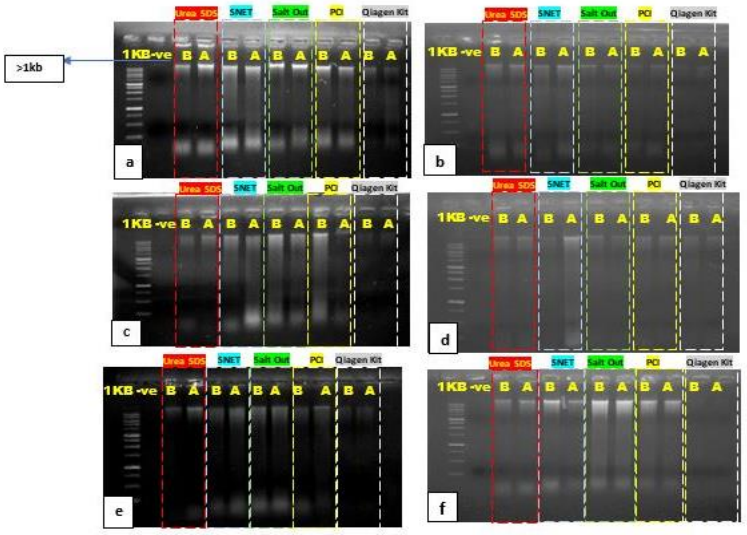

Fig. 6: DNA extraction from fresh as well as 3-months frozen fish samples cooked varyingly by different extraction methods. C= control; NMR= non-marinated and non-fried; NWCO = non-marinated and fried in cooking oil; NWMO= non-marinated and fried in mustard oil; NMCO= marinated and fried in cooking oil and NMMO= marinated and fried in mustard oil). For each photograph from left to right KB Ladder, -ve control, B= Fresh fish samples, A= After 3 months frozen samples processed for DNA extraction by respective method.

Effects of differently processed fish samples (NMR, NWCO, NWMO, NMCO and NMMO) on the extracted DNA fragment size are shown in Fig. 6. All the bands were found to be of similar sizes, greater than 10kb but as can be seen from the figure their compactness and brightness varied a lot. The traditional methods produced comparatively brighter and more compact bands compared to the kit. This may be because of low DNA concentration (ng/µl) extracted from kit. Likewise, Akkurt (2012) reported that although the same amount of sample was used in all isolation methods, yet the traditional methods produced brighter bands as compared to kit employed [30].

A degree of smear formation was observed in almost all the samples. A usual smear pattern formation had also been reported by Sakalar *et al*. (2012) [18]. Piskata et al. (2019) attributed the levels of DNA integrity and yields to the method

of processing. While considering the processed products, the smear formation occurred invariably for any extraction method used [41]. This can be because of heat treatment. Anjali *et al*. (2019) reported a comparable quality yield with some degraded DNA in deep-fried products and documented the order of success as in the same study, the DNA quality was produced as raw>frozen>cooked samples [46]. Armani *et al*. (2013) also reported diverse results in frozen storage conditions [38]. Bauer *et al*. (2003) also documented the fragmented DNA in processed food [14]. Irrespective to the methods used, heat-processed samples (100 ºC to 120 ºC), usually yield low DNA. The DNA band's brightness and compactness can also be affected by other factors, for instance, it takes few minutes or hours to degrade DNA after sampling from live organisms and its degradation will be continued despite being preserved [39]. It becomes even more rapid in open environmental conditions after the activities of endonucleases and exonucleases in the cells [40].

Application of some traditional methods was found better than the commercial kits. Both the DNA yield and purity were higher with traditional methods which is parallel to the study of Lefort *et al*. (1998) who also proposed that commercial DNA extraction kits are widely preferred in recent era because of their momentous advantages of practicality and speedy results. However, their disadvantages like expensiveness, low DNA yield and purity's non-repeatability factors are now being well recognized by the workers [47].

IV. CONCLUSION

The DNA stability tests exposed that heat treatments and freezing caused variable levels of DNA degradation in the fish tissue. In spite of significant degradation, traditional methods were found to be more practical, easier and economical than the outcomes of a commercial kit. SNET and Salt out extraction methods were found superior in terms of DNA yield and its purity respectively. These methods quality as efficient DNA extraction methods for fish tissues fidelity investigations in genetics studies. Thus, this amplicon can efficiently detect adulteration in raw and processed fish meat and have potential to increase confidence level of export of fish and the products. Such scientifically fried products export will definitely contribute.

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