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Comparative analyses of genomic DNA extracted from freshwater fish tissues preserved in formaldehyde and alcohol in different periods of time

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ABSTRACT

High yields of extracted DNA from animal tissues for a number of molecular research work are depended on acquiring tissues that should not highly degraded and must give a sufficient yield of DNA. Current experiments were performed on various tissues preservations, so, later on, the DNA extraction can be done to get high quality and quantity of DNA. 90% alcohol, 8% formalin, and −40°C deep freezing were used for 5 days, 20 months, and 28 months each to stay fish tissues. DNA was extracted from preserved tissues after specified intervals; quantification was performed using Nanodrop spectrophotometer and polymerase chain reaction amplifications through random primers. Genomic DNA extraction method was used for good quantity and reproducibility for molecular markers studies in *Sperata seenghala* population. Phenol-chloroform-isoamyl alcohol method agreed clear, reproducible, and high quality of bands preserved in 90% alcohol and the purity near was 1.8. However, formalin-preserved samples gave the low quantity of DNA as 8.0 ng/µl with 1.35 ratios was not acceptable for molecular work. Two methods proved successful results as "tissues was preserved in 90% alcohol" and "−40°C preserved in Deep Freezer," whereas formalin-based preservation method failed with respect to molecular work.

1. INTRODUCTION

In the past two decades, advances in molecular biology research have led to extraction, amplification of targeted gene, and consequently, sequencing of DNA from different tissues, blood, non-invasive biological samples including formalin-fixed and ethanol-preserved specimens [1,2]. DNA analysis is a tool for the study of facets of biology, not least of all fields such as evolutionary biology, biosystematics, biodiversity, population biology, conservation biology, and ecology [3]. For the study of population biology and conservation biology, mostly samples were collected from remote areas and preserved in different chemicals [4]. Some chemicals are not appropriate for the DNA studies because they create some modifications in tissue chemicals or degradation of DNA which hinder the molecular biology experiments.

Tissues preservation is most important to make samples keep on original for long time worth for getting a high yield of genomic DNA for molecular studies. Safety of tissue samples for genomic DNA extraction is important as it can protect these potentially to give a high yield of genomic DNA [5]. Usually formaldehyde is most commonly used chemical for preservation of tissues in laboratories. Ethanol solutions are one of the methods for tissue preservation for DNA analysis. Ethanol is appropriate to the storage of vertebrate tissue and has been used successfully in DNA hybridization and sequencing [5,6].

The extraction of the high quality of genomic DNA may be difficult in formalin-fixed tissues as of cross-linking between proteins and DNA as formalin induces DNA destruction and nucleotide modification [7,8]. The rapid reaction of formalin with double helical DNA generally is flexible but over the long term, especially with denaturation of the DNA, a variety of reactions can occur, many of which have not been characterized [8,9].

In the present study, three different preservatives formaldehyde, ethanol 90% solution, and freezing at −40°C were used to examine and determined the effects of DNA of the samples at different time intervals. The aim of this study was to find which method of tissue storage is suitable for extraction of optimum quality of DNA for molecular and phylogenetic studies because the availability of fresh samples in taxonomical studies is limited.

2. MATERIALS AND METHODS

2.1. Collection of Fish Samples

Five specimens of *Sperata seenghala* were collected from Upper Lake Bhopal brought to the laboratory for performing experimental work.

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Liver and muscle tissues were taken out for extraction of genomic DNA. Tissues preserved in 8% formalin, 90% alcohol, and −40°C deep freezer for 5 days, 20 months, and 28 months.

2.2. Genomic DNA Isolation and Quantification

Isolation of genomic DNA was performed as the protocol of Janarthan and Vincent [10] using phenol:chloroform:isoamyl-alcohol (25:24:1) method.

Qualitative and quantitative estimation of extracted genomic DNA was done by NanoDrop ultraviolet spectrophotometer (ND-1000) by calculating the ratio of absorbance at 260–280 nm wavelength. Pure genomic DNA shows ratios as 1.8 at 260 nm v/s 280 nm with respect to protein contamination, because protein tends to absorb at 280 nm wavelength. Final dilutions were made as required for polymerase chain reaction (PCR) amplification ±50 ng/µl of DNA samples.

2.3. PCR Amplification

Two primers RAn-5 and RAn-6 with accession numbers AM750052 and AM765829, respectively, were used for final PCR amplification who gave positive and scorable results. A 25 µl of reaction mixture composed of 12.50 µl Red Dye, 1.0 µl primer, 11.00 µl of sterile distilled water, and 1.0 µl template DNA was prepared for PCR amplification with preheating for 5 min at 94°C. PCR was run for 45 cycles consisted of a 94°C denaturation for 0.45 min, 37°C annealing for 1.0 min, and 72°C elongation for 1.5 min in thermal cycler (Model EP Gradient, Eppendorf, Germany) with a final extension of 72°C for 10 min.

2.4. Gel Electrophoresis and Visualization of DNA Pattern

Amplified DNA fragments were separated by gel electrophoresis on 1.2% agarose gel concentration containing ethidium bromide for visualization of DNA fragments. Low range DNA ladder was also used for each gel with the known interval of 100, 200, 300, 600, 1000, 1500, 2000, 2500, and 3000 bp. Gel was visualized and photographed on gel documentation system (Alpha-Innotech, USA) for scoring of DNA fingerprints and finally obtained molecular weights.

3. RESULTS AND DISCUSSION

Molecular research studies need for genomic DNA extraction from animal tissues including aquatic animals for performing molecular taxonomy, development of species-specific markers, recombinant DNA technology, and transgenic studies. Even few methods exist genomic of DNA extraction from cultured cells and blood, but in the absence of these facilities, how the samples are collected from the field and preserve them in a proper manner so that a desired quality as well quantity of DNA can be obtained from the preserved samples. The major problems challenged by forensic laboratories or researchers engaged in molecular biology are to obtain the samples in proper conditions to performed molecular work. Most of the biological samples brought for DNA analysis are either partially degraded or completely degraded, and many times, it is not possible to extract DNA from them, and therefore, the present research carried on *S. seenghala*.

In this study, tissues preserved in different preservatives for 5 days gave following results with respect to quantification of DNA. 8% formalin preservative for 5 days, DNA quality was obtained from 8.07 ng/ul to 15.77 ng/ul with absorbance ratio from 1.30 to 1.53 of 260/280 wavelengths [Table 1]. The obtained results were unsatisfactory with respect to quality and quantity as they do not fall in the normal range. In case of −40°C preservative tissue samples, the DNA was recorded from 410.79 ng/ul to 645.79 ng/ul with the ratio between 1.98 and 2.13 of 260/280 [Table 1 and Figures 1-5] showed satisfactory results and may called results are up to the mark, because it lies close to the normal value for obtaining better results in DNA amplification. Laith [11] studied on the effect of formalin, alcohol, and freezing on some body proportion of a marine fish *Alepes djeddaba,* observed

Table 1: Quantification of extracted DNA from tissues of *S. seenghala* preserved in 90% alcohol, 8% formalin, and – 20°C deep freezer.

Samples ID	Samples preserved for 5 days			Samples preserved for 20 months			Samples preserved for 28 months		
	Date	ng/µl	260/280	Date	ng/µl	260/280	Date	ng/µl	260/280
A. 90% Alcohol									
90% A-01	30.11.2010	816.14	1.69	6/7/2012	34.34	2.11	19.03.2013	1.62	62.60
90% A-02	30.11.2010	140.97	1.30	6/7/2012	66.68	2.09	19.03.2013	1.44	137.20
90% A-03	30.11.2010	1902.44	1.80	6/7/2012	219.30	2.06	19.03.2013	1.56	43.20
90% A-04	30.11.2010	661.20	1.47	N/A	N/A	N/A	19.03.2013	1.02	866.70
90% A-05	30.11.2010	1248.56	1.79	N/A	N/A	N/A	N/A	N/A	N/A
B. 8% formalin									
8% F-01	30.11.2010	11.70	1.33	6/7/2012	0.69	0.78	20.03.2013	1.30	9.60
8% F-02	30.11.2010	8.92	1.35	6/7/2012	1.59	2.31	19.03.2013	1.40	12.70
8% F-03	30.11.2010	8.07	1.42	6/7/2012	2.53	1.44	20.03.2013	2.49	1.40
8% F-04	30.11.2010	9.09	1.30	N/A	N/A	N/A	19.03.2013	1.37	9.20
8% F-05	30.11.2010	15.77	1.53	N/A	N/A	N/A	20.03.2013	1.07	2.20
C. Dried ice									
Dried ice-01	30.11.2010	473.20	1.89	6/7/2012	445.77	1.99	20.03.2013	1.86	218.60
Dried ice-02	30.11.2010	531.89	1.80	6/7/2012	267.11	2.04	20.03.2013	1.53	392.60
Dried ice-03	30.11.2010	645.79	1.86	6/7/2012	103.46	1.36	20.03.2013	1.83	37.80
Dried ice-04	30.11.2010	410.79	1.93	N/A	N/A	N/A	20.03.2013	1.57	602.40
Dried ice-05	30.11.2010	542.01	1.93	N/A	N/A	N/A	N/A	N/A	N/A

S. seenghala: Sperata seenghala

greatest shrinkage of specimens preserved in 8% formalin-tap water, while the least shrinkage was in the fishes stored in 70% alcohol tap water. The present investigation also shows that the tissues preserved in formalin not gave good results.

Tissues preserved in different preservatives for 5 days and gave the following results with respect to reproducibility of the DNA fingerprints obtained by RAn-05 and RAn-06. In 90% alcohol for 5 days amplified with random amplified polymorphic DNA (RAPD), primer RAn-05 gave 16 reproducible bands with the molecular weights of 982.61, 576.92, 290.00, 130.70, 965.22, 553.8, 380.77, 250.00, 965.22, 553.85, 392.31, 260.00, and 115.38 bp. In case of 8% formalin preservative we 10 reproducible bands with respect of molecular weights as 300.00, 100.00, 311.54, 115.38, 323.08, 130.77, 965.22, 600.00, 300.00, and 146.15 bp [Table 2]. Similarly, for dried ice, 10 amplicons with the molecular weights of 1075.00, 461.54, 323.08, 161.54, 1075.00, 617.39, 450.00, 311.54, 1100.00, and 473.08 bp were obtained [Table 2 and Figure 6].

RAPD primer RAn-06 was also screened for extracted DNA of all three preservatives tissues samples. The frequencies of the bands obtained by RAn-06 are given in Table 3. It showed that the 90% alcohol 18 bands was obtained with the molecular weights of 1208.33, 729.59, 1166.67, 748.15, 425.81, 1145.83, 733.33, 416.13, 188.89, 1312.50, 1125.00, 940.74, 718.52, 493.55, 194.44, and 183.33 bp with minimum molecular weight as 183.33 and maximum as 1354.17 bp. 8% formalin preservative gave 5 bands which showed molecular weights 866.67. However, dried ice produced 16 bands with the molecular weights of 1354.17, 1166.67, 762.96, 512.90, 435.48, 309.68, 188.89, 1187.50, 762.96, 493.55, 425.81, 1187.50, 762.96, 483.87, 100.00, and 194.44 bp [Figure 7].

Formaldehyde was used in many industries, hospitals, and research as a sterilizing and preserving agent. In the current study, it has concluded that 90% alcohol preservative can be used as ideal preservative because alcohol has the property to bind with proteins. Thus, it binds with the proteins of DNA, and in this way, we got the pure form of DNA. In contrast to the alcohol, 8% formalin cannot be considered as an ideal

Table 2: Frequency of amplicons and their molecular weight obtained through RAPD marker RAn-5.

Sample preservatives	Total number of amplicons obtained	Molecular weight in bp	Range of molecular weight	
			Minimum	Maximum
90% alcohol	16	982.61, 576.92, 290.00, 130.70,965.22, 53.8,380.77, 250.00, 965.22,553.85,392.31,260.00, 115.38	115.38	982.61
8% formalin	10	300.00, 100.00, 311.54, 115.38, 323.08, 130.77 965.22, 600.00, 300.00, 146.15.	100.00	965.22
Dried-ice	10	1075.00, 461.54, 323.08, 161.54, 1075.00, 617.39, 450.00, 311.54, 1100.00, 473.08	161.54	1100.00

RAPD: Random amplified polymorphic DNA

Table 3: Frequency of amplicons and their molecular weight obtained through RAPD marker RAn-6.

Sample preservatives	Total number of band obtained	Molecular weight in bp	Range of molecular weight	
			Minimum	Maximum
90% alcohol	18	1208.33, 729.59, 1166.67, 748.15, 425.81, 1145.83, 733.33, 416.13, 188.89, 1312.50, 1125.00, 940.74, 718.52, 493.55, 194.44, 183.33	183.33	1354.17
8% formalin	5	N/A, N/A, N/A, 866.67, N/A, N/A	866.67	N/A
Dried-ice	16	1354.17, 1166.67, 762.96, 512.90, 435.48, 309.68, 188.89, 1187.50, 762.96, 493.55, 425.81, 1187.50, 762.96, 483.87, 100.00, 194.44	100.00	1354.17

RAPD: Random amplified polymorphic DNA

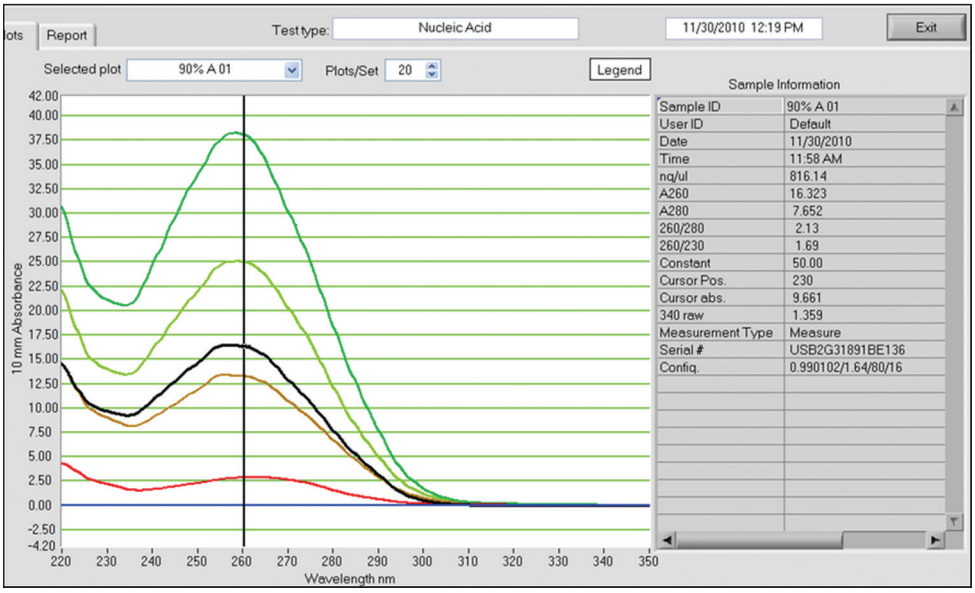

Figure 1: Qualitative and quantitative status of extracted DNA from preserved samples in 90% alcohol for 5 days.

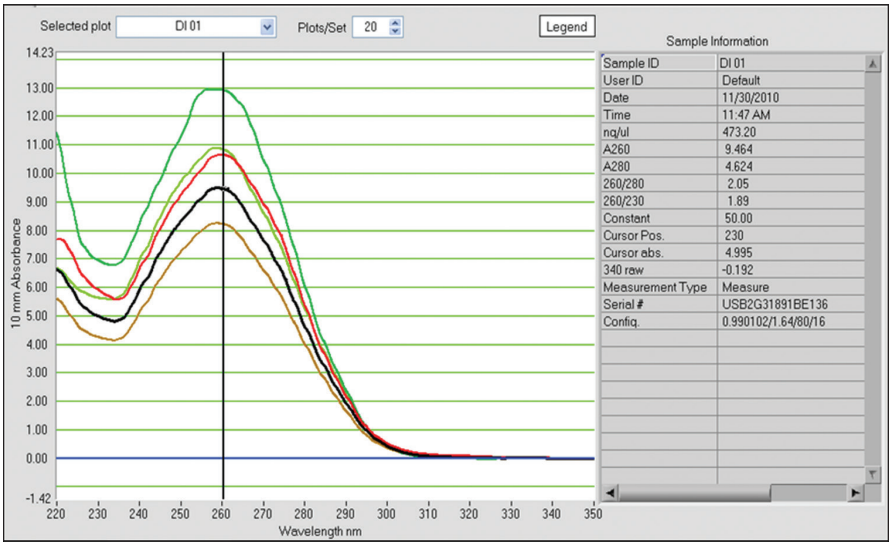

Figure 2: Qualitative and quantitative status of extracted DNA from preserved samples in 8% formalin for 5 days.

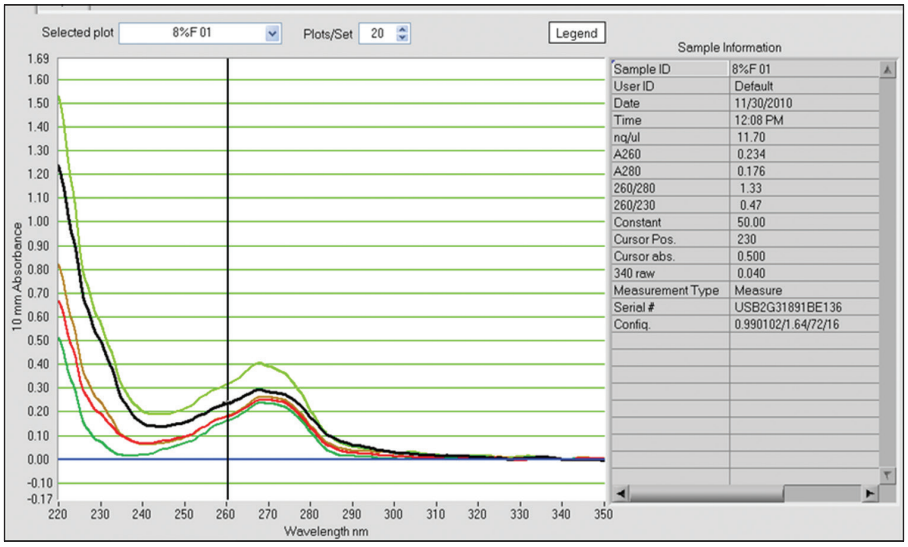

Figure 3: Qualitative and quantitative status of extracted DNA from preserved samples in dried ice for 5 days.

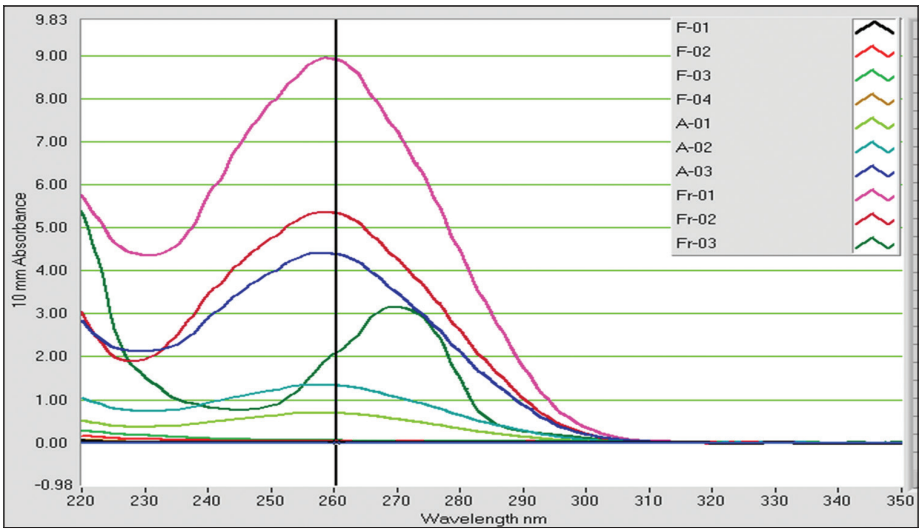

Figure 4: Qualitative and quantitative status of extracted DNA from preserved samples in 90% alcohol, 8% formalin for 20 months and fresh samples.

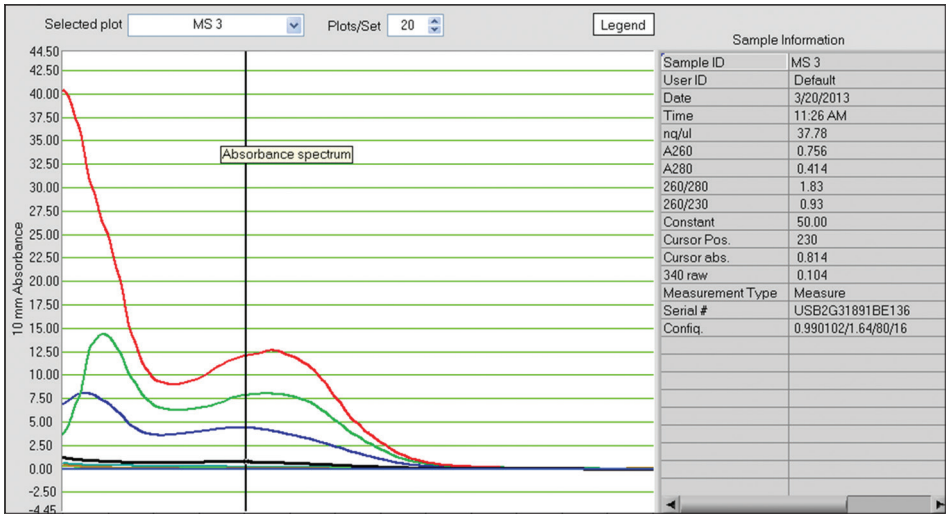

Figure 5: Qualitative and quantitative status of extracted DNA from preserved samples in 90% alcohol, 8% formalin for 28 months and fresh samples.

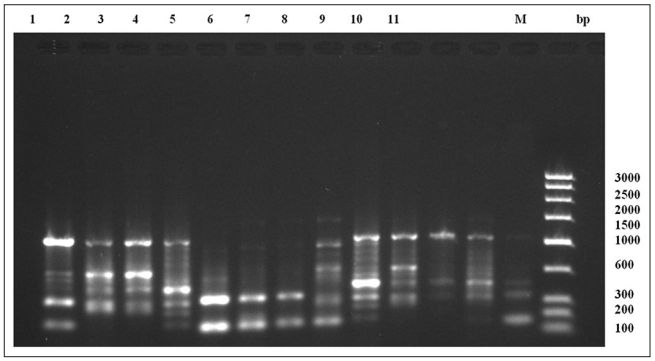

Figure 6: Random amplified polymorphic DNA-polymerase chain reaction fragment patterns obtained by RAn-05 from tissues of *Sperata seenghala* preserved for 5 days. 1–4 = samples with 90% alcohol preservative, 5–8 = samples with 8% formalin preservative, 9–11 = samples with dried ice preservative. M is molecular marker (bp) of low range DNA ladder.

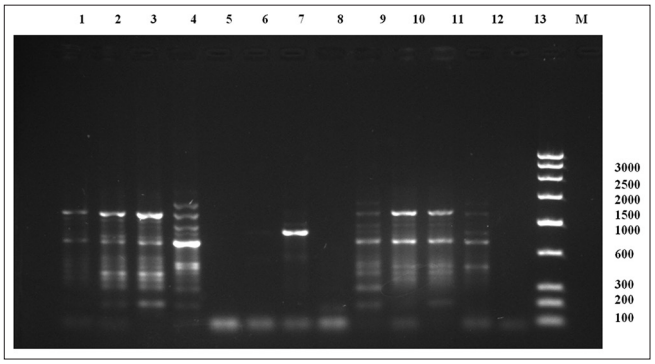

Figure 7: Random amplified polymorphic DNA-polymerase chain reaction fragment patterns obtained by RAn-06 from tissues of *Sperata seenghala* preserved for 5 days. 1–4 = samples with 90% alcohol preservative, 5–8= samples with 8% formalin preservative, 9–11= samples with dried ice preservative. M is molecular marker (bp) of low range DNA ladder.

because it has a tendency of degradation of DNA at room temperature. Thus, qualitatively and quantitatively wise, it does not given satisfactory results [Figure 10]. Comparable type of effects of formaldehyde was

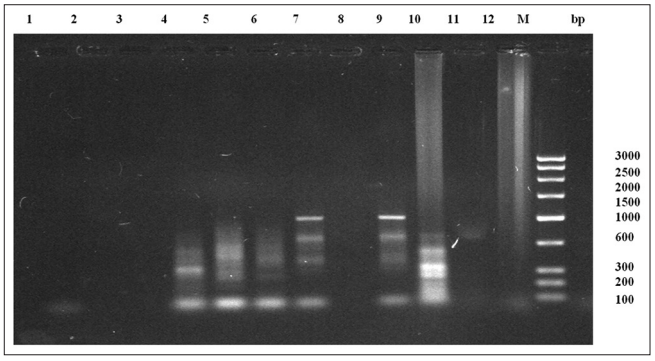

Figure 8: Random amplified polymorphic DNA-polymerase chain reaction fragment patterns obtained by RAn-05 from tissues of *Sperata seenghala* preserved for 20 months. 1–6 = tissues preserved in 8% formalin, 07–09= tissues preserved in 90% alcohol, 10–12 tissues preserved in dried ice. M is molecular marker (bp) of low range DNA ladder.

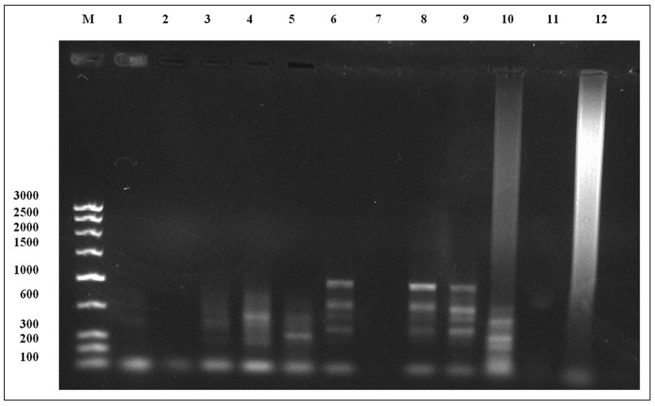

Figure 9: Random amplified polymorphic DNA-polymerase chain reaction fragment patterns obtained by RAn-06 from tissues of *Sperata seenghala* preserved for 20 months. 1–5 = tissues preserved in 8% formalin, 6–9 = tissues preserved in 90% alcohol, 10–12 tissues preserved in deep freezer. M is molecular marker (bp) of low range DNA ladder.

also observed by Shields and Carlson [12] when they studied on effects of formalin and alcohol preservatives on length and weight of juveniles

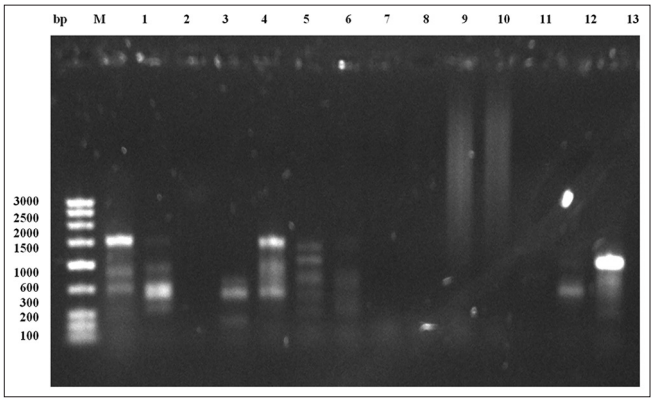

Figure 10: Random amplified polymorphic DNA-polymerase chain reaction fragment patterns obtained by RAn-05 from tissues of *Sperata seenghala* preserved for 28 months. 1–4 = tissues preserved in 90% alcohol, 5–8 = tissues preserved in 8% formalin, 10–12 tissues preserved in deep freezer. M is molecular marker (bp) of low range DNA ladder.

of salmon. Tissues sample storage in ethanol was the best preservation for extraction of DNA as studied for storage and shipping of tissue samples for DNA analyses from earthworms [13,14]. Our investigation was showed good preservative as ethanol which supported by the study carried by Straube and Juen [13] on earthworms for shipping and transport of tissues samples for extraction of genomic DNA.

The results of present studies concluded that −40°C deep freezer and 90% alcohol are fruitful/good preservative for tissues to be used the extraction of genomic for molecular studies. In 8% formalin, we observed degradation in DNA quantity right from the beginning of experiment, i.e., from 5th day to 28 months.

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