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NON-DESTRUCTIVE GENETIC SAMPLING IN TWO CYPRINID FISH SPECIES OF SOUTHERN PART OF WESTERN GHATS-INDIA

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ABSTRACT

Non- destructive genetic sampling for DNA isolation in two cyprinid endemic fishes of the species *Danio aequipinnatus* and *Puntius tambraparniei* was attempted. The total genomic DNA was isolated from fin clips, scales, liver and muscles by using Phenol: choloroform method by different storage method like Ethanol/EDTA and air dry in the different cell lysis buffer like TNES- urea and TNES. In *Puntius tambraparniei* showed higher quantity of genomic DNA (402.36µg) in the liver tissues by using the TNES cell lysis buffer in the ethanol/ EDTA storage method. *Danio aequipinnatus* also showed the high quantity of genomic DNA (415.26µg) in the muscle tissue by using TNES cell lysis buffer in the Ethanol/ EDTA storage method.

Key words- Non-destructive, genetic sampling, DNA extraction, fin clips, scale, muscle, cyprinid fish.

INTRODUCTION

In recent years many vertebrate species are at risk and their methods of conservation is possible without destruction and based on this DNA based studies gain importance to gain information on the diversity and population analysis (O'Brien 1994). DNA analysis will help to study the phylogeny, determination of population size and level of genetic polymorphism within and between populations. Liver and muscle tissues are used for DNA analysis and this method implies the sacrifice of the animals and hence it called as destructive method. The non-destructive sources of DNA are hair, faeces, urine, shed feathers, snake skin, sloughed whale skin, eggshells and even skulls. However, this method usually results in a low quantity and poor quality of DNA. Non-destructive sampling also includes the use of blood, skin and scales (Hilsdorf *et al*. 1999). Muscle tissues and blood samples are used for DNA isolation without sacrifying the fish especially large population of threatened fishes. (Cummings *et al*. 1994) and (Estoup *et al*. 1996)

Even this can be overcome by using fins and scales. Hence an attempt has been made to compare the destructive method of extracting DNA from muscle and liver tissues and a non-destructive method of extracting DNA from fins and scales of two freshwater fish species of India.

One is an ornamental fish *Danio aequipinnatus* which has the natural distribution in clear water streams/rivers of India, and another species is *Puntius*

tambraparniei an endemic species confined to its distribution range to Tamiraparani river basin alone. (Silas 1953, Arunachalam and Sankaranarayanan 2000).

MATERIALS AND METHODS

Sample Collection:

Cyprinids species *Danio aequipinnatus, Puntius tambraparniei* were collected from river and cannels, middle reach of the rivers Gadana and Ramanadhi which are the two such basins of the Tamiraparani river system. Fish sampling was performed by using cast net and gill nets.

Extraction of DNA

The genomic DNA was isolated by phenol-chloroform method based on Sam brook *et al*. (1989). DNA was achieved from fish caudal (or) anal fins and from fish scales by changing the protocol previously described for tissue preservation and DNA extraction from muscle (or) liver (Asahida *et al*. 1996, Sam brook and Russell 2001). Total DNA was obtained from individually belonging to two different fish species *Puntius tambraparniei and Danio aequipinnatus.*

Approximately 100-500 mg of fins (1-2cm^2) or scales are initially stored in 95% ethanol- 100µl EDTA pH 8.0 (Dessauer *et al*. 1996). 100 mg freshly air-dried samples Fins and scales are collected from *Puntius tambraparniei and Danio aequipinnatus.* The samples are cut into small pieces using sterile blade. The samples are placed in 4ml of a TNES, TNES- urea -digestion buffer inside of 15 ml tube. 30µl of *RNase* (10mg/ml) is added to the tube. The samples are incubated at 42°C for 1h. After this period, 30µl of *Proteinase K* (10mg/ml) is added and tissues are maintained at 42°C for at least 10 hours. The DNA is then isolated by adding 4 ml of phenol: choloroform: isoamylalcohol (25:24:1) to the tubes. After inverting the tubes for 15 minutes, they are rotated for 15 minutes at 10,000rpm. The top aqueous layer is removed to a new tube. The DNA is precipitated in 1M Nacl and volume of cooled absolute ethanol, inverting the tubes several times. The DNA is recovered by 10,000 rpm centrifugation. Then the DNA pellet is washed briefly in 70% ethanol, air dried and resuspended in an appropriate volume of TE buffer. After completely soluble, the DNA is stored at -20°C. The DNA integrity is checked on 1% Agarose gel stained with ethidium bromide. A spectrophotometer (Spectronic® Genesys™ 2) is used to evaluate the total amount of obtained DNA. The quantification of DNA was done by UV spectrophotometric analysis. The quantity of DNA was measured by obtaining the absorbance reading at 260nm and the purity of DNA was checked by calculating the ratio of absorbance readings at 260nm and 280nm.

Total amount of DNA = (Absorbance at 260 nm) X 50 X (Dilution Factor) µg/ml

$$\text{Purity of DNA} = \frac{\text{Absorbance at 260 nm}}{\text{Absorbance at 280 nm}}$$

Separation of the DNA by using Agarose Gel Electrophoresis

After the isolation, the DNA samples were taken out and mix with 7µl Bromophenol blue (sample loading dye) and a 15µl of mixed DNA product was loaded in 1% Agarose gel (50ml) containing Ethidium bromide at the concentration of 20µl per 50ml of gel. The electrophoresis was carried out for 1 to 2 hours at 50 volts.

Gel Documentation

After electrophoresis gel was placed in the UV transilluminator and bands were visualized and were photographed using digital camera.

RESULTS

Fin samples (n=3) of *Puntius tambraparniei* showed higher quantity of genomic DNA using TNES/urea in Ethanol/EDTA and TNES/urea in air dried methods, 225.625µg/ml and 150.23µg/ml respectively; where as in TNES without urea solution shows the absence of DNA. Scale samples (n=3) of *Puntius tambraparniei* showed higher quantity of genomic DNA using TNES/urea in Ethanol/EDTA and air-dried methods, 120.75µg/ml and 101.25µg/ml respectively; where as in TNES without urea solution shows the absence of DNA. Liver samples (n=2) of *Puntius tambraparniei* showed higher quantity of genomic DNA using TNES method than TNES/urea, 402.36µg/ml and 186.34µg/ml respectively. Muscle samples (n=2) of *Puntius tambraparniei* showed higher quantity of genomic DNA using TNES method than TNES/urea, 369.48µg/ml and 134.48µg/ml respectively (Table. 1-2).

Fin samples (n=3) of *Danio aequipinnatus* showed higher quantity of genomic DNA using TNES/urea in Ethanol/EDTA and TNES/urea in air-dried methods, 260.42µg/ml and 175.25µg/ml respectively; where as in TNES without urea solution shows the absence of DNA. Scale samples (n=3) of *Danio aequipinnatus* showed higher quantity of genomic DNA using TNES/urea in Ethanol/EDTA and TNES/urea in air-dried methods, 135.00µg/ml and 125.36µg/ml respectively, where as in TNES without urea solution shows the absence of DNA. Liver samples (n=2) of *Danio aequipinnatus* showed higher quantity of genomic DNA using TNES and TNES/urea method, 369.45µg/ml and 210.25µg/ml respectively. Muscle samples (n=2) of *Danio aequipinnatus* showed higher quantity of genomic DNA using TNES and TNES/urea method, 415.26µg/ml and 154.52 µg/ml respectively (Table. 3-4).

Most of the isolated DNA of the two species showed no sign of degradation and the spectrophotometer comparison of absorbance at 260 - 280nm provided a DNA/ RNA relationship of (1.6-1.9) indicating good DNA quality. The DNA concentration ranged from 25-500ng/µl, with on average concentration 200ng/µl and the obtained DNA volume (approximately 1ml) was high enough to be employed on several molecular experiments. Although the present methodology was applied on samples of two fish species, similarity in fin (or scales) anatomy suggests that the technique will work on samples of different taxa.

DISCUSSION

As stated by some authors (Chen *et al*. 1995; Strassmann *et al*. 1996; Pinto *et al*. 2000), tissue homogenization in liquid nitrogen can be an efficient method to isolate significant amounts of DNA, especially on hard consistent tissues. However, in our experiments the use of nitrogen maceration with fins and scales did not give any further improvement in the DNA isolation. Better results were achieved in the present study by mixing the scales or small pieces of the fins with a cell lysis solution containing urea. The initial 8M urea concentration of the buffer, suggested by Asahida *et al*. (1996), was gradually decreased to 4M, which allowed a better preservation of the material and a non-degraded isolated DNA. Urea treatment seems to be a necessary step to breakdown hard tissues such fins and scales, since it is quite denaturing for protein and at least it disrupts most likely any protein multicomplexes. Another improvement on the DNA isolation process

was provided by a pretreatment with *RNase*, which allowed us to obtain DNA samples with lower quantities of RNA that could interfere in the accurate DNA quantification and on further amplification procedures. The concentration and time/temperature for *Proteinase K* incubation were also very important to obtain high-quality DNA. The use of lower concentrations of this enzyme resulted in poor quality-DNA, as it failed to completely digest the tissue. A better dissociation of the tissues was also obtained when the digestion was done at 42°C. Higher incubation temperatures (50°C or more) were inefficient and temperatures lower than 42°C resulted in a partially digested tissue. Experiments using a final concentration of 0.075 mg/ml of *Proteinase K* provided tissues that were totally digested after a 10 hours-incubation. Less-time incubation was not efficient. After tissue digestion, a phenol:chloroform:isoamyl alcohol purification step was utilized, as suggested by Taggart *et al*. (1992) and Sambrook and Russell (2001). The use of phenol-chloroform proved to be essential to obtaining pure DNA samples from fish fins and scales. Crude extractions could result in a DNA contaminated with proteins that may not be stable for long-term storage. However, repeated DNA extractions with phenol-chloroform were not necessary. Single and double washes gave same results, avoiding protein residues.

The described technique was applied on air-dried and ethanol/ EDTA-fixed fin clips and scales and also on ethanol/ EDTA-preserved liver and muscle tissues. In addition, DNA samples were also obtained from liver and muscle using nitrogen maceration and by the use of a digestion buffer without urea, as described in Sambrook and Russell (2001). The use of a lysis solution without urea showed to be not appropriate for fin clips and scales. It is evident that fins and scales, represent a DNA source as suitable as other tissues and also the DNA amount isolated from the fins was also high, when compared to the amount obtained from liver or muscle. Therefore, the extraction of DNA from fish fins or scales offers an extremely a positive alternative to conventional DNA isolation techniques, representing a minimally destructive sampling approach.

An adequate preservation of tissue samples is a prerequisite in field locations and for long-period analyses. Despite the successful isolation of DNA from different tissues of *Puntius tambraparniei* and *Danio aequipinnatus*, some differences were observed in relation to the material storage. Samples of fins and scales preserved on ethanol/EDTA proved to be more suitable as a DNA source, when compared to air-dried samples stored for 1 to several days. Preservation of nucleic acids depends primarily on the inhibition of tissue nucleases and denaturation, which can be achieved with EDTA and ethanol, respectively (Dessauer *et al*. 1996).

The ethanol/EDTA storage solution also permits to maintain the softness of the tissue, facilitating its further dissociation in the digestion buffer. However, the long term storage of tissues on TNES- Urea buffer, as suggested by Asahida *et al.* (1996), was not appropriate for fins or scales due to a high DNA breakdown.

The protocol outlined in this work offers a cost-efficient and suitable alternative to conventional DNA isolation techniques, representing a non-destructive sampling approach to isolate high-quality DNA from fish. The total amount of isolated DNA (25–500µg) is also sufficient for several other molecular procedures that often demand more DNA than the pictogram

range. It can be considered that a genetic stock of several fish species could be easily achieved by using the described methodology.

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Table 1: Comparison of different tissues of Puntius tambraparniei

Sample tissue	Weight (g)	Storage method	Cell lysis buffer	Purity of DNA	Total DNA amount (µg)
Fin	0.25	Ethanol/EDTA	TNES-urea	1.65	225.625
Fin	0.25	air-dry	TNES-urea	1.73	150.523
Fin	0.25	Ethanol/EDTA	TNES	No result	No result
Scales	0.25	Ethanol/EDTA	TNES-urea	1.69	120.75
Scales	0.25	air-dry	TNES-urea	1.63	101.23
Scales	0.25	Ethanol/EDTA	TNES	No result	No result
Liver	0.25	Ethanol/EDTA	TNES-urea	1.64	186.34
Liver	0.25	Ethanol/EDTA	TNES	1.73	402.36
Muscle	0.25	Ethanol/EDTA	TNES-urea	1.69	134.48
Muscle	0.25	Ethanol/EDTA	TNES	1.81	369.48

Table 2: Comparison of different tissues of Puntius tambraparniei

Sample tissue	Weight (g)	Storage method	Cell lysis buffer	Absorbance at	
				260nm	280nm
Fin	0.25	Ethanol/EDTA	TNES-urea	0.4512	0.2734
Fin	0.25	air-dry	TNES-urea	0.3010	0.1739
Fin	0.25	Ethanol/EDTA	TNES	0	0
Scales	0.25	Ethanol/EDTA	TNES-urea	0.2415	0.1428
Scales	0.25	air-dry	TNES-urea	0.2024	0.1242
Scales	0.25	Ethanol/EDTA	TNES	0	0
Liver	0.25	Ethanol/EDTA	TNES-urea	0.3726	0.2272
Liver	0.25	Ethanol/EDTA	TNES	0.8047	0.4651
Muscle	0.25	Ethanol/EDTA	TNES-urea	0.2689	0.1591
Muscle	0.25	Ethanol/EDTA	TNES	0.7389	0.4082

Table 3: Comparison of different tissues of *Danio aequipinnatus*

Sample tissue	Weight (g)	Storage method	Cell lysis buffer	Purity of DNA	Total DNA amount µg
Fin	0.25	Ethanol/EDTA	TNES-urea	1.73	260.42
Fin	0.25	air-dry	TNES-urea	1.89	175.25
Fin	0.25	Ethanol/EDTA	TNES	No result	No result
Scales	0.25	Ethanol/EDTA	TNES-urea	1.82	135.00
Scales	0.25	air-dry	TNES-urea	1.63	125.36
Scales	0.25	Ethanol/EDTA	TNES	No result	No result
Liver	0.25	Ethanol/EDTA	TNES-urea	1.67	210.25
Liver	0.25	Ethanol/EDTA	TNES	1.79	369.45
Muscle	0.25	Ethanol/EDTA	TNES-urea	1.80	154.52
Muscle	0.25	Ethanol/EDTA	TNES	1.79	415.26

Table 4: Comparison of different tissues of *Danio aequipinnatus*

Sample tissue	Weight (g)	Storage method	Cell lysis buffer	Absorbance at	
				260nm	280nm
Fin	0.25	Ethanol/EDTA	TNES-urea	0.5211	0.3005
Fin	0.25	air-dry	TNES-urea	0.3505	0.1854
Fin	0.25	Ethanol/EDTA	TNES	0	0
Scales	0.25	Ethanol/EDTA	TNES-urea	0.27	0.1483
Scales	0.25	air-dry	TNES-urea	0.2507	0.1538
Scales	0.25	Ethanol/EDTA	TNES	0	0
Liver	0.25	Ethanol/EDTA	TNES-urea	0.4205	0.2517
Liver	0.25	Ethanol/EDTA	TNES	0.7389	0.4127
Muscle	0.25	Ethanol/EDTA	TNES-urea	0.3090	0.1716
Muscle	0.25	Ethanol/EDTA	TNES	0.8305	0.4639

Fig. 1 Experimental fishes for DNA extraction

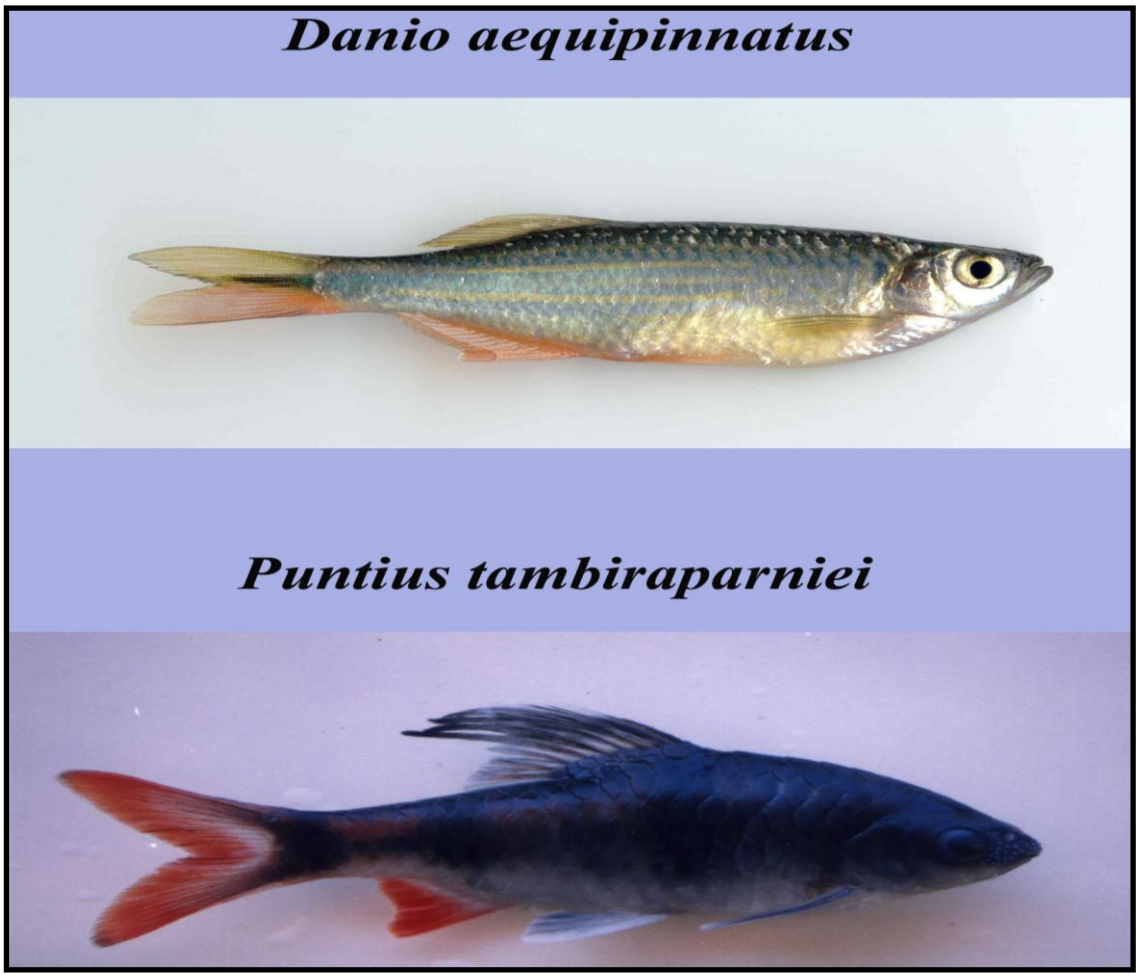

Fig. 2 - 2 % Agarose gel of DNA samples obtained from fish fin and scales. (Lanes 1 – 6). Lanes 1 and 3 total DNA isolated from fish fin clips. *Puntius tambraparniei* and *Danio aequipinnatus* and Lane 2 and 4 total DNA isolated from scales of *Puntius tambraparniei* and *Danio aequipinnatus*. Lane 5 and 6 total DNA isolated from muscles of *Puntius tambraparniei* and *Danio aequipinnatus.*