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A non-invasive technique for rapid extraction of DNA from fish scales

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DNA markers are being increasingly used in studies related to population genetics and conservation biology of endangered species. DNA isolation for such studies requires a source of biological material that is easy to collect, non-bulky and reliable. Further, the sampling strategies based on non-invasive procedures are desirable, especially for the endangered fish species. In view of above, a rapid DNA extraction method from fish scales has been developed with the use of a modified lysis buffer that require about 2 hr duration. This methodology is non-invasive, less expensive and reproducible with high efficiency of DNA recovery. The DNA extracted by this technique, have been found suitable for performing restriction enzyme digestion and PCR amplification. Therefore, the present DNA extraction procedure can be used as an alternative technique in population genetic studies pertaining to endangered fish species. The technique was also found equally effective for DNA isolation from fresh, dried and ethanol preserved scales.

Keywords: DNA extraction, Fishes, Non-invasive, Scales

DNA markers are being increasingly used for gathering information on the diversity, conservation biology and population analyses of different organisms[1-3]. Such information is useful for planning conservation strategies for great number of fish species, around 800[4], which have been designated as threatened in recent years, due to various anthropogenic stresses.

Species and population genetic assessment requires easy, fast, less expensive and reliable DNA extraction methodologies[5-7]. Among different procedures of tissue sampling to obtain DNA, non-invasive sampling seems to be very attractive and need of the day, since it allows genetic analysis of several individuals without much handling or sacrificing them[8-11]. The DNA isolation from non-invasively collected tissues is particularly useful, when large populations or threatened species have to be studied. Liver and muscles are the most common tissues used as sources of DNA, but for collection of liver the animal needs to be sacrificed. Another tissue, which is frequently used in vertebrates for DNA extraction is peripheral blood, but its use in fish present greater difficulties[12]. Although, DNA can successfully be obtained from muscles [11] or blood samples of fish[13-15] without the sacrifice of the animals, it is usually difficult to perform a blood or muscle sampling on many fishes. Therefore, large-size individuals, specialized staff and high sampling speed would be necessary for samples' survival. The other noninvasive source of sampling for DNA can be hair, faeces, urine, shed feathers, egg shells etc. However, this strategy usually results in a low quantity and poor quality DNA and also does not provide individual identification, which limits its potential application[7].

DNA isolation from fish fins or scales provides a suitable non-invasive procedure which can overcome the difficulties encountered during tissue sampling from other tissues and allows the maintenance of the individuals without much disturbance. In the present paper, an improved and a very rapid DNA extraction method from the fish scales has been described that used a modified lysis buffer. This DNA extraction method provides high-quality and high-quantity DNA that can serve as a template in polymerase chain reaction (PCR) and restriction digestion experiments.

Materials and Methods

Sample collection—For the present study the scales have been used from five specimens each of seven different fresh water fish species namely *Catla catla, Labeo rohita, L. calbasu, L. bata, Cirrhinus mrigala*, *Channa punctatus* and *Cyprinus carpio*. The scales were collected non-invasively by gentle scrapping on the caudal portion of the body with a

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forceps, and the detached scales were collected in 2 ml tube. The scales were either used fresh or preserved in sufficient amount of 90% ethanol.

DNA extraction—DNA was extracted, both from fresh scales as well as from more than a month ethanol preserved scales by using the following method. The protocol was followed according to Wasko *et al*.[7] with modifications. Approximately, 50 mg of scales were taken from each species and dried on a filter paper. The scales were then cut into small pieces and placed in a 2 ml-Eppendorf tube containing 940 µl lysis buffer (200 m*M* Tris-HCl, *p*H 8.0; 100 m*M* EDTA, *p*H 8.0; 250 m*M* NaCl), 30 µl Proteinase K (10 mg/ml) and 30 µl 20% SDS. The contents in the tubes were incubated at 48°C for 45-50 min in a water bath. The appropriateness of the incubation temperature was studied in a separate experiment, by incubating scales sample from *C. punctatus* at different temperatures viz. 42°, 44°, 46°, 50°, 52° and 54°C. After incubation, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the tube containing lysed scale cells. The contents were then mixed properly by gently inverting the tube for 10 min to precipitate the proteins and other part of the nucleic acids. The tube was then rotated for 10 min at 9,200 *g*. The top aqueous layer was transferred to a new 1.5 ml-Eppendorf tube, leaving interphase and lower phase. The DNA was then precipitated by adding equal volume of isopropanol and 0.2 volumes of 10 *M* ammonium acetate and inverting the tubes gently several times. The precipitated DNA was then pelleted by centrifugation at 13,200 *g* for 10 min. The supernatant was removed by pouring out gently, taking care to avoid loss of DNA pellet. The pellet was then washed briefly in 500 µl chilled 70% ethanol, air-dried and resuspended in 200 µl sterile water/TE buffer (Qualigen).

After ensuring complete solubility of DNA, the purity factor (A_{260}/A_{280} nm) was measured spectrophotometrically and its integrity was checked by loading 10 µl DNA preparation (2 µl extracted DNA, 2 µl dye and 6 µl sterile water) on 0.7% agarose gel and stained with ethidium bromide. The quantity and quality of the DNA were compared by loading 0.2 µl Lambda *Hind III* DNA standard marker (provided by M/s Bangalore Genei, India, stock conc. 500 ng/µl) and DNA isolated from blood of *Channa punctatus* using Sigma kit (cat # NA 2000) in the same gel. The DNA quantifications were done using Syngene Gene Genius gel documentation system. The extracted DNA samples were then stored at −20°C till their further use.

Restriction digestion—For checking the quality, the DNA samples were digested with *HaeIII* (10 U/ µl) restriction enzyme (provided by M/s Bangalore Genei, India). The reaction volume was set up for 10 µl, which contained sterile water, 2 µl 10X RE buffer (provided with the enzyme), 200 ng template DNA and added 2 U (0.2 µl) *HaeIII* restriction enzyme. The reaction mixture was incubated at 37°C for 60 min for restriction digestion followed by 15 min incubation at 70°C to stop the reaction. The restriction digested products were tested on 1.2% agarose gel.

PCR amplification—Polymerase chain reactions (PCRs) for amplification of genomic DNA extracted from fish scales of different species were carried out with three random decamer primers (OPAS 12, OPAS 13 and OPAS 14) in a 25 µl reaction volume. The sequences of these OPAS primers were as follows:

OPAS 12: 5'- TGACCAGGCA-3' (Mol. Wt. 3037)
OPAS 13: 5'-CACGGACCGA-3' (Mol.Wt. 3022)
OPAS 14: 5'- TCGCAGCGTT-3' (Mol.Wt. 3019)

The PCR reaction contained 20 ng genomic DNA, 2.5 µl of 10X PCR buffer (Fermentas), DDW, 2.0 mM $MgCl_2$ (Fermentas), 0.5 µl of 10 mM dNTPs mix (Fermentas), 5-6 pmol of each OPAS 12-14 decamer random primers (Operon, QIAGEN) and 0.625 U Taq DNA polymerase (Fermentas). The amplification was carried out in the Eppendorf Master Gradient Thermal Cycler. The PCR conditions were initial denaturation at 94°C for 4 min followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 1 min followed by final extension at 72°C for 10 min. The 8 µl amplified product was analyzed on 2.0 % agarose gel.

Statistical analysis— The data on DNA yield in different species was analyzed by using one-way ANOVA with MS-EXCEL software.

Results and Discussion

Genomic DNA of high quality and quantity is required to analyze genetic diversity by using molecular markers. It becomes one of the major concerns for DNA based techniques, especially when a large number of samples need to be processed. A number of simplified protocols for DNA extraction have been reported, such as salting out procedure[16], microwave based extraction[17], silica-guanidinium

thiocyanate method[18,19], CTAB procedure[20], boiling method[21], and Chelax-based extraction[22], but majority of these methods were developed for plant samples that contain cell wall of polysaccharides and poly phenol compounds.

In fish, most of the DNA isolation is done from blood. But when the small and rare/endangered species from remote/ rare location are encountered, it is not desirable to extract blood. As per IUCN red list[23], 56% of the 252 endemic freshwater Mediterranean fish are threatened with extinction. As per CAMP Workshop[24], in India, 45 fish species are categorized as critically endangered (CR), 1 extinct in the wild (EW), 91 are endangered (EN), 81 are vulnerable (Vu), 66 are lower risk near threatened (LR-nt), 16 are Lower risk least concern (LR-lc) and 26 are data deficient (DD). In such situations, collection of scales in butter paper/plastic bag can serve the purpose of DNA extraction. Fish fin and scales are reliable non-invasive source of DNA and have been used earlier to isolate DNA from some fish species[14, 25-31]. Since the collection of few scales does not harm the fish, DNA based studies on genetic diversity, mating systems and parentage determination can easily be done with minimum disturbance especially in endangered and ornamental species. In the present communication, a very rapid (requires around 2 hr), simple, reproducible and less expensive method for DNA extraction from fresh and ethanol preserved fish scales has been described. The present protocol is faster even than the ultra-fast method of DNA extraction proposed by Cambareri and Kinsey[32]. The amount of scale required is very small, even single scale could provide sufficient amount of DNA. Using present technique, DNA isolation, RE digestion and PCR amplification can be performed on the same day. Many papers have described methods of DNA extraction from fish scales[7,14,29-31,33-34]. However, the method described by Nelson *et al*.[34] was too complicated for DNA isolation from a large number of individuals while the quality of DNA isolated by Yue and Orban[31] using protocol of Eutoup *et al*.[14] was inappropriate for PCR amplification using primers. Some of the protocols of DNA isolation from scales incubated cells at 37°C overnight[7] which increased the duration of DNA isolation. However, the short duration technique for scale, proposed by Yue and Orban[31], used Chelex 100 and Silica for extraction which increases the cost of DNA isolation, and the preparation of silica takes 10-12 hours time which increase the duration of the protocol.

The quantities of DNA isolated from fish scales from different species have been presented in Table 1. The quantity of DNA ranged from 25-100 ng/µl, which is generally sufficient for PCR amplification and molecular genetic approaches. The concentration of isolated DNA in the present study was quite high as compared to Yue and Orban[31] who isolated 10-105 ng of DNA/mg of scale and at par to Wasko *et al*[7]. The variation in DNA concentration was reported in the present study, which may be due to the fact that different amounts of tissue present on the scales[29] or due to differences in the quantity of gDNA in the sample[5]. Moreover, the variation in DNA concentration may also be due to considerable variation in the size of scale and amount of dermis and epidermis cells on the outside and not in the collagen of the matrix of the scales[29]. The level of degradation of DNA quality is also related to age of the samples.

The isolated DNA fragment was more than 23 kb in size, since it has resemblance with upper most bands of λ Hind III DNA marker of 23,130 base pair in the gel (Fig 1.). The isolated DNA had no sign of degradation and the spectrophotometer comparison of absorbance at A_{260}/A_{280} nm provided a purity factor of 1.6–2.0, indicating its good quality. The DNA extracted with this method was very stable and could be stored at 4°C temperature for months together without any adverse effect on its concentration and its use for PCR. Further, the present protocol was found suitable for a heterogeneous group of fish species. Dried scales are a valuable resource for population genetic studies[33,35]. This technique has been tried for extraction of DNA from dried scales, however, the DNA recovery in dried scales was less than the fresh and ethanol preserved scales (data not shown).

Table 1— Yield of DNA isolated from scales of different fish species

S.N.	Fish species	DNA Yield (ng/mg of scale)
1.	*Catla catla*	143.2±11.06[a]
2.	*Labeo rohita*	336.8±21.85[b]
3.	*Labeo calbasu*	216.0±11.80[c]
4.	*Labeo bata*	266.4±17.23[d]
5	*Cirrhinus mrigala*	113.6±6.52[e]
6.	*Channa punctatus*	260.8±15.25[d]
7.	*Cyprinus carpio*	403.2±12.61[f]
8.	DNA isolated from blood of *Channa punctatus* using Sigma kit	263.2±3.88[d]

Values with different alphabets differ significantly (P<0.05).

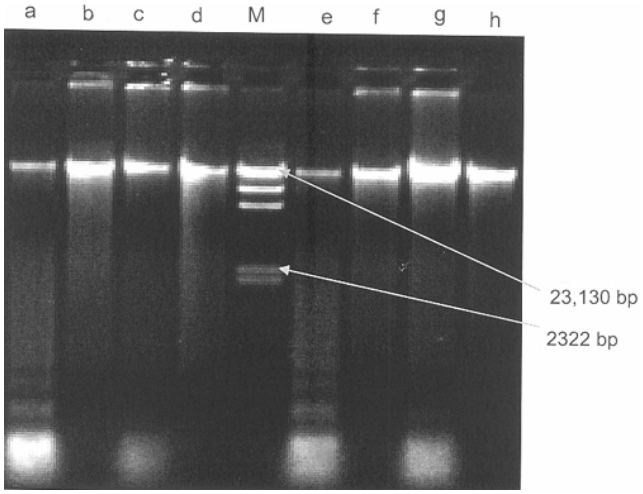

Fig. 1— Agarose (0.7 %) gel electrophoretic profile of DNA samples obtained from fish scales. (a) *C. catla*, (b) *L. rohita*, (c) *L. calbasu,* (d) *L. bata,* (M) *Lambda Hind III* DNA marker, (e) *C. mrigala* (f) *C. punctatus* (g) *C. carpio* (h) *C. punctatus* DNA isolated from blood using Sigma kit (Cat # NA2000).

Wasko *et al*.[7] found an improvement in the DNA isolation with pretreatment by RNAse, which allowed obtaining DNA samples with lower quantities of RNA but they observed that it could interfere with accurate DNA quantification and further amplification procedures. In the present protocol, RNAse pre-treatment was not given. As regards the appropriateness of the incubation temperature, the purity factor of DNA samples ranged from 0.77 (42°C) to 1.74 (48°C) with higher OD values at 48°C as compared to lower and higher temperatures (Fig 2). Thus, the incubation temperature at 48°C was found to be appropriate for quick digestion of scales, without compromising for DNA quality and quantity. Wasko *et al*.[7] have also reported earlier that the higher incubation temperatures (50°C or more) were inefficient and temperatures lower than 42°C resulted in a partially digested tissue. The appropriate concentration of Proteinase K was required to obtain high-quality DNA. In the present study 0.3 mg/ml final concentration of Proteinase K was used in the experiment and the tissue protein was digested in 45-50 min, whereas, Wasko *et al*.[7], in their experiments used a final concentration of 0.075 mg/ml of Proteinase K which took 10 hr-incubation to digest the tissues. However, high amount of protein and other materials present in the scales[31] required more proteinase K for quick digestion of protein. After scale digestion, a phenol-chloroform-isoamyl alcohol purification step was utilized, as suggested by Taggart *et al*.[26] and Sambrook and Russell[36]. The use of phenol-chloroform-isoamyl alcohol was found to be essential in 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, removing protein residues[7].

Isolated DNA was also digested with restriction enzyme *HaeIII*, a frequent cutter, as shown in Fig. 3.

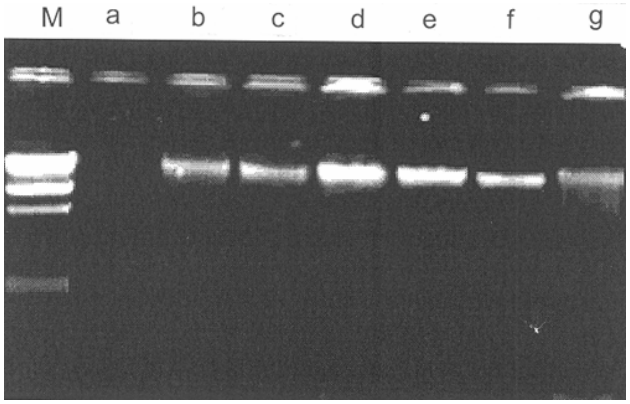

Fig. 2— Agarose (0.7 %) gel electrophoretic profile of DNA samples obtained from *C. punctatus* scales at different incubation temperature. M is *Lambda Hind III* DNA marker, (a) at 42°C, (b) at 44°C, (c) at 46°C, (d) at 48°C, (e) at 50°C, (f) at 52°C, (g) at 54°C.

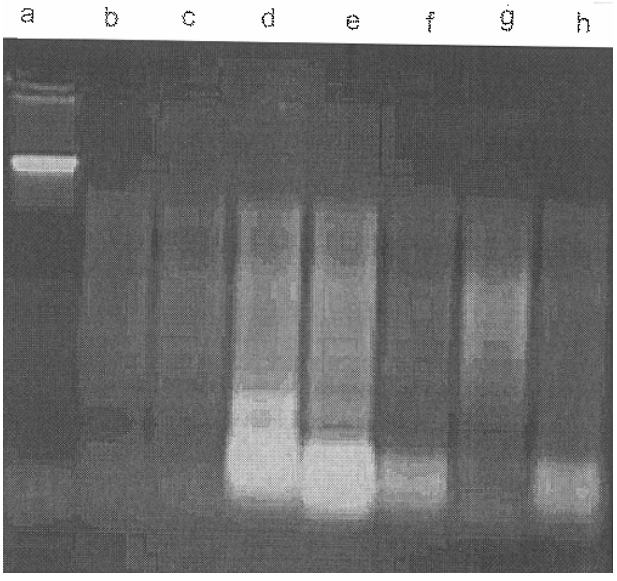

Fig. 3— Agarose (1.2 %) gel electrophoretic profile of *HaeIII* restriction enzyme digested DNA of (a) control (200 ng of *L. rohita* DNA without RE treatment) (b) *C. catla*, (c) *L. rohita*, (d) *L. calbasu,* (e) *L. bata* (f) *C. mrigala* (g) *C. punctatus* (h) *C. carpio*.

HaeIII, the most commonly used enzyme in forensic science, cuts the DNA everywhere the bases are arranged in the sequence GGCC. *HaeIII* cuts human DNA into approximately 12 million different restriction fragments ranging in size from a few hundred to 10,000 or more base pairs in length (http://www.genelex.com/paternitytesting/paternitybook3. html). The template DNA was completely digested with the enzyme in all the scale samples studied, which showed the isolated DNA was of high quality. The purity of DNA samples, confirmed by RE digestion, shows that it could also be used for cloning experiment and DNA profiling.

The protocol developed required no additional chemical or equipment and extraction can be done even without low temperature high speed centrifuge. Generally, a number of protocols require boiling of tissues during extraction[31,37], but here boiling of scale is not required. This procedure also does not require any pretreatment of scales to remove components such as protein and other cellular debris from the scale as required in other procedures[18,19,31]. It is important that the isolated DNA should be dissolved in sterile water which contains no PCR inhibitor such as EDTA in TE buffer. Most of the isolated DNA extracted by this technique showed no sign of degradation.

Modern studies of population genetics increasingly rely on DNA markers amplified by polymerase chain reaction for detecting genetic variations within and among populations. DNA samples isolated by the present method from scales of seven fish species were successfully used in amplification of sequences using three random primers (Operon, QIAGEN), shown in Fig. 4. Reproducible fragment of different lengths were amplified cleanly in almost all the species with the three primers. Moreover, despite inconsistencies in the yields of the isolated genomic DNA, PCR amplification indicated that the quality of the extracted DNA is good enough to allow PCR amplification of desired length fragment without further purification. Further, the rate of amplification success demonstrated that preserved scales are particularly useful for temporal population studies that depend upon large sample sizes for analysis[30].

Thus, the results obtained with restriction digestion and PCR amplification indicated suitability of this DNA extraction technique for its use in field oriented population and conservation genetic studies involving a wide range of fish species. The procedure would also be useful for rapid genetic screening of fishes.

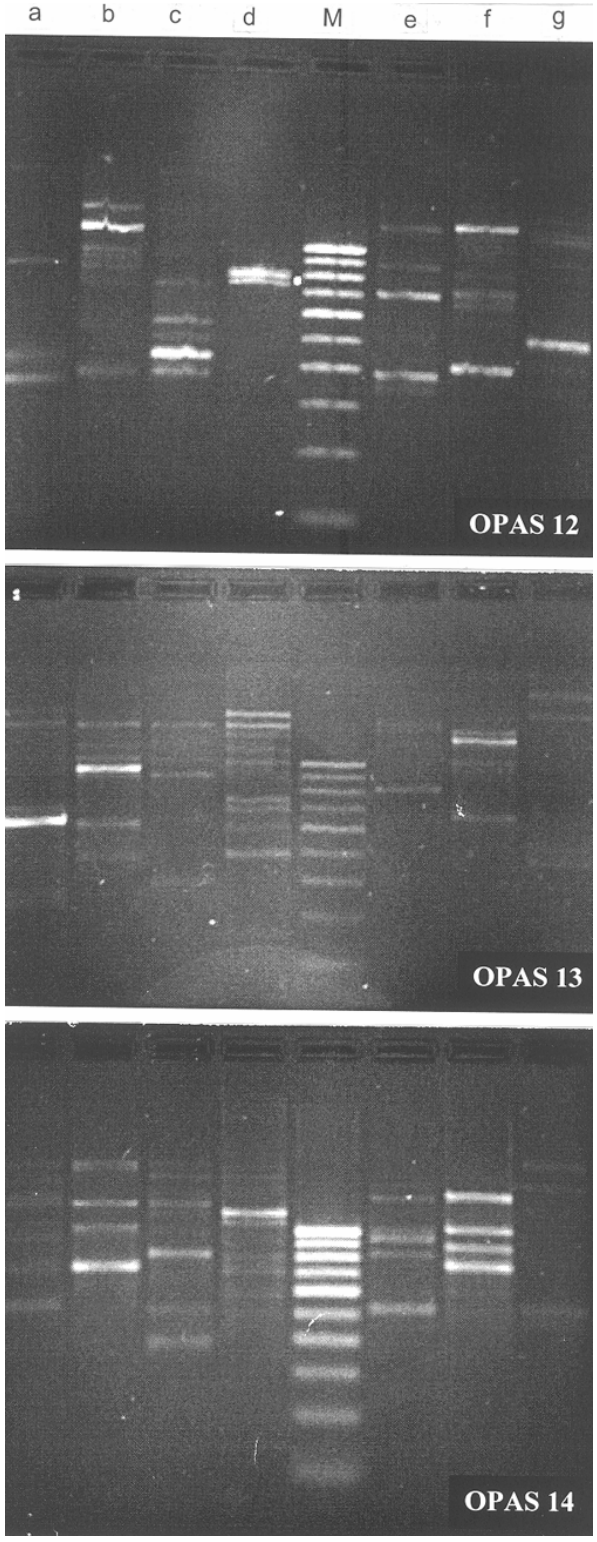

Fig. 4— Agarose (2 %) gel electrophoretic profile of PCR products with (i) OPAS 12, (ii) OPAS 13 and (iii) OPAS 14 decamer random primer using template DNA from scale of (a) *C.Catla*, (b) *L.rohita*, (c) *L.calbasu*, (d) *L.bata*, (M) 100bp molecular weight marker, (e) *C. mirgala* (f) *C. punctatus*, (g) *C. carpio*.

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