Non-destructive genetic sampling in fish. An improved method for DNA extraction from fish fins and scales

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DNA-based studies have been one of the major interests in conservation biology of endangered species and in population genetics. As species and population genetic assessment requires a source of biological material, the sampling strategy can be overcome by non-destructive procedures for DNA isolation. An improved method for obtaining DNA from fish fins and scales with the use of an extraction buffer containing urea and further DNA purification with phenol-chloroform is described. The methodology combines the benefits of a non-destructive DNA sampling and its high efficiency. In addition, comparisons with other methodologies for isolating DNA from fish demonstrated that the present procedure also becomes a very attractive alternative to obtain large amounts of high-quality DNA for use in different molecular analyses. The DNA samples, isolated from different fish species, have been successfully used on random amplified polymorphic DNA (RAPD) experiments, as well as on amplification of specific ribosomal and mitochondrial DNA sequences. The present DNA extraction procedure represents an alternative for population approaches and genetic studies on rare or endangered taxa.

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In recent years, a great number of vertebrate species have been designated at risk and DNA-based studies on threatened or endangered species have been one of the major interests in gathering information on the diversity, conservation biology and population analyses (O'BRIEN 1994; AVISE 1996; SNOW and PARKER 1998).

Species and population genetic assessment requires a reliable source of biological material. Among different procedures to obtain DNA, non-invasive sampling seems to be very attractive, since it allows genetic analysis of several individuals without having to catch, handle or even observe them (HÖSS et al. 1992; TABERLET and BOUVET 1992; MORIN et al. 1993). The source of DNA can be hairs, feces, urine, shed feathers, snake skin, sloughed whale skin, eggshells and even skulls in owl pellets. However, this strategy usually results in a low quantity and poor quality DNA and also does not provide individual identification, which limits its potential. In contrast, non-destructive sampling, that implies the use of tissues (blood, skin, scales, muscle biopsy) without critical damages to the animals, can exploit the full potential of DNA analyses including individual and sex determination, relatedness estimates, pedigree reconstruction, determination of the effective population size and the level of genetic polymorphism within and between populations (TABERLET et al. 1999).

Liver and muscle tissue represent the most common used sources of fish DNA. However, non-destructively DNA isolation is desirable, especially when large populations or threatened species have to be analyzed. Although DNA can be successfully obtained from muscle (HILSDORF et al. 1999) or blood samples of fish (CUMMINGS and THORGAARD 1994; ESTOUP et al. 1996; MARTINEZ et al. 1998) without the sacrifice of the animals, adult individuals of some species are often too small to be effectively sampled by muscle biopsy or drawing blood.

The sampling strategy can be overcome by non-destructive procedures for DNA isolation from fish fins or scales. In the present paper, an improved DNA extraction method is described, using a lysis buffer containing a high urea concentration and further DNA purification with phenol-chloroform. The DNA-sampling method provides high-quality and high-quantity DNA useful as template in polymerase chain reaction (PCR) experiments and allows the maintenance of the individuals without disturbance in their health or behavior.

MATERIAL AND METHODS

DNA was achieved from fish caudal or anal fins and from fish scales by changing standard protocols previously described for tissue preservation and DNA extraction from muscle or liver (ASAHIDA et al. 1996;

SAMBROOK and RUSSELL 2001). Total DNA was obtained from individuals belonging to different fish species (*Brycon cephalus*, *Brycon orbignyanus*, *Leporinus elongatus*, *Astyanax scabripinnis*, *Hypostomus* sp. and *Geophagus brasiliensis*). Approximately 100–300 mg of fins (1 to 2 cm^2) or scales were initially stored in 95 % ethanol–100 μM EDTA pH 8.0 (DESSAUER et al. 1996). Freshly air-dried fins and scales of *Geophagus brasiliensis* were also processed for comparison. Ethanol-fixed tissues were then dried on a filter paper, cut into small pieces and placed in 4 ml of a TNES-digestion buffer (10 mM Tris-HCl pH 8.0; 125 mM NaCl; 10 mM EDTA pH 8.0; 0.5 %SDS; 4M urea) inside a 15 ml tube. Thirty μl of RNase (10 mg/ml) was added to the tubes and the tissues were incubated at 42°C for 1 h. After this period, 30 μl of Proteinase K (10 mg/ml) was added and the tissues were maintained at 42°C for at least 10 h. The DNA was then isolated by adding 4 ml of phenol:chloroform:isoamyl alcohol (25:24:1) to the tubes. After inverting the tubes for 15 min, they were rotated for 15 min at 10.000 rpm. The top aqueous layer was removed to a new tube and the DNA was precipitated in 1M NaCl and two volumes of cooled absolute ethanol, inverting the tubes several times. The DNA was recovered by 10.000 rpm centrifugation. Further, the DNA pellet was washed briefly in 70 % ethanol, air dried and resuspended in an appropriate volume of TE buffer (10 mM Tris HCl pH 8.0; 1 mM EDTA pH 8.0). After completely soluble, the DNA was stored at −20°C.

The DNA integrity was checked on 1 % agarose gel stained with ethidium bromide. A spectrophotometer (GeneQuant II – Amersham Pharmacia Biotech) was used to evaluate the total amount of obtained DNA and the computer program Kodak Digital Science 1D was used to quantify the DNA, by direct comparisons with a standard marker (Low DNA Mass Ladder – Gibco.Brl). DNA samples were used as templates on PCR with specific primers for amplification of random polymorphic DNA (RAPD Analysis Beads – Amersham Pharmacia Biotech), following the manufacturer instructions. In addition, total DNA was used to amplify a mitochondrial DNA region (D-loop), as described in BERNATCHEZ et al. (1992), and a ribosomal DNA (5S rDNA) (MARTINS and GALETTI 1999). All amplification products were visualized by 1.4 % agarose gel electrophoresis and compared with a standard marker (1Kb Plus Ladder– Gibco. Brl).

RESULTS AND DISCUSSION

The isolation of high-quality DNA for genetic analysis has become one of the major concerns for DNA-based techniques, especially when a large number of samples must be processed. Fish fins and scales are a reliable non-destructive source of DNA and these materials have been used to isolate DNA from some species (SHIOZAWA et al. 1992; TAGGART et al. 1992; WHITMORE et al. 1992; ZHANG et al. 1994; ESTOUP et al. 1996; NIELSEN et al. 1999; ADCOCK et al. 2000). However, there are some difficulties due to the consistency and small size of these tissues which can led to a low amount and poor quality of total recovered DNA.

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, specially 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 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 (Fig. 1) 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

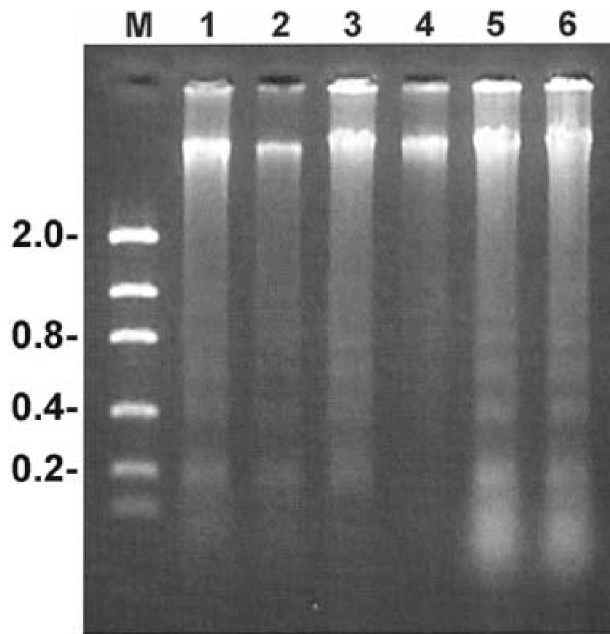

Fig. 1. 1.4 % agarose gel of DNA samples obtained from fish fin and scales. Lanes 1–3 and 5–6: total DNA isolated from fish fin clips. (1) *Brycon orbignyanus*, (2, 5, 6) *Brycon cephalus*, (3) *Leporinus elongatus*. Lane 4: total DNA isolated from scales of *Geophagus brasiliensis*. Lanes 1–4 correspond to samples treated with RNase. Lanes 5–6 correspond to samples with no RNase treatment. M, molecular weight marker (Kb).

were not necessary. Single and double washes gave same results, avoiding protein residues.

Some DNA samples can be visualized on Fig. 1. The obtained results demonstrate the success of the applied technique. Most of the isolated DNA had no sign of degradation and the spectrophotometer comparison of absorbance at 260–280 nm provided a DNA/RNA relationship of 1.6–2.1, indicating a good DNA quality. The DNA concentration ranged from 25–500 ng/μl, with an average concentration of 200 ng/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 six fish species, similarity in fin (or scales) anatomy suggests that the technique will work on samples of different taxa.

Comparisons of DNA samples isolated from different tissues of *Geophagus brasiliensis* also demonstrate the feasibility of the methodology. 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. The results, summarized on Table 1, demonstrate that fins and scales, in special fin clips, represent a DNA source as suitable as other tissues – the DNA amount isolated from 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 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 *G. brasiliensis*, some differences were observed in relation to the material storage (Table 1). 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. How-

Table 1. *Comparison among different tissues of a specimen of Geophagus brasiliensis (weight: 6.18 g; size: 7.5 cm) and total amount of isolated DNA. Tissues were weighted before the storage.*

Sampled tissue	Weight (g)	Storage method	Cell lysis buffer	Total DNA amount (μg)
fin	0.10	ethanol/EDTA	TNES-urea	83.26
fin	0.10	air-dry	TNES-urea	54.08
fin	0.10	ethanol/EDTA	TNES	no result
scales	0.10	ethanol/EDTA	TNES-urea	39.62
scales	0.10	air-dry	TNES-urea	29.18
scales	0.10	ethanol/EDTA	TNES	no result
liver	0.10	ethanol/EDTA	TNES	269.12
liver	0.10	ethanol/EDTA	TNES-urea	88.34
muscle	0.10	ethanol/EDTA	TNES	109.82
muscle	0.10	ethanol/EDTA	TNES-urea	35.64

ever, 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 report 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, which is extremely important in polymerase chain reaction-based techniques, since a low amount of DNA or a degraded DNA and the presence of PCR inhibitors are the main factors to difficult the amplification of long sequences (KWOK 1990). DNA samples obtained from fins were successfully used as templates to amplify random polymorphic DNA (RAPD) and specific nuclear and mitochondrial DNA sequences, as the 5S rDNA and the mitochondrial D-loop region, respectively (Fig. 2). The total amount of isolated DNA (25–500 μg) is also sufficient for several other molecular procedures that often demand more DNA than the picogram range. It can be considered that a genetic stock of several fish species could be easily achieved by using the described methodology.

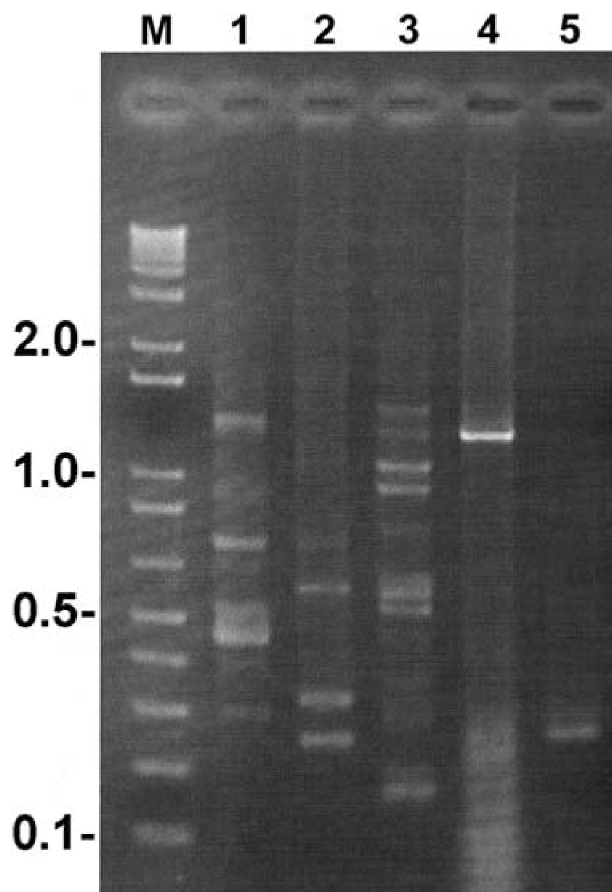

Fig. 2. 1.4 % agarose gel of PCR products using total DNA of *Brycon cephalus*. Lanes (1) RAPD/primer 1, (2) RAPD/primer 2, (3) RAPD/primer 4, (4) D-loop mtDNA, (5) 5S rDNA. M, molecular weight marker (Kb).

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