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ARTICLE

Fixed cytogenetic cells suspension: an alternative for obtaining DNA of birds

Denise Monnerat Nogueira[1,3] and Andréa de Andrade Rangel de Freitas[2]

[1] Departamento de Genética, Instituto de Biologia Universidade Federal Rural do Rio de Janeiro (UFRRJ), Rodovia BR 465 - Km 07, CEP 23890-000, Seropédica, RJ, Brazil.
E-mail: denisemn@ufrrj.br
[2] Graduação em Medicina Veterinária, Universidade Federal Fluminense (UFF), Rua Vital Brazil Filho 64, Niterói, CEP 24230-340, RJ, Brazil.
[3] Author for correspondence.

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ABSTRACT: Fixed cytogenetic cells suspension: an alternative for obtaining DNA of birds. Birds are especially sensitive to biological sampling and the stress related to this procedure can influence important clinical parameters and even threaten the life of the animal. In genetic studies preference has been given to less invasive sampling to obtain DNA. Good quality and quantity of genomic DNA are crucial steps for successful amplification by the polymerase chain reaction (PCR) and, therefore, for research and diagnostic purposes. Here, we extracted DNA from small volumes of cell suspension fixed and frozen for up to nine years, previously used for cytogenetic analysis of different Psittaciformes species. We compared two protocols of DNA extraction (Cell lysis and Phenol/chloroform). Only the phenol/chloroform method provided adequate DNA for PCR amplification, providing suitable DNA for molecular sexing, suggesting that it can also be used for other genetic analyses and avoiding recapture to collect tissue samples.

KEY-WORDS: CHD gene; Molecular sexing; Psittacidae, Psittaciformes, feather pulp

INTRODUCTION

The most recent survey published by the Brazilian Ornithological Records Committee in 2011 (CBRO 2011) has shown that Brazil is home to 1,832 bird species, representing one of the richest avifauna of the world. Both cytogenetic analysis and molecular biology have contributed to the identification of cryptic species, comprehension of the evolutionary mechanisms among different groups, establishment of the taxonomic relations among different taxa, sex identification for reproduction of birds in captivity, and studies about sex ratios in wild populations.

The description of the short-term culture technique of feather pulp by Sandness (1954) enabled the study of rare wild living birds or birds from zoos representing the less invasive sampling which provides the best results for the study of avian karyotypes (Christidis 1989). Such cells, after properly cultured, can persist in fixative at -20° C for a long time, resulting in good quality metaphases when defrosted (Christidis 1989, Coleman & Tsongalis 1997).

There are several non-destructive and non-invasive methods of tissue sampling that can also be used for molecular genetic analysis of birds, such as: blood samples (Haig *et al*. 1997, Paterson & Snyder 1999, Bouzat 2001), feather pulp (Marsden & May 1984, Viala *et al*. 2006), fresh adult feathers (Bello *et al*. 2001, Viala *et al*. 2006), feces (Robertson *et al*. 1999), urine (Nota & Takenaka 1999), and egg shells (Chilton & Sorenson 2007).

When working with birds, it becomes a priority to adopt less invasive sampling procedures, reducing the amount of physical restraint and the associated dangerousness generated by stress. It is also recommended to optimize the use of remnant tissues collected for further analyses, especially in the case of endangered species (Gaunt & Oring 1999).

In human beings with Down syndrome, the DNA extracted from bone marrow cells and lymphocytes of peripheral blood cultures, kept frozen for four years after cytogenetic analysis, allowed the execution of other important genetic evaluations on those patients (Amorim *et al*. 2007).

Our objective was to verify if the cells obtained for cytogenetic analysis from young feather pulps of six Psittaciformes species and stored in fixative solution at -20° C for up to nine years could be used to extract good quality DNA for molecular studies. We also compared the phenol/chlorophorm and cell lysis methods for obtaining DNA to determine the most suitable technique for reusing the samples in other research projects, avoiding recaptures.

MATERIAL AND METHODS

For DNA extraction, we used a short-term culture of young feather pulps from eight individuals of six Psittaciformes species, following Sandness (1954) with some modifications. Samples were collected at RIOZOO Foundation, Rio de Janeiro, Brazil, and cultured for cytogenetic analyses between 1999 and 2006. Following the analysis, the remnant tissue was stored at -20 ºC in fixative solution (methanol: acetic acid 3:1) (Coleman & Tsongalis 1997) for up to nine years. The species and their respective years of cell culture and fixation were: *Pionites leucogaster* (Kuhl 1820), 1999; *Diopsittaca nobilis* (Linnaeus 1758), 1999; *Anodorhynchus hyacinthinus* (Latham 1790), 1999a; *Anodorhynchus hyacinthinus* (Latham 1790), 1999b; *Ara ararauna* (Linnaeus 1758), 2001; *Ara chloroptera* (Gray 1859), 2001; *Amazona aestiva* (Linnaeus 1758), 2003; and *Ara ararauna* (Linnaeus 1758), 2006. After defrosting, the samples were centrifuged at 1000 rpm for 10 min. The supernatant was discarded and about 50 to 100 µg of the cell pellet was transferred to a 1.5 mL plastic tube which was held open and upside down for 4 h over a paper towel to ensure that the fixative evaporated completely. Two DNA extraction protocols were tested: phenol/chlorophorm (Sambrook *et al*. 1989) and cell lysis (Khatib & Gruenbaum 1996). At the end of the extraction, 100 µL of ultra-pure water were added to each sample. The suspension was placed in a boiling water bath at 37º C for 2 h to attain the complete DNA dissolution. DNA quantification of phenol/chlorophorm extractions were performed using a fluorometer. The DNA obtained from both methods was submitted to a polymerase chain reaction (PCR) using the primers P2 (5′-TCTGCATCGCTAAATCCTTT-3') and P8 (5′- CTCCCAAGGATGAGRAAYTG-3′) (Griffiths *et al*. 1998). These sequences are currently used for the molecular sexing of birds, and in the present study allow the comparison of the results with those obtained by cytogenetic analysis. Each 10 µL reaction contained 2 µl of DNA (5 ng/µL), 1 µL of each primer (10 µM), 5 µL of Green Master Mix (Promega®), and 1 µL of nuclease-free water (Promega®). The reaction profile used was 94 °C for 2 min, then 40 cycles of 94 °C for 15 s, 50 °C for 20 s, 72 °C for 25 s, followed by 72 °C for 1 min. Samples of a male *Agapornis* spp. and a female *Nymphicus hollandicus* were used as a positive control. We also used a negative control for all reactions containing water instead of DNA to check for contaminants. PCR products were analyzed by eletrophoresis using 2% agarose gel stained with 5% ethidium bromide solution (10 mg/ml) on a transiluminator with UV light.

RESULTS

We studied eight individuals of Psittaciformes. In all samples analyzed, only the phenol/chlorophorm method provided good quantity and quality of DNA to PCR amplification. Concentration varied strongly between extracts, from 13 to 224 ng/µL (Table 1), with a mean value of 107.5 ng/µL.

Six out of eight samples analyzed showed satisfactory PCR amplification. Samples from *Pionites leucogaster* 1999 and *Ara ararauna* 2001 did not provide constant results, presenting both evident and weak amplification.

The sex of all individuals whose samples showed PCR amplification (Figure 1, Table 1) was compatible with the previous cytogenetic results.

TABLE 1. Species studied and year of cytogenetic cell culture with the respective DNA concentration extracted using the phenol/chloroform method, quantified with a fluorometer. Also shown, the sex of the individuals obtained by the Polymerase Chain Reaction (PCR) with P2 and P8 primers (Griffiths *et al*., 1998).

Species (year of cell culture)	DNA quantification (ng/µL)	Molecular sexing
Ara ararauna (2001)	170	female
Diopsittaca nobilis (1999)	108	female
Amazona aestiva (2003)	224	male
Anodorhynchus hyacinthinus (1999 a)	56	female
Anodorhynchus hyacinthinus (1999 b)	149	female
Pionites leucogaster (1999)	13	female
Ara ararauna (2006)	39	male
Ara chloroptera (2001)	101	female

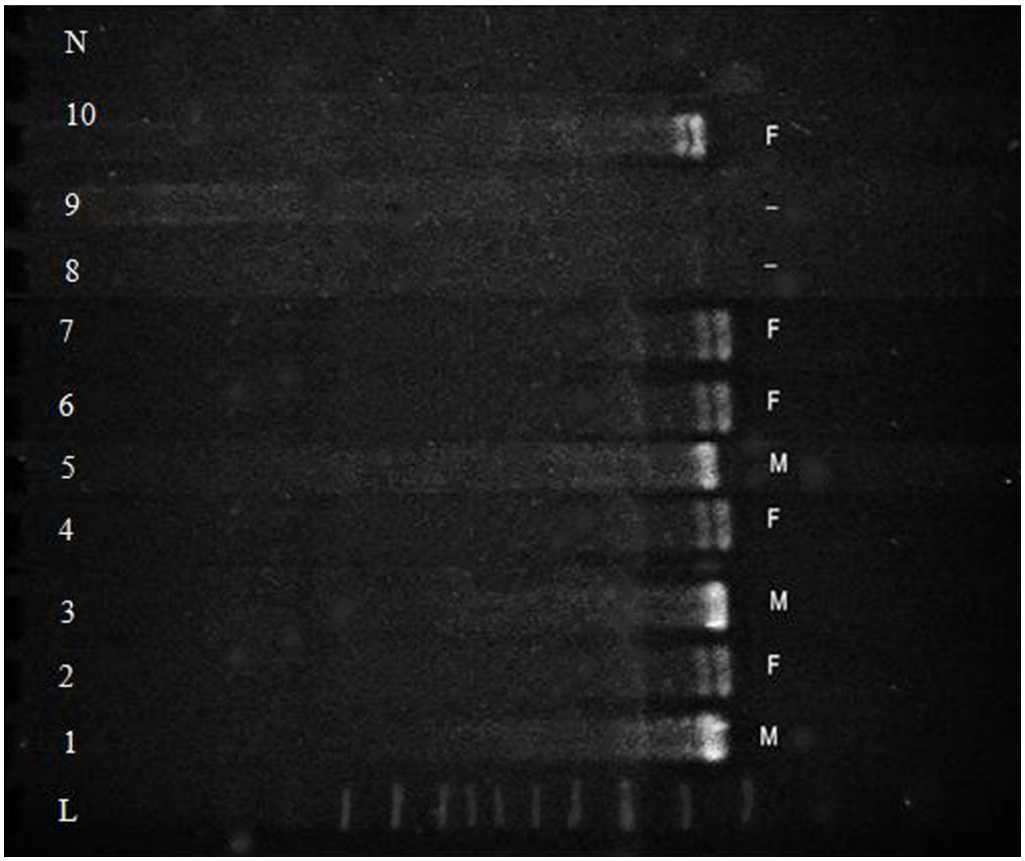

FIGURE 1. PCR products analyzed in 2% agarose gel amplified with P2 and P8 (Griffiths *et al*., 1998) primers from DNA extracted from cells of young feather pulps stored in fixative. The year of cell culture preparation is in parentheses. L - 100-bp DNA ladder; 1 - *Agapornis* spp. (male control); 2 - *Nymphicus hollandicus* (female control); 3 - *Ara ararauna* (2006); 4 - *Diopsittaca nobilis* (1999); 5 - *Amazona aestiva* (2003); 6 - *Anodorhynchus hyacinthinus* (1999a); 7 - *Anodorhynchus hyacinthinus* (1999b); 8 - *Pionites leucogaster* (1999); 9 - *Ara ararauna* (2001); 10 - *Ara chloroptera* (2001); N - Negative control, M - Male, F - Female, – Not amplified.

DISCUSSION

Several samples did not allow for conclusive results in some trials, which may be related to the small DNA concentration, such as in the case of *Pionites leucogaster* 1999 and the poor DNA quality of *Ara ararauna* 2001. The cell lysis technique, although faster and cheaper, did not prove to be a good alternative for DNA extraction from fibroblasts frozen in fixative solution, yielding higher impurity levels that can interfere with primer annealing (Roux 1995). The phenol/chlorophorm extraction is longer, more expensive, and more toxic (Fernandes *et al*. 2004). However, due to the use of proteinase K and successive washes and centrifugations, it yields DNA of excellent quality that can be used for several molecular analyses (Sambrook *et al*. 1989). In this report, we describe the DNA extraction from fibroblasts fixed for cytogenetic analyses and stored at -20 ºC for up to nine years. This storage period is longer than the one previously described for DNA extraction from lymphocytes of peripheral blood and bone marrow cells of human beings with Down Syndrome, whose samples had been kept frozen for four years (Amorim *et al*. 2007). Although blood, and recently, feathers, are the choice tissues to access genomic DNA in birds, the young feather cells fixed and frozen for cytogenetic analyses constitute an important alternative for molecular studies of birds, including endangered species, where such sample collection usually represents a restrictive component for scientific research.

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