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Avian DNA extraction: An economical and efficient alternative for Farmer-fixed samples

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Abstract. Cytogenetics laboratories often accumulate vast collections of cells fixed in Farmer's solution (3 parts methanol to 1-part glacial acetic acid), stored long-term in freezers. While many of these samples are unsuitable for conventional cytogenetic analyses, they hold potential for molecular applications, especially as ethical restrictions around the collection of biological material through invasive procedures (e.g., biopsies, tissue excision, bone marrow aspiration) become increasingly stringent. However, extracting DNA from these cells presents significant challenges, such as structural fragility induced by the fixative and potential genetic material degradation, which can compromise subsequent analyses, including PCR. This study developed and standardized a protocol for extracting DNA from Farmer-fixed avian cells using accessible and low-cost reagents. The method proved economical and efficient, even for decades-old samples, recovering DNA suitable for cytogenomic and molecular studies. This approach significantly advances sustainable practices in science by utilizing long-stored samples that might otherwise be discarded, this approach provides a cost-effective strategy that reduces the need for new collections and aligns with current ethical guidelines in molecular genetics research. Compared to commercial kits, the protocol demonstrated economic viability while expanding the use of biological collections in genetic research and evolutionary studies.

Keywords: DNA extraction, cell preservation, cytogenomics, sustainable methods, 3Rs principles.

INTRODUCTION

Biological sample fixation is widely used to preserve cells and tissues, ensuring structural integrity essential for subsequent analyses (Tan and Yiap, 2009). In the field of cytogenomics, understood here as the integration of cytogenetic and genomic approaches, the primary fixation method employs Farmer's solu-

tion, a 3:1 mixture of methanol and acetic acid, which dehydrates and stabilizes cells (Coleman and Tsongalis, 1997; Amorim et al., 2007). While effective for long-term preservation, fixation alters the chemical and physical properties of cells, making them more prone to fragmentation and chemical or cross-DNA contamination. These factors can compromise DNA quality and hinder molecular analyses (Schrader et al., 2012; Floridia et al., 2023).

Long-term storage exacerbates these challenges, as variations in temperature, exposure to contaminants, and infrequent fixative replacement can degrade genetic material (Pereira, 2015). Nonetheless, using fixed samples offers significant bioethical advantages. These samples enable genetic analysis without requiring new collections, contributing to species conservation, minimizing impact on wild populations, and adhering to the 3Rs principles (replacement, reduction, and refinement) (Díaz et al., 2020; Hubrecht and Carter, 2019). While commercial DNA purification kits are available, most target blood samples are costly, have limited shelf lives, and are impractical for processing large sample volumes (Kulkarni et al., 2020).

This study proposes a protocol adapted and optimized for Farmer-fixed avian cells, making use of accessible, low-cost reagents. Effective for cytogenomic and evolutionary research, the approach also emphasizes sustainable practices by preserving vital genetic data from existing biological collections. Furthermore, this work introduces an optimized protocol tailored for fixed avian samples, with potential applicability to other vertebrates. By utilizing affordable reagents, the protocol not only ensures efficiency but also promotes the expanded use of preserved biological collections in future studies. This method facilitates the preservation of essential genetic data while advancing sustainable and ethical practices in data collection (Srinivasan, 2002; Miyaki, 2001).

MATERIALS AND METHODS

Cell suspensions were obtained from avian samples collected between 1998 and 2024 from Antarctic research stations (Carlini and Orcadas Bases) and Brazilian biomes (Pampa and Atlantic Forest) (Table 1). Lymphocyte cultures were prepared from blood collected with heparinized syringes following Moorhead et al. (1960). Cultures were incubated at 39°C for 72 hours in RPMI 1640 medium supplemented with 20% fetal bovine serum, 0.25 mL penicillin/streptomycin, and 0.2 mL phytohemagglutinin. Colchicine (0.05%) was added one hour before harvest. Hypotonic treatment (0.075 M KCl) and fixation with Farmer's solution were performed as described in Garnero and Gunski (2000). Samples were stored at -20°C. The bone marrow was suspended in 10 mL of Hank's balanced solution (HBSS), together with 0.1 mL of 0.05% colchicine, and incubated at 37°C for 1 hour. After this period, the suspension was centrifuged at 120 g for 8 minutes and the supernatant was removed. Then, 10 mL of hypotonic solution (0.075 M KCl) was added and the sample was incubated again at 37°C for 30 minutes. After incubation, the sample was centrifuged again at 120 g for 8 minutes, discarding the supernatant. The cell pellet was fixed with a solution of methanol and acetic acid (3:1) and centrifuged at the same speed. This fixation procedure was repeated three times to ensure the integrity of the preparations (Table 1). Finally, the samples were stored in a freezer at -20°C.

DNA Extraction

Cell suspensions were centrifuged at 12,000 g for 5 minutes at 4°C, the supernatant was removed, and the cells were resuspended in 500 µL of 10% phosphate-buffered saline (PBS) at 4 °C (Amorim et al., 2007), repeating washes up to three times to remove as much fixative residue as possible. Add 20 µL Proteinase K (20 mg/mL) and lyse cells with 400 µL lysis buffer (0.1 M Tris-HCl, 0.1 M EDTA, 1% SDS, 0.06 M NaCl) at 56°C for 30 minutes. RNase A (20 µL at a concentration of 4 mg/mL) was added to all samples, except for the *Colaptes melanochloros* specimen from Santana da Boa Vista. Precipitated DNA with 2 volumes of ice-cold ethanol; incubated at -20°C overnight.

Afterwards, centrifuged at 15,000 g for 10 minutes at 4°C; washed pellet twice with 70% ethanol and once with 90% ethanol. The material was centrifuged at 15,000 g for 8 min at 4ºC, dry pellet at 45ºC for ~15 minutes and eluted in 50 µL of ultrapure water. Store at -20ºC. DNA quality and quantity were assessed using a Nanovue spectrophotometer and agarose gel electrophoresis. PCR amplification targeted avian 18S rDNA (559 bp) with specific primers, evaluated under standard cycling conditions.

RESULTS

The DNA was isolated from samples collected and fixed up to 26 years ago on scientific bases in Antarctica, as well as more recent samples collected in 2015, 2022, and 2024 in the Pampa Biome and the Atlantic Forest. As is characteristic of materials fixed and stored for long periods, the extracted DNA showed a significant degree of degradation, as shown in Figure 1.

Table 1. Details of the analyzed samples: bird species, method of obtaining metaphases, origin, geographical location and year of collection.

Species	Method of obtaining metaphase	Origin of the sample	Coordinates	Year of collection
Daption capense	Peripheral blood leukocyte culture	Carlini Scientific Station, Antarctica	62° 14′ S, 58° 40′ O	1998
Pygoscelis papua	Peripheral blood leukocyte culture	Carlini Scientific Station, Antarctica	62° 14′ S, 58° 40′ O	1998
Catharacta lonnbergi	Peripheral blood leukocyte culture	Orkney Base, Antarctica	60° 44′ 17″ S, 44° 44′ 17″ O	1999
Chionis alba	Peripheral blood leukocyte culture	Orkney Base, Antarctica	60° 44′ 17″ S, 44° 44′ 17″ O	1999
Macronectes giganteus	Peripheral blood leukocyte culture	Orkney Base, Antarctica	60° 44′ 17″ S, 44° 44′ 17″ O	1999
Leucocarbo bransfieldensis	Peripheral blood leukocyte culture	Orkney Base, Antarctica	60° 44′ 17″ S, 44° 44′ 17″ O	1999
Turdus subalaris	Bone marrow culture	Porto Vera Cruz, Atlantic Forest	27° 44′ 09″ S, 54° 54′ 03″ O	2015
Colaptes campestris	Bone marrow culture	Porto Vera Cruz, Atlantic Forest	27° 44′ 09″ S, 54° 54′ 03″ O	2015
Colaptes melanochloros	Bone marrow culture	Santana da Boa Vista, Pampa Biome	30º 52’ S 53º 07’ O	2022
Colaptes campestris	Bone marrow culture	Porto Vera Cruz, Atlantic Forest	27° 44′ 09″ S, 54° 54′ 03″ O	2015
Turdus amaurochalinus	Bone marrow culture	Santana da Boa Vista, Pampa Biome	30º 52’ S 60° 41′ 47,88″ O	2022
Elaenia chilensis	Bone marrow culture	São Gabriel, Pampa Biome	30° 20′ 09″ S, 53º 07’ O	2024

Cost analysis highlighted significant savings, with the protocol costing $0.05-$0.10 per sample compared to $2.50-$3.00 for commercial kits, such as the ReliaPrep™ gDNA Tissue Miniprep System. This difference represents a saving of up to 98%, making the reagent-based protocol a viable option for large-scale studies or in laboratories with budget constraints. For comparison purposes, DNA was also extracted using the ReliaPrep™ gDNA Tissue Miniprep System (Promega). Some degree of degradation was observed in both the samples isolated using the protocol developed in this study and those extracted using the commercial kit. However, the samples extracted with the commercial kit showed better integrity. In the samples extracted using the protocol developed, no significant differences were observed between the samples collected in 2024 and those collected between 1998-1999. Notably, the oldest sample, corresponding to Daption capense and stored for 26 years, showed integrity comparable to the most recent samples and to those extracted with the commercial kit (Figure 1). Despite the degree of degradation observed and the low amount of DNA obtained (Figure 1), these factors did not prevent efficient PCR amplification. The expected amplicon of around 559 bp was detected with good resolution in all the samples analyzed (Figure 2).

DISCUSSION

DNA obtained from cell suspensions represents a practical and viable alternative for genomic studies, particularly when derived from samples originally prepared for cytogenetic analyses, as demonstrated by Amorim et al. (2007). Contrary to the findings of Nogueira and Freitas (2013), this study shows that the cell lysis technique is an effective method for extracting avian DNA from fixed materials, offering significant advantages over commercial kits due to its affordability. This methodology is especially beneficial for challenging scenarios, such as accessing samples from remote regions like Antarctica or rare specimens, including endangered species.

The use of fixed material samples provides both scientific and ethical benefits, allowing genetic studies to proceed without the need for new sample collections. This aligns with research ethics guidelines that emphasize reducing the number of animals used in scientific studies. Leveraging biological material stored for

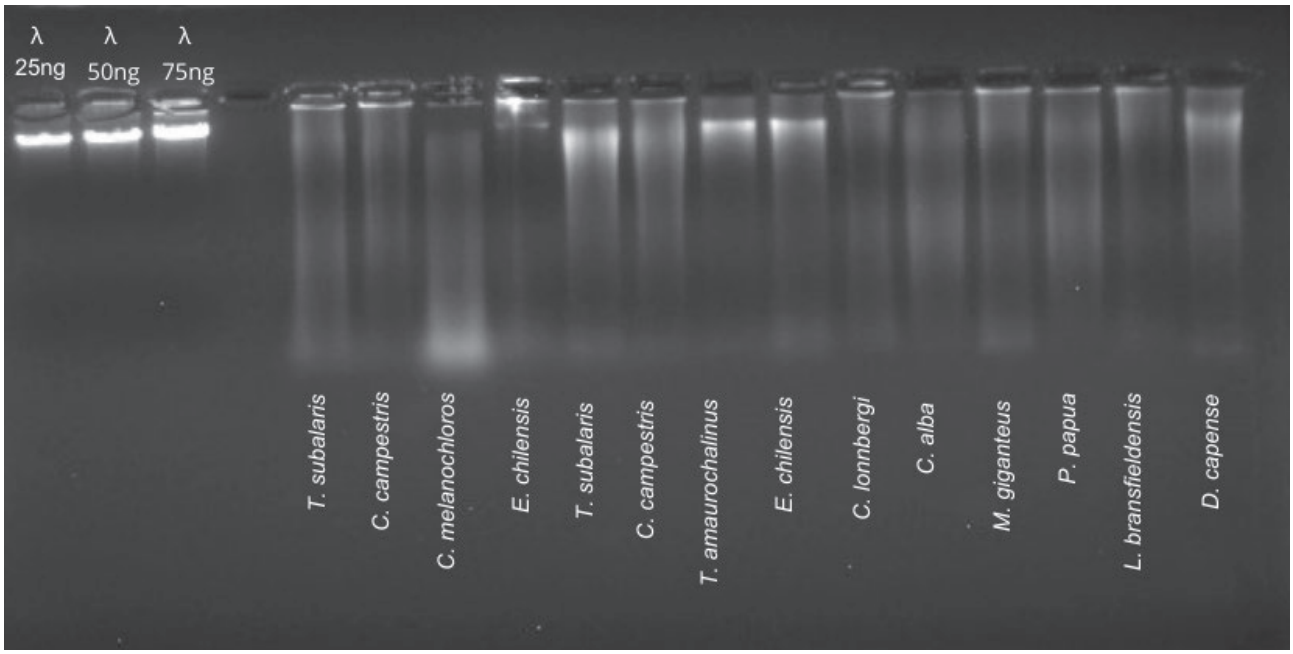

Figure 1. 1% agarose gel showing DNA integrity. Lanes 1–3: Lambda DNA standards (25 ng, 50 ng, and 75 ng; 10 μL each). Remaining lanes: DNA extracted using the protocol developed in this study, all loaded with equal volumes (10 μL). Differences in band intensity reflect variation in extraction efficiency among species. Samples correspond to *Turdus subalaris, Colaptes campestris, Colaptes melanochloros, Elaenia chilensis, Catharacta lonnbergi, Chionis alba, Macronectes giganteus, Pygoscelis papua, Leucocarbo bransfieldensis, e Daption capense*. DNA extracted with a commercial kit corresponds to *T. subalaris, C. campestris, Turdus amaurochalinus,* and *E. chilensis*.

extended periods reduces the impact on populations of endangered species and adheres to the 3Rs principles (replacement, reduction, and refinement) (Hubrecht and Carter, 2019; Díaz et al., 2020). These principles play a crucial role in minimizing animal suffering and decreasing the reliance on new animal collections for research purposes.

However, extracting DNA from Farmer-fixed samples presents inherent challenges. Cells treated with hypotonic solutions and fixatives such as methanol and acetic acid become structurally fragile and susceptible to contamination, potentially compromising DNA quality and hindering analyses like PCR. Residues from heparin and the culture medium can persist despite thorough washing, interfering with PCR efficiency depending on their concentration (Schrader et al., 2012). Nevertheless, in this study, no significant interference from heparin or residual culture medium was observed, as corroborated by Beránek et al. (2022). These authors reported that heparin, when present in appropriate concentrations, interferes less with PCR reactions compared to other anticoagulants like EDTA and citrate. Floridia et al. (2023) further emphasized that variations in anticoagulants, including heparin, minimally affect gene expression in quantitative PCR, highlighting their applicability in diverse experimental contexts.

The low protein content observed in the extracted samples is attributed to the denaturing effects of the fixative and the action of Proteinase K, which degrades proteins during the extraction process. Prolonged storage can exacerbate genetic material degradation; however, no significant differences were noted between recently collected samples and those stored since 1998. Proper maintenance of the fixative is essential, as inadequate replacement can compromise cell preservation and further degrade DNA. These findings highlight the importance of strict storage and handling conditions to maintain the integrity of genetic material and minimize degradation impacts on subsequent analyses.

CONCLUSION

This study validates the viability of extracting DNA from Farmer-fixed avian cells for molecular research, demonstrating significant cost and ethical advantages. The proposed method effectively extracts DNA from Farmer-fixed avian cells, offering a cost-effective alternative to commercial kits. Its application aligns with

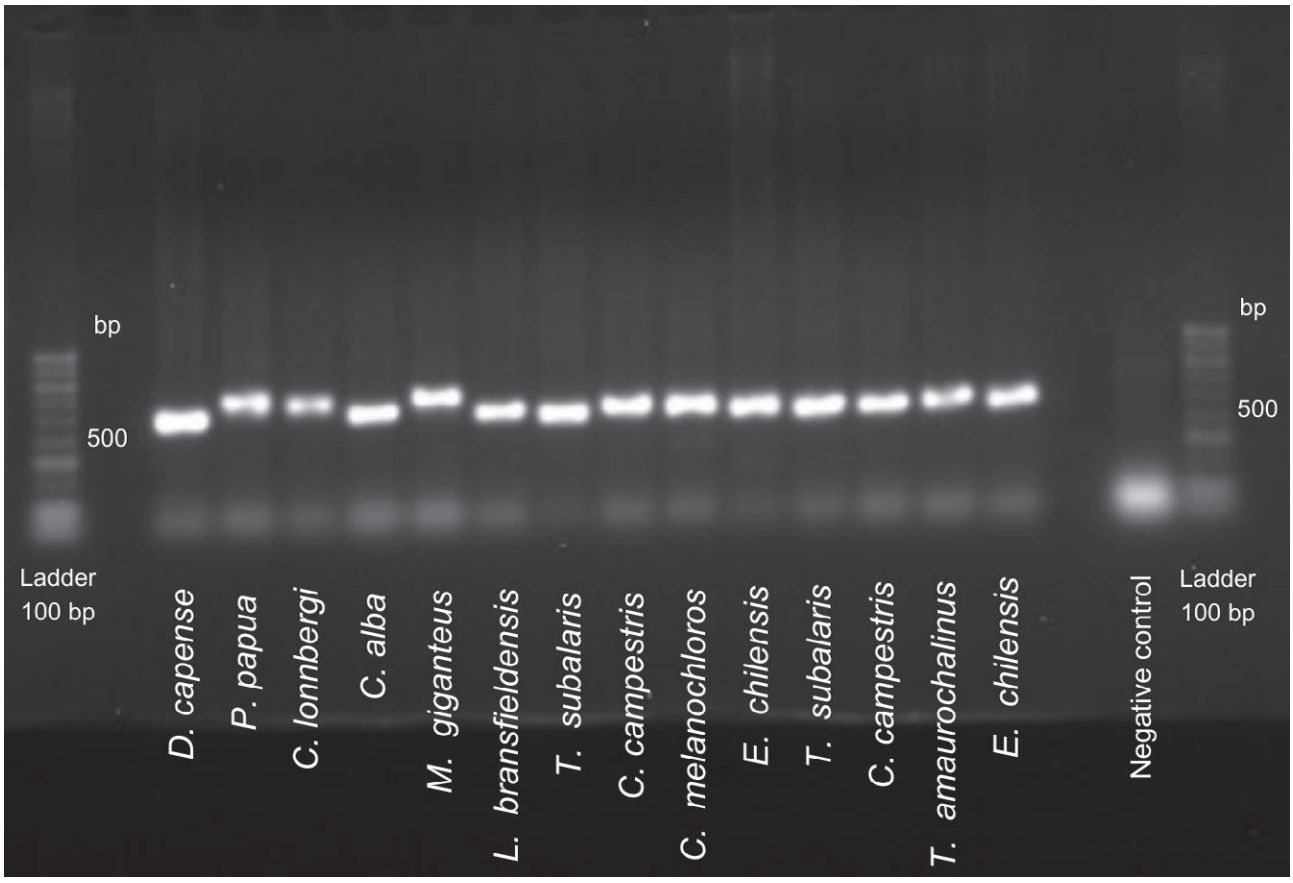

Figure 2. A 1.2% agarose gel with PCR products obtained from amplified DNA using specific primers for the avian 18S rDNA gene. The first and last wells contain a 100 bp ladder. The first 10 samples correspond to extracted DNA using the protocol developed in this study, while the next 4 samples refer to extracted DNA using a commercial kit. The penultimate well corresponds to the negative control of the reaction.

ethical research principles, reducing the need for new sample collections and minimizing ecological impact. Despite degradation challenges, the protocol consistently yielded DNA suitable for PCR, emphasizing its potential for cytogenomic studies. Additionally, adherence to strict storage conditions can further enhance DNA integrity in future studies. By utilizing existing biological collections, this approach provides a sustainable framework for advancing genetic and evolutionary studies in avian species.

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STATEMENT OF ETHICS

The study protocol was reviewed and approved by the Ethics Committee on the Use of Animals, under approval numbers CEUA 019/2020 and CEUA 024/2023. Additional approvals were obtained from the Biodiversity Authorization and Information System (SISBIO), with authorization numbers 61047-3, 33860-2, and 81564-1. Some samples were collected with authorization from the Argentine Antarctic Institute.

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AUTHOR CONTRIBUTIONS

L.O.M., A.V.G., and R.J.G. contributed to the conception and design of the study. Methodology was carried out by L.O.M. and L.R.P. Formal analysis, data curation, and investigation were conducted by L.O.M. and F.P.T. Visualization was prepared by L.O.M. and A.S.K. The original draft was written by L.O.M. and H.S.S. Review and editing were performed by H.S.S., L.R.P., F.P.T., A.S.K., A.V.G., and R.J.G. Funding acquisition was handled by R.J.G. and A.V.G.

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SUPPLEMENT MATERIAL

Table 1. Information on the quality of the extracted DNAs

Species	Extraction Protocol	A260/A280	A260/A230	Concentration (ng/ µl)
Daption capense	lysis	1.6	1.8	236.5
Pygoscelis papua	lysis	1.5	2.1	466
Catharacta lonnbergi	lysis	1.6	1.8	2424
Chionis alba	lysis	1.6	1.9	713
Macronectes giganteus	lysis	1.7	2.0	1163
Leucocarbo bransfieldensis	lysis	1.7	2.0	1558
Turdus subalaris	lysis	1.7	1.6	1890
Colaptes campestris	lysis	1.5	1.5	2009
Colaptes melanochloros	lysis	1.6	1.5	368.5
Elaenia chilensis	lysis	1.7	1.8	1126
Turdus subalaris	Commercial Kit	1.6	1.7	417
Colaptes campestris	Commercial Kit	1.8	1.8	1138
Turdus amaurochalinus	Commercial Kit	2.1	3.0	42
Elaenia chilensis	Commercial Kit	1.8	1.9	161.5