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OPTIMIZING DNA EXTRACTION METHODS FOR MAJOR HISTOCOMPATIBILITY COMPLEX GENE STUDIES IN BLACK FRANCOLIN (*Francolinus francolinus*) USING SOFT TISSUE SAMPLES

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ABSTRACT

Background: Robust immunogenetic profiling of wild birds requires high-quality genomic DNA (gDNA) isolated from multiple tissue types. The Major Histocompatibility Complex (MHC) serves as a key immunogenetic indicator for evaluating adaptive capacity and population health in birds. However, successful multi-locus polymerase chain reaction (PCR) and high-throughput sequencing of these highly variable genes depend critically on the quantity and purity of extracted DNA. To date, there is a lack of literature on DNA extraction from Black francolin (*Francolinus francolinus*), as well as information about the comparison of different extraction methods using different tissue types. Hence, this study evaluated three DNA extraction techniques (Phenol-Chloroform-Isoamyl Alcohol extraction, PCI; Silica-Membrane Spin-Column purification, SC; Magnetic Bead-based isolation, MB) using skeletal and cardiac muscle tissues from the Black francolin.

Methods: DNA yield and quality were assessed via spectrophotometry, agarose gel electrophoresis, and functional validation through PCR amplification of MHC Class II exon 2. An assessment was conducted on the hands-on time and cost-effectiveness of each protocol, and the degree of DNA degradation was also assessed using the TaqMan PCR method.

Results: The PCI yielded a high DNA concentration. However, spectrophotometric and PCR analyses showed moderate DNA quality. The SC produced the best DNA purity with a moderate cost-benefit ratio and low time consumption in comparison to other extraction methods. PCR amplification of MHC Class II exon 2 from skeletal muscle was specific and reproducible, with no primer-dimers or non-specific amplification, across all extraction methods. Despite lower yields, SC and MB methods produced sharp ~270 bp amplicons comparable to PCI, indicating that DNA integrity rather than quantity determines successful amplification.

Conclusions: The SC extraction technique was identified as the most effective DNA extraction approach for eliminating myofibrillar proteins and PCR inhibitors, making it superior for accurate MHC genotyping in the Black francolin.

Keywords: DNA Extraction, Phenol-Chloroform, Spin-Column, Magnetic Beads, Major Histocompatibility Complex, *Francolinus francolinus*

1 INTRODUCTION

Avian immunogenetics is the study of the relationship between an avian host and pathogen; it is one of the key factors in understanding the processes of coevolution, adaptation, and resistance in wild birds (Edwards et al., 2010). The collection of genes responsible for adaptive immunity, in particular, the Major Histocompatibility Complex (MHC) Class I and Class II genes, has shown extraordinary polymorphism, duplication events, and variability of gene families (Promerová et al., 2011; Minias et al., 2015). The MHC encompasses the most polymorphic and evolutionarily conserved gene families in vertebrates, and these genes encode cell-surface glycoproteins that bind foreign peptides and present them to T cells (O'Connor et al., 2019), making its diversity a central focus.

Despite its relevance, obtaining high-quality MHC genomic fragments from wild or preserved bird specimens often faces challenges at the initial stage: DNA extraction. While whole blood is a rich source of nucleated cells in birds, many studies instead rely on post-mortem tissue samples, such as skeletal or cardiac muscle, collected from field salvage, roadkill, or museum archives (Shen et al., 2014; Wakeling, 2004).

To obtain good DNA extracts from wild birds, it is necessary to consider possible problems associated with the isolation of nucleic acids from different tissues. Thus, skeletal and cardiac muscle tissues obtained from animals during post-mortem pathological dissection or from taxidermy collections contain various components (such as collagen, actin, myosin, myoglobin, lipids, and endonucleases) that are part of the cellular and intracellular structures of muscle tissue (Sambrook & Russell, 2001; Seutin et al., 1991). If they are not completely separated at the isolation stage can act as inhibitors of DNA polymerase during the polymerase chain reaction (Schrader et al., 2012), resulting in poor DNA sequencing.

Given that MHC genotyping typically involves sensitive and sequence-specific PCR, even trace contaminants such as RNA, lipids, residual salts, proteins, or organic solvents can inhibit polymerase activity, leading to technical issues such as failed amplifications, false negatives, or allelic dropout in heterozygous individuals. Therefore, DNA amplification relies on the efficacy of DNA extraction protocols, which should yield a substantial amount of high-quality DNA. In addition to being efficient, appropriate extraction methods should also be user-friendly, economical, and time-saving, and can be applied universally to minimize the need for several extraction procedures and calibrations in PCR, DNA quantification following gel electrophoresis, or spectrophotometric quantification (Ren et al., 2025)

Different methods are available for extracting DNA (Li and Lin, 2016; Tan et al., 2019; Jun et al., 2026), but only a few can be utilized to isolate DNA from wild birds such as Black Francolin (*Francolinus francolinus),* and their effectiveness for avian muscle tissues in immunogenetic applications remains poorly documented. Most often, the standard Phenol-Chloroform-Isoamyl Alcohol (PCI) solution has been used for the isolation of nucleic acids, which allows the maximum amount of RNA and DNA to be isolated from a wide range of tissues (Sambrook & Russell, 2001). However, the procedure is associated with a number of difficulties due to the need to handle toxic substances in separating organic and aqueous phases. In recent years, methods and compositions for solid-phase extraction of nucleic acids have been developed to address the handling of toxic substances, among which columns with a spin filter and magnetic beads functionalized with carboxyl groups have proven to be effective (Tan & Yiap, 2009; Berenstein et al., 2014) in isolating DNA with high efficiency, simplicity, and less time-consuming. However, there has been limited comparison of various existing DNA extraction procedures in a comprehensive manner, particularly in Black Francolin (*Francolinus francolinus*), and information about the comparison of these methods using different tissue types is not available in the literature.

Hence, in this study, we evaluate the purity, quality, and quantity of DNA extracted from *Francolinus francolinus* using two commercial methods and one non-commercial extraction method by the NanoDrop spectrophotometric technique, and conventional PCR was used to assess the amplification level of the isolated DNA using degenerate MHC Class II exon

2 primers targeting conserved flanking sequences, as it encodes the hypervariable peptide-binding region (PBR) responsible for recognizing and binding foreign antigens. Also, we also assessed the handling technique, time consumption, and cost per preparation of the extraction protocols.

2 MATERIALS AND METHODS

2.1 Sample collection and preservation

The birds were purchased from reputable farms and allowed to acclimatize for a period of 2-weeks at the Experimental Poultry Unit, Teaching and Research Farm, Oyo State College of Agriculture and Technology, Igboora. Prior to sample collection, all animals were humanely euthanized by inducing anaesthesia (isoflurane at 4-5% in oxygen) until loss of consciousness, followed by intravenous injection of sodium pentobarbital (120 mg/kg) to ensure a rapid and stress-minimized death, which was confirmed by absence of heartbeat and reflexes. Tissue samples (skeletal muscle and cardiac muscle) were collected immediately post-mortem from 30 *Francolinus francolinus* individuals following the approved institutional ethical guidelines. Approximately 25 mg each of gastrocnemius (skeletal muscle, n = 15) and the ventricular myocardium (cardiac muscle, n = 15) were obtained and were preserved, immersed in 95% molecular-grade ethanol, and stored at 20 °C until DNA extraction.

2.2 DNA extraction methods

Genomic DNA was extracted from tissue samples in triplicate using three distinct DNA extraction methods. Two commercial methods were utilised in the analysis: DNeasy Blood and Tissue Kit (abbreviated as SC; Silica-membrane spin-column, Qiagen, Germany) and Magnetic Bead-based isolation (abbreviated as MB; Sera-Mag™ Carboxylate-Modified Magnetic Speed Beads). One non-commercial method was also evaluated: phenol:chloroform:isoamyl alcohol (PCI)-based method, which was slightly modified in our laboratory. The three DNA extraction methods that were used for this study are described below and summarised in Table 1 along with their key features. All extracted DNA was suspended in a final volume of 50 µL elution buffer, and all extractions were carried out by a single individual in order to fairly assess and compare the performance of these extraction techniques.

2.2.1 Silica-Membrane Spin-Column (SMSC) method

Genomic DNA was extracted from 25 mg of tissue using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, with minor adaptation to the sample type according to Haesendonck et al., (2015). Briefly, tissue was digested in 180 µL of Buffer ATL and 20 µL of Proteinase K (20 mg/mL) at 56 °C for 3 hours. Then, 200 µL of Buffer AL was added, and the mixture was vortexed and incubated at 70 °C for 10 minutes to complete lysis. Followed by the addition of 200 µL absolute ethanol, the lysate was transferred to a silica spin column and centrifuged at 8000 x g for 1 min. The column was washed sequentially with 500 µL Wash Buffer 1 (8,000 × g, 1 min), then with 500 µL Wash Buffer 2, and centrifuged at 17,000 × g for 3 min to remove residual wash buffer. The column was transferred to a clean 1.5 mL tube, and DNA was eluted with 50 µL of pre-warmed (56 °C) Buffer AE for 5 minutes at room temperature and centrifuged at 8,000 × g for 1 minute.

2.2.2 Magnetic Bead-Based Isolation

The samples (25 mg) were homogenized in 200 µL of specialized lysis buffer containing 20 µL of Proteinase K using a micro-pestle and incubated at 56 °C for 2 hours for complete digestion. Then, 20 µL of well-mixed carboxyl-modified paramagnetic beads were added along with 200 µL of binding buffer (containing polyethylene glycol and high salt concentration) and mixed by gentle rotation at room temperature for 10 minutes to allow the DNA to bind to the magnetic beads. The tubes were placed on a magnetic rack for 3 minutes to immobilize the beads, and the supernatant was removed and discarded. While still on the rack, the bead-DNA complex was washed twice with 500 µL of freshly prepared 80% ethanol, with 30 seconds of incubation per wash. The beads were then left on the rack for 10 minutes to dry to remove residual ethanol. DNA was eluted by resuspending the beads in 50 µL of 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and incubated for 5 minutes at 55 °C. The eluate was recovered after 2 minutes of magnetic separation and transferred into a clean nuclease-free tube.

2.2.3 Organic Extraction (Modified PCI)

The tissue samples were homogenized and incubated in 500 µL of lysis buffer containing 10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, and 0.5% SDS, supplemented with 20 µL Proteinase K, and incubated overnight (~14 hours) at 56 °C with shaking at 600 rpm until complete digestion, following Li and Lin (2016). The lysate was first extracted with 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0), vortexed for 15 seconds, and centrifuged at 12,000 × g for 10 minutes at 4 °C to separate phases. The upper aqueous phase was carefully transferred to a new tube, and 450 µL of chloroform:isoamyl alcohol (24:1, v/v) was added, followed by centrifugation at 12,000 × g for 8 minutes.

Table 1: Key characteristics of the three selected DNA extraction procedures used in this study

	Kit name	Tissue lysis	Extraction buffer	Washing and drying	Elution buffer	DNA purification	Key advantages
Non-Commercial	PCI	20 µL Proteinase K; SDS	10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.5% SDS, 1 mL PCIA (25:24:1, pH 8.0)	2 volumes cold ethanol + 0.1 volume 3 M sodium acetate (pH 5.2); 2x 70% ethanol wash, and air-dried	50 µL TE buffer	Isopropanol	High-molecular-weight DNA recovery, and does not require a commercial column
Commercial method	SC	20 µL Proteinase K	180 µL Buffer AL + 200 µL absolute ethanol	500 µL Wash Buffer 1, 500 µL Wash Buffer 2	50 µL AE Buffer	Silica spin column	Simple, reproducible, and suitable for routine genomic DNA extraction
	MB	20 µL Proteinase K	200 µL Buffer AL, 200 µL absolute ethanol, 20 µL Sera-Mag beads	2 × 500 µL 80% ethanol, then air-dried	50 µL TE buffer	Isopropanol / Sera-Mag™ Speed Beads	Eliminates silica membranes and organic-solvent extraction, requires limited centrifugation, and is readily scalable to automated/high-throughput workflows

PCI = Phenol-Chloroform-Isoamyl Alcohol extraction; SC = Silica-Membrane Spin-Column purification; MB = Magnetic Bead-based isolation

The aqueous phase was moved to a clean tube, and nucleic acids were precipitated by adding 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 100% ethanol. Samples were incubated at −20 °C for 2 hours, then centrifuged at 16,000 × g for 15 minutes at 4 °C. The resulting DNA pellet was washed twice with 1 mL of cold 70% ethanol, air-dried in a laminar flow hood for 20 minutes, and dissolved in 50 µL of sterile 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.3 Spectrophotometer measurements

DNA quality and yield were measured after extraction using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, ThermoFisher, Waltham, MA, USA). The assessment of protein contamination was conducted by determining the ratio of absorbance at 260 nm to that at 280 nm. Similarly, organic or salt residue contamination was assessed by calculating the absorbance ratio at 260 nm to that at 230 nm. Each experimental trial was conducted with a sample volume of 1 µL, using the elution buffers from commercially available kits acting as the control and sterile double-distilled water (ddH_2O) as the blank.

2.4 PCR amplification

To assess the usability of the extracted DNA in downstream applications, we amplified the MHC Class II exon 2 region, as it encodes the hypervariable peptide-binding region (PBR) responsible for recognizing and binding foreign antigens (Alcaide et al. 2014; Pikus et al. 2022), using the degenerate primers (Table 2) targeting conserved flanking sequences.

Table 2: Primers and probe specific for MHC Class II exon 2

Target	Probe names	Sequence (5' – 3')	Annealing temp (°C)	Amplicon length (bp)
MHC Class II exon 2	MHC-F	GACTGCTYWTBYSNGSNGG	61.00	~ 270
	MHC-R	CTCGCCGCTKBYSNGSNGC	63.00	

Temp = temperature

For the PCR, the reaction mixture was prepared in a 25 µL reaction volume containing 2 µL template DNA, 12.5 µL of 2× ReadyMix PCR Master Mix, 1 µL of each primer (10 µ mol/L), and 8.5 µL nuclease-free water. PCR was conducted using the Veriti 96-Well Thermal Cycler (Thermo Scientific, ThermoFisher, Waltham, MA, USA). The process of DNA amplification was conducted utilizing the following conditions: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 45 s, 72°C for 45 s. The amplification process concluded with a final extension step at 72 °C for 7 min (Table 3). A PCR control was conducted using distilled water in place of DNA during the PCR reaction in order to assess and monitor any contamination in the PCR reagent. Structural integrity was evaluated by loading 5 µL of each extract from each PCR amplicon with 1.0 µL of 6× gel loading dye onto a 1.0 % agarose gel prepared in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) containing 0.5 µg/mL ethidium bromide and electrophoresed for a duration of 60 minutes at a voltage of 90 V. A high-molecular-weight DNA ladder (0.5 - kb) was included in adjacent lanes for reference. The DNA fragments were visualized under a UV transilluminator using the Bio-Rad ChemiDoc™ Touch Imaging system, focusing on the presence of high-molecular-weight DNA (>10 kb), signs of shearing (low-molecular-weight fragments), and potential RNA contamination.

Table 3: PCR program for amplification of all used genes

	Step		Temperature	Time
PCR	Initial denaturation		95 °C	5 min
	35 cycles	denaturation	95 °C	30 s
		Annealing	58 °C	45 s
		Extension	72 °C	45 s
	Final extension		72 °C	7 min
	Cooling		4 °C	∞

2.5 Statistical Analysis

The data from the DNA extraction procedures were evaluated using a general linear model procedure, following the Psifidi et al. (2010) and Ren et al. (2025) models with certain changes. All parameters for each tissue's DNA evaluation (DNA yield and two spectrophotometer measurements for purity) were evaluated individually by testing for normality

(Shapiro-Wilk test) and an F-test of equality of variances (for the homogeneity of variances). Post-hoc analyses were conducted using least squares mean tests and a Bonferroni adjustment for multiple testing to compare various DNA extraction procedures for each evaluation criterion. The significance level of $p < 0.05$ was deemed statistically significant. All analyses were conducted using GraphPad Prism 10.6.0(890) (GraphPad Software, Inc., La Jolla, CA, USA).

3 RESULTS

3.1 The yield and quality of the extracted DNA

The mean of DNA yield (concentration) and absorbance ratios (A_{260}/A_{280} and A_{260}/A_{230}), which reflect the DNA concentration and purity, are shown in Table 4 and Fig. 1, along with statistical comparisons. Based on the DNA concentration statistics, the PCI method produced the highest DNA amounts for the skeletal and cardiac muscle. Even though the difference between the two tissue samples was not statistically significant, both resulted in significantly ($p < 0.05$) higher DNA yield compared to the commercial methods (SC and MB) having DNA concentrations below 20 ng/µl for both tissue samples. In contrast, based on the A260/A280 nm ratio, the SC extraction method had the highest values while the MB method had the lowest values for skeletal and cardiac muscle, respectively. However, A260/A230 nm ratios revealed a different pattern, with the PCI method having the highest values, indicating its effectiveness at removing organic salts and carbohydrates, while the MB methods had markedly lower values for skeletal and cardiac tissues, respectively, suggesting possible residual salts or guanidine-containing reagent contamination. These results revealed that the extraction protocols differed in both DNA yield and spectrophotometric purity, with PCI combining high DNA yield with slight protein contamination or traces of organic solvents such as phenol, as revealed by the A_{260}/A_{280} ratio.

Table 4: Quantitative profiles of extracted DNA from *Francolinus francolinus* tissues

EP	Tissue	DNA yield (ng/µL)	A_{260}/A_{280}[1]	A_{260}/A_{230}[1]
PCI	Skeletal	134.20 ± 11.89^{a}	1.58 ± 0.04^{b}	1.96 ± 0.07^{a}
	Cardiac	118.50 ± 10.90^{a}	1.54 ± 0.05^{b}	1.91 ± 0.08^{a}
SC	Skeletal	15.20 ± 2.37^{b}	1.72 ± 0.03^{a}	1.42 ± 0.05^{b}
	Cardiac	12.44 ± 1.99^{b}	1.69 ± 0.04^{a}	1.38 ± 0.07^{b}
MB	Skeletal	12.80 ± 2.23^{b}	1.02 ± 0.07^{c}	0.55 ± 0.05^{c}
	Cardiac	10.10 ± 1.65^{b}	0.98 ± 0.06^{c}	0.51 ± 0.04^{c}

[1]A_{260}/A_{280} and A_{260}/A_{230} are ratios of absorbances measured at 260 nm and 280 nm, and at 260 nm and 230 nm, respectively. [a,b,c] Comparison of each column's values. Values (means ± SD, n = 5) with different letters showing significant differences ($p < 0.05$). EP = Extraction Protocol; PCI = Phenol-Chloroform-Isoamyl Alcohol extraction; SC = Silica-Membrane Spin-Column purification; MB = Magnetic Bead-based isolation

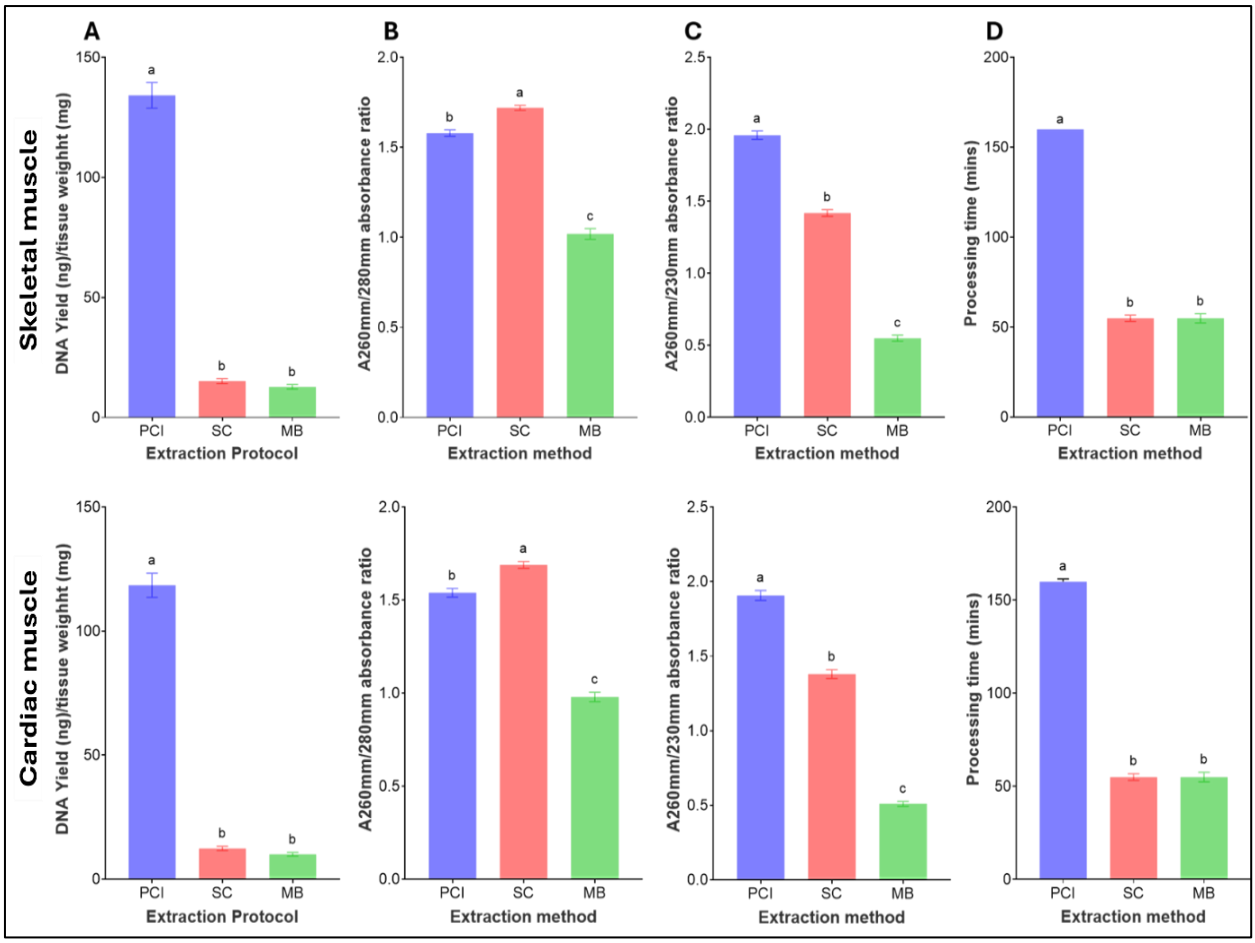

Figure 1: DNA yield, purity, and processing time values obtained from different sample groups in triplicate using the different extraction methods. Standard deviations from three separate experiments using two different tissue types are represented by error bars in the column charts. A: Chart showing DNA yields obtained for all extraction methods evaluated. B: Chart showing absorbance ratios at 260/280 for all extraction methods assessed. C: Chart showing absorbance ratios at 260/230 for all evaluated extraction techniques. D: Processing time for all extraction techniques examined. ^{a,b,c} Comparison of the values in each column; values with different superscripts are significantly different ($p < 0.05$)

3.2 Evaluation of Genomic DNA Integrity

The integrity of the extracted genomic DNA (gDNA) was assessed by agarose gel electrophoresis alongside a high molecular weight marker (Fig. 2). Across all samples, bright and distinctly stained bands of high molecular weight (HMW) DNA above the 10 kb marker were detected with no visible low-molecular-weight smearing. This indicates high-quality DNA extracts, which aligns with the DNA yields and quality obtained (Table 2; Fig. 1), and that all the extraction methods successfully recovered intact DNA from the tissue types with minimal degradation or shearing.

However, variations in band intensity and quality were observed across the different extraction methods and tissue types. Among the skeletal muscle samples (lanes 2-4), the PCI extraction (lane 2) produced an intensely bright, slightly overloaded HMW band with minor trailing, indicating a substantially higher DNA concentration compared to the other samples. In contrast, SC (lane 3) and MB (lane 4) exhibited clean, compact, and well-defined HMW bands, demonstrating efficient extraction, albeit with slightly lower intensity.

A notable difference was observed as Lane 6 displayed a prominent vertical smear trailing downwards from the HMW band. This smearing is likely attributable to increased mechanical resistance encountered during the homogenization of tightly packed cardiac myofibrils, resulting in physical shearing of the DNA. Conversely, Lanes 7 and 8 produced sharp, discrete HMW bands with negligible smearing, indicating that these extraction methods yielded highly intact gDNA from the cardiac tissue.

However, in the cardiac muscle samples (lanes 6-8), the SC and MB methods maintained DNA integrity by showing no signs of shearing or degradation. The PCI method (lane 6), however, produced a slight vertical smear at approximately 2 kb, indicating physical shearing and partial degradation, which is likely due to the increased mechanical resistance

during the homogenization of tightly packed cardiac myofibrils, compared to the more loosely structured skeletal muscle fibres. These outcomes underscore the importance of purification in ensuring consistent primer binding, especially when analysing diverse avian samples collected under varying preservation conditions (Adeola et al., 2021).

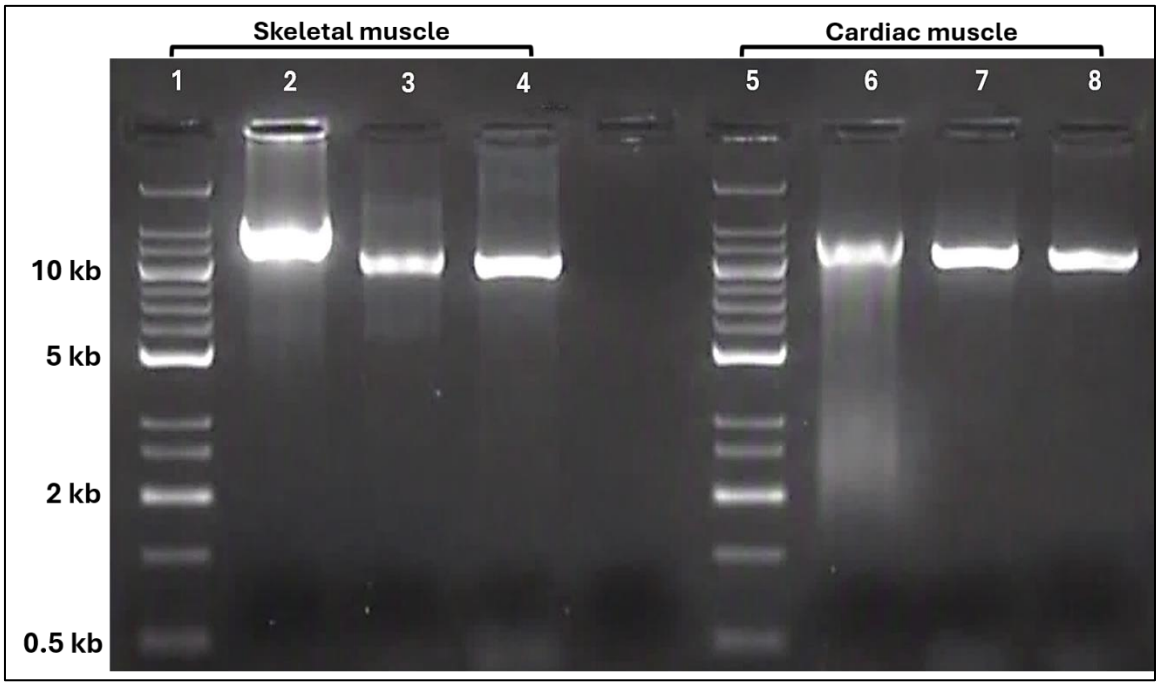

Figure 2: Representative results from gel electrophoresis analysis of genomic DNA from two different tissue samples extracted by three methods. DNA Marker (lanes 1 and 5), Skeletal tissue (PCI = lane 2; SC = lane 3; MB = lane 4), Cardiac tissue (PCI = lane 6; SC = lane 7; MB = lane 8), (PCI: Phenol-Chloroform-Isoamyl Alcohol method, SC: Silica-Membrane Spin-Column purification method, MB: Magnetic Bead-based isolation method).

3.3 Gel electrophoresis of the amplified DNA products

To further assess the suitability of the extracted DNA for downstream applications, PCR amplification was performed using a degenerate primer targeting the MHC Class II exon 2 region (~270 bp), followed by agarose gel electrophoresis alongside a 1000 bp molecular weight marker (Fig. 3). A distinct, bright band of the expected size (~270 bp) was successfully amplified for all samples across all three extraction methods (PCI: lanes 2 and 3; SC: lanes 4 - 6; MB: lanes 7-9). No primer-dimers or non-specific amplification products were detected, indicating high specificity of the primer and high quality of the DNA templates. The PCI extraction method (lanes 2 and 3) displayed slightly higher band intensities compared to the SC (lanes 4-6) and MB (lanes 7-9) methods. However, all the extraction methods produced sharp, discrete amplicon bands with no visible signs of smearing or shearing, and band intensities were relatively uniform across all lanes, demonstrating consistent PCR efficiency regardless of the extraction method or tissue type.

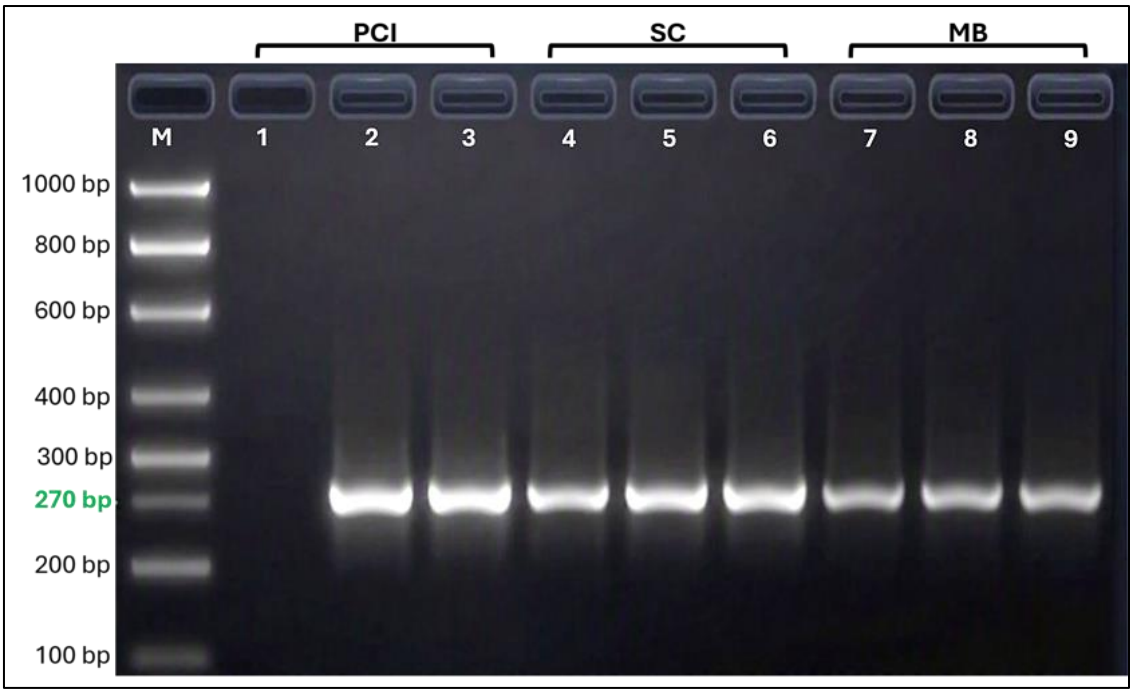

Figure 3: Representative results from gel electrophoresis analysis of the MHC class II gene from skeletal tissue samples extracted by three methods. DNA Marker (lane 1), (PCI = lane 2; SC = lane 3, MB = lane 4), Cardiac tissue (PCI = lane 6; SC = lane 7, MB = lane 8), (PCI: Phenol-Chloroform-Isoamyl Alcohol method, SC: Silica-Membrane Spin-Column purification method, MB: Magnetic Bead-based isolation method).

3.4 Economic, labour, and time assessments of the extracted DNA

An analysis was also conducted to compare the various DNA extraction approaches' material costs and throughput time (Table 5). The amount of manual labour needed to extract DNA was identical for all three protocols, and each protocol was straightforward and uncomplicated to follow. When each extraction method exclusively focuses on extracting one sample within a specific time frame, the commercial methods (SC and MB extraction procedures) were the shortest, taking approximately 55 minutes, while the PCI extraction processes, which utilize the isopropyl alcohol precipitation protocol, resulted in an extraction time of approximately 160 minutes. Finally, substantial differences were observed in the relative cost per sample when comparing the extraction protocols used in this study. As anticipated, the costly techniques were the MB extraction method, followed by the SC and PCI extraction methods.

Table 5: Estimation of the time and material cost required for each extraction procedure

EP	Tissue	Processing Time	Relative Cost / Sample
PCI	Skeletal	~ 160 min	Lowest (1.0 ×)
	Cardiac	~ 160 min	Lowest (1.0 ×)
SC	Skeletal	~ 55 min	Moderate (3.0 ×)
	Cardiac	~ 55 min	Moderate (3.0 ×)
MB	Skeletal	~ 55 min	Highest (4.5 ×)
	Cardiac	~ 55 min	Highest (4.5 ×)

EP = Extraction Protocol; PCI = Phenol-Chloroform-Isoamyl Alcohol extraction; SC = Silica-Membrane Spin-Column purification; MB = Magnetic Bead-based isolation

4 DISCUSSION

The selection of a suitable DNA extraction method involves weighing several factors, including yield, purity, ease of use, and cost. In avian immunogenetics, it is particularly important to isolate high-quality genomic DNA free from PCR inhibitors to enable reliable amplification of specific loci such as MHC Class I and II. The substantial differences in DNA yield among the extraction protocols indicate that extraction efficiency was strongly dependent on the applied protocol

as well as the unique buffer system, pre-processing phase, and isopropyl alcohol cold treatment processes (Ren et al., 2025).

The PCI generated the highest yields of 134.20±11.89 and 118.20±10.90 from skeletal and cardiac tissue, respectively (Figure 1), which are approximately 8 - 13-fold greater DNA concentrations than the SC and MB protocols, and likely due to thorough cell lysis during extended Proteinase K treatment and effective separation of phases during liquid-liquid extraction (Sambrook & Russell, 2001). In addition, its relatively low A_{260}/A_{280} ratio (1.58±0.04 and 1.54±0.05 for skeletal and cardiac, respectively) suggested an incomplete removal of residual peptides, proteins, phenolic compounds, or other organic contaminants because a small amount of residual organic solvents above 0.2% v/v can interfere with Taq polymerase activity (Schrader et al., 2012), and this aligns with the earlier reports for Nigerian avian studies, where high yields from dense tissues often mask underlying impurities (Oke *et al.,* 2023). This combination indicated that the PCI protocol is more efficient at recovering DNA while minimizing the accumulation of compounds contributing to absorbance at 230 nm. Similar method-dependent differences have been reported in tissue-derived DNA from fish, where extraction protocols produced distinct combinations of DNA yield and purity depending on tissue type and purification chemistry (Lutz et al., 2023). While this method yields abundant DNA suitable for long-term storage or large-scale applications, additional purification may be necessary before use in sensitive enzymatic reactions.

In contrast, the Silica Spin-Column approach yielded DNA with higher purity, as indicated by a more favourable A_{260}/A_{280} ratio of 1.72±0.03 and 1.60±0.04 for skeletal and cardiac, respectively (Figure 1), which falls below the threshold of 1.8-2.0 for purified DNA (Vesty et al., 2017) and signal allelic dropout (Minias *et al.,* 2018). Although total yield was lower at 20 ng/µL, the silica membrane efficiently removes contaminants such as proteins, lipids, and cellular debris during washing steps (Tan & Yiap, 2009) and delivers cleaner, inhibitor-free templates essential for robust amplification of complex avian genetic markers (Westerdahl et al., 2000). Reduced yields are common with solid-phase techniques, often due to binding capacity limits or incomplete DNA elution, which are sufficient for routine PCR and next-generation sequencing applications (Desjardins & Conklin, 2010). However, the corresponding A_{260}/A_{230} ratios of 1.42±0.05 and 1.38±0.07 for skeletal and cardiac, respectively, were substantially lower than the expected range, which suggests that the samples retained appreciable amounts of substances such as residual salts, guanidinium-containing reagents, carbohydrates, lipids, or other extraction-associated components, depending on the chemistry of the extraction procedure (Versmessen et al., 2024). Comparable variation in A_{260}/A_{230} and A_{260}/A_{280} ratios across extraction protocols conforms to the report by Sancho-Blanco et al. (2022) that extraction chemistry and subsequent purification steps influence the spectrophotometric quality of genomic DNA from animal-derived samples.

The Magnetic Bead protocol demonstrated consistent results across replicates, with an average yield of 15 ng/µL and well-preserved high-molecular-weight DNA fragments (Fig. 2). However, the low A_{260}/A_{280} of 1.02 and 0.98 and A_{260}/A_{230} of 0.51 - 0.55 ratios for skeletal and cardiac, respectively indicate the presence of residual impurities, likely from insufficient removal of salts or binding agents, which can be improved by optimizing the wash steps, such as increasing the number or volume of 80% ethanol rinses, to effectively clear the contaminants before elution (Berenstein et al., 2014).

The observed reduction in DNA yield, with minor fragmentation observed (approximately 2 kb) in cardiac muscle samples, underscores the influence of tissue architecture on nucleic acid recovery, as cardiac myocardium is rich in dense collagen and tightly interlaced myofibrils, necessitating intense mechanical disruption during homogenization resulted in increased physical processing and shearing of high-molecular-weight genomic DNA (Lee et al., 2012; Belviso et al., 2022). Despite this, the majority of extracted DNA remained intact above 10 kb and retained sufficient structural integrity for reliable amplification of target genes. However, visualization of the crude DNA on an agarose gel alone may lead to incorrect conclusions when assessing the DNA integrity in muscle tissues. Therefore, comparing the level of amplifiable endogenous genes among the same sample remains a valuable strategy for evaluating extraction techniques.

The successful amplification of the MHC Class II exon 2 region from skeletal muscle is notable for yielding specific and reproducible results, with no primer-dimers or non-specific amplification products across the extraction methods, because of the well-documented difficulty in amplifying the region (Edwards et al. 1995). The specificity observed in our amplification indicated that the degenerate primer used successfully targeted the conserved regions flanking the polymorphic antigen-binding site, enabling consistent amplification despite the sequence variation, coupled with frequent gene duplication, recombination, and gene conversion, which makes the design of universal primers that reliably amplify across species and individuals challenging (Alcaide et al., 2014; Minias et al., 2023).

The uniformity in amplification success, despite the substantial differences in DNA yield and purity between the methods, suggests that the primary determinant of successful PCR is the functional integrity of the DNA template, because PCR, as a highly sensitive enzymatic reaction, requires only a small number of intact DNA template molecules, and excessive DNA input can inhibit amplification through the presence of co-purified inhibitors (Canal et al., 2010).

The successful amplification of MHC class II exon 2 from skeletal muscle has broader implications for studies of avian immunogenetics, as characterisation of MHC diversity is central to understanding host-pathogen coevolution, sexual selection, and immune response in natural populations (O'Connor et al. 2019). The employed degenerate primer strategy, which targets conserved coding regions of the β chain, represents a practical approach for generating species-specific MHC clones and for extending such studies to non-model avian species. The high specificity and absence of non-specific products in our reactions further demonstrate that high DNA yield and primers can effectively evade amplification of non-classical MHC genes and pseudogenes, a common challenge in avian MHC typing (Promerová et al. 2012).

In terms of workflow performance and cost, the methods varied considerably (Table 3). The PCI-based protocol demanded approximately 160 minutes of hands-on time, factoring in an overnight digestion step, repeated centrifugations, and liquid-phase separation procedures. By comparison, commercial spin-column and magnetic bead approaches streamline processing, reducing active labour to around 55 minutes per batch. Economically, the PCI method is the most affordable, costing one-third of the baseline, which makes it a practical choice for laboratories with limited resources or for analysing large sample sizes. Spin-column kits are about three times more expensive per sample, while magnetic bead kits cost 4.5 times as much. Although these commercial kits entail higher material costs, they offer advantages in processing speed, enhanced operator safety, and improved DNA purity, which are beneficial for sensitive downstream applications such as enzymatic analyses.

Abbreviations

- PCI Phenol-Chloroform-Isoamyl Alcohol extraction
- SC Silica-Membrane Spin-Column purification
- MB Magnetic Bead-based isolation
- PCR Polymerase Chain Reaction
- DNA Deoxyribonucleic acid
- MHC Major Histocompatibility Complex
- PBR Peptide-binding Region

5 CONCLUSIONS

In summary, this study demonstrates that the purity of extracted DNA is more critical than its total amount for MHC genotyping in *Francolinus francolinus* and depends on the extraction process used. The silica spin-column protocol is best suited for sensitive enzymatic techniques such as MHC locus amplification and next-generation sequencing, offering high optical quality, consistent yield, and a fast processing time of approximately 55 minutes. In contrast, phenol-chloroform-isoamyl alcohol (PCI) extraction is preferable when high DNA yield, long-term genomic storage, or cost efficiency are priorities, as long as the subsequent analyses can accommodate lower purity or incorporate extra purification steps. Magnetic bead-based methods enable quick and highly reproducible extractions, though their accuracy in spectrophotometric measurement improves when optimized washing procedures are used to eliminate residual salts.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

The authors declare no competing interests

Statement of ethical approval

All animal procedures and the number of animals used in this study were reviewed and approved by the Ethics Committee of Animal Use and Care of the Oyo State College of Agriculture and Technology, Igbo-ora, Oyo State, Nigeria. A veterinarian assisted with manual handling of the animals; a short-acting anaesthetic was administered, and tissue

samples were collected under humane conditions using appropriate equipment. All procedures were performed in accordance with guidelines for the care and use of animals (NIH publication No. 85-23, revised in 1985).

Author contributions

The research conducted in this study was a collaborative effort involving all authors. OAA originated the study, conducted the experiments, data collection, statistical analysis, and drafted the manuscript. OAA and ABA were responsible for designing the primers. ABA contributed to the statistical analysis, prepared all tables and figures, reviewed the manuscript, and interpreted the results.

Data availability

Data are provided within the manuscript or supplementary information files.

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