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OPTIMISATION OF WILD BIRDS' FECAL EXTRACTION USING MACHEREY-NAGEL NUCLEOSPIN DNA STOOL KIT

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Abstract: Current DNA extraction methods for bird faeces samples might not provide sufficient DNA quantity, which in turn affects downstream analyses. This study aimed to improve the current method of DNA extraction from the faeces of wild birds - a total of seven (n = 7) samples for analysis and protocol comparison. Three different methods in this study were compared: (1) The original method as per the manufacturer's instructions, (2) a supplementary chicken-specific protocol provided by the manufacturer, and (3) modifications to the protocol conducted in the laboratory. After extensive troubleshooting, DNA yield was significantly increased by utilising the modified protocol, which includes ethanol evaporation with a heat block, a direct 30-minutes freezer step during contaminant precipitation replacing ice, and a warm elution buffer for improved DNA elution. These findings offer valuable guidance for developing a refined and more specific methodology for collecting and processing bird faeces samples, improving the reliability of DNA-based studies in microbiota research in the bird gut.

Keywords: Optimisation, microbiome, Bornean wild birds, bird faeces, DNA.

Introduction

Studies utilising animal faeces samples to understand diet and microbial composition have been increasing (Ingala *et al*., 2018), with research into this relationship being formally documented since at least 2010 (Yildirim *et al*., 2010). Many studies provide valuable points of view on various DNA extraction methods for animal faeces samples, whether using boiling extraction methods or commercial kits, in molecular genetic research (Vo & Jedlicka, 2014; Hart *et al*., 2015; Afrin *et al*., 2018; Gaur *et al*., 2019). However, comparison studies have revealed that some extraction methods effective for mammalian faeces may be less effective for avian faeces samples (Eriksson *et al*., 2017). What is known is that the effectiveness of DNA extraction methods varied significantly due to various environmental factors and the specific characteristics of avian faeces matter, including dietary influences (Hou *et al*., 2021). However, a study by Edwards *et al*. (2023) found that the gene-based microbiota profiles from avian faeces were largely unaffected by different DNA preservation and extraction methods, indicating robust and consistent results across varied protocols. Despite the findings from previous studies, DNA extraction methods involving avian faeces matter still require optimisation (Eriksson *et al*., 2017; Hou *et al*., 2021).

Indeed, the purity and concentration of extracted DNA are crucial for molecular analysis, particularly in metagenomics. The diversity of samples and the varying analytical methods used for measuring DNA extraction efficiency and purity make meaningful comparisons difficult (Miller *et al*., 1999). This specific parameter can introduce systematic errors, particularly in DNA extraction efficiency and purity. Therefore, the extraction method chosen significantly affects the quality and quantity of DNA isolated from faecal samples, which can lead to biased Next-Generation Sequencing (NGS) results (Vesty *et al*., 2017; Fiedorova *et al*., 2019). Optimising DNA extraction techniques such as SPRI-based and magnetic bead-based methods enhances the purity and quantity of DNA from avian faeces

samples, ensuring reliable NGS results (Vo & Jedlicka, 2014; McGaughey *et al*., 2018).

Researchers in Southeast Asia examined the avian gut microbiota using ileal and cecal content (Susanti *et al*., 2020; Susanti *et al*., 2023), cloacal swabs (Joakim *et al*., 2022), and faecal samples (Shaufi *et al*., 2015; Rosa & Rivera, 2021). These samples were extracted with commercial DNA extraction kits, either the Qiagen QIAamp Fast DNA Stool Mini Kit (Shaufi *et al*., 2015; Susanti *et al*., 2020; Joakim *et al*., 2022; Susanti *et al*., 2023) or Qiagen MO BIO's PowerSoil DNA Isolation kit (Rosa & Rivera, 2021). The results were consistent and valuable despite using different sample types and extraction kits.

This study aims to optimise DNA extraction from faeces samples to ensure high-quality and sufficient DNA for microbiota and metagenomic analysis. The protocol of a commercial DNA extraction kit was thus modified to enhance DNA yield from selected wild bird faeces samples. Three DNA extraction methods were evaluated and compared using faeces samples from seven Bornean wild bird species. The concentration of the extracted DNA products was analysed to determine the success of the optimisation.

Materials and Methods

Faeces samples were collected from wild birds using the modified paper bag method (Knutie & Gotanda, 2018). Each sample was swabbed with a sterile cotton swab from the modified weighing boat. Subsequently, the swabbed faeces material was transferred into a cryotube containing 99.8% molecular-grade ethanol for storage. All collected samples were then transported to the laboratory and stored in a -80°C freezer before DNA extraction. In this study, seven wild bird species were selected, with only one individual from each species (Table 2.0) being used for multiple DNA extractions.

Genomic DNA (gDNA) extraction from the faeces samples was performed using the commercial Macherey-Nagel NucleoSpin^{O} DNA Stool Kit. However, the extraction kit protocol was not entirely suited for the faeces samples from wild birds, so adjustments were made to obtain the appropriate quality and quantity of gDNA needed for the metagenomic NGS. Three DNA extraction protocols, the original, the chicken, and the optimised protocol were compared to identify which one best aligns with the required DNA concentration for metagenomic NGS.

Method 1: Original Protocol from the Manufacturer's Guide (Macherey-Nagel, 2023)

A table form of method 1 is shown in Table 1.

Method 2: Chicken Protocol (Supplementary Protocol from Manufacturer) + Manufacturer's Recommendation for Elution at Step 9

The chicken protocol is a supplementary protocol supplied by the manufacturer (Macherey, n.d.). This protocol is somewhat similar to the original protocol but includes additional preparations at the following steps:

Table 1: The original protocol for DNA extraction from the manufacturer's protocol

1.	**Sample preparation**	Mix 180 mg to 220 mg of wild bird stool material with 850 uL Buffer ST1 in an MN Bead Tube Type A and shake for 2 s to 3 s.
2.	**Sample lysis**	Incubate the MN Bead Tubes for 5 minutes at 70°C, then, vortex for 10 minutes.
3.	**Contaminant precipitation**	Centrifuge at 13,000 RPM for 3 minutes. Transfer 600 uL of the supernatant to a new, sterile 2 mL microcentrifuge tube with a lid. Add 100 uL Buffer ST2, close the lid and vortex for 5 s. Incubate for 5 minutes at 2°C to 8°C (on ice). Centrifuge at 13,000 RPM for 3 minutes.

4.	**Lysate filtration**	Transfer 550 uL of cleared lysate (avoiding the pellet) onto the **NucleoSpin® Inhibitor Removal Column (*red ring*)** seated in a collection tube. Centrifuge at 13,000 RPM for 1 minute and discard the column.
5.	**Binding adjustments**	Add 200 uL Buffer ST3 and close the lid. Vortex for 5 s.
6.	**DNA binding**	Load 700 uL sample onto **NucleoSpin® DNA Stool Column (*green ring*)** seated in a collection tube. Centrifuge at 13,000 RPM for 1 minute. Discard the flowthrough and place the column back into the collection tube.
7.	**Washing silica membrane**	Add 600 uL Buffer ST3 to the NucleoSpin® DNA Stool Column and centrifuge 13,000RPM for 1 minute. Discard flowthrough and reuse the collection tube. Add 550 uL Buffer ST4 to the NucleoSpin® DNA Stool Column and centrifuge 13,000RPM for 1 minute. Discard flowthrough and reuse the collection tube. Add 700 uL Buffer ST5 to the NucleoSpin® DNA Stool Column, vortex for 2 s, and centrifuge at 13,000 RPM for 1 minute. Discard flowthrough and reuse the collection tube. Add 700 uL Buffer ST5 to the NucleoSpin® DNA Stool Column and centrifuge at 13,000 RPM for 1 minute. Discard flowthrough and reuse the collection tube.
8.	**Drying silica membrane**	Centrifuge at 13,000 RPM for 2 minutes.
9.	**DNA elution**	Place the NucleoSpin® DNA Stool Column into a new, sterile 1.5 mL microcentrifuge tube. Add 40 uL of Buffer SE (elution buffer) and centrifuge at 13,000 RPM for 1 minute. Discard the NucleoSpin® DNA Stool Column and vortex the microcentrifuge tube for 2 s.

A. Step 1

I. Use the same NucleoSpin® Bead Tube (Type A), but only with 200 mg of faeces sample.

II. Use a larger volume of Buffer ST1 (1 mL).

B. Step 2

I. Start with mechanical disruption by vortexing for 10 minutes at maximum speed.

II. Centrifuge the samples briefly by pulsing to spin down foam for 5 s before proceeding to chemical lysis.

III. Add 20 uL of Proteinase K to the sample solution and shake horizontally for 2 s to 3 s.

IV. Increase the incubation time to 30 minutes while maintaining the temperature at 70°C.

V. Invert the tubes every 10 minutes to ensure the solution is properly mixed.

C. Step 9

I. After adding elution buffer to the centre of the membrane, incubate for 1 minute before centrifugation.

II. Transfer the eluate back to the column and incubate for an additional 1 minute before centrifugation.

Method 3: (Modifications to the Original Protocol)

The original protocol was modified for wild bird faeces samples through the following steps:

A. Step 1

I. Since the samples were initially stored in 99.8% molecular grade ethanol, the ethanol must be evaporated using a heat block and allowed to air dry.

II. Utilise less than 200 mg of the sample (the optimal range based on our trials was 100 mg to 150 mg).

III. Add 20 uL of Phosphate-buffered Saline (PBS) to the sample.

IV. Add 1,000 uL of Buffer ST1 to the sample.

B. Step 2

I. This step is similar to the chicken protocol, except that mechanical disruption is performed using the BeadBugÔ Benchtop Homogenizer machine.

C. Step 3

I. Rather than placing the tubes containing samples on ice in a bucket or beaker, store them directly in the freezer for 30 minutes.

D. Step 9

I. Use a warm Buffer SE for elution to facilitate the DNA elution from the column membrane. The Buffer SE can be heated in the microwave for 1 minute or placed in a water bath until it reaches 70°C.

II. Following the recommendations of the manufacturer, incubate for 1 minute after adding Buffer SE to the centre of the silica membrane before centrifugation.

III. Following the manufacturer's recommendations, transfer the DNA eluate back to the column, incubate for an additional 1 minute, and then proceed to centrifugation.

After extracting DNA from the faeces samples, the extracted samples were then analysed using the Nanodrop DeNovix DS-11 spectrophotometer to estimate the DNA concentration (in ng/uL) and purity was assessed by calculating the absorbance ratio at 260 nm and 280 nm. The DNA extraction readings were taken five times for each extraction attempt across all three different protocols (the original protocol, the chicken protocol, and the modified protocol). If there is no DNA or the yield is below 5 ng/uL, the protocol should be repeated with a new extraction sample until the minimum concentration can be achieved (Liu & Harada, 2013). The obtained results were then tabulated. The data was then analysed using ANOVA, with a p-value < 0.05, indicating statistical significance to assess whether there were significant differences in the sample concentration readings among the three protocols.

Results and Discussion

Data was represented simply by taking five DNA concentration readings for each extraction protocol across the seven wild bird species (Table 2). The table showed an increase in mean concentration from each extraction protocol across the seven wild bird species.

Using the original protocol, many of the DNA concentration readings were consistently low, with most readings falling below 3 ng/µL (Table 2). The DNA extraction from the faeces sample of the Eurasian Tree Sparrow (*Passer montanus*) yielded the lowest mean DNA concentration, followed by the Spotted Dove (*Streptopelia chinensis*) and the Oriental Magpie-robin (*Copsychus sauLaris*). The mean concentrations for the three faeces samples barely reached 1.0 ng/uL (Table 2). Meanwhile, the same samples were extracted using the chicken protocol, including an additional step recommended by the manufacturer for elution. Most of the readings remained below 6 ng/µL (Table 2), with some mean concentration readings even falling below 2 ng/µL. Therefore, additional troubleshooting was conducted to

Table 2: The concentration results are based on different protocols

Samples	Trials	Concentration (ng/uL)		
		Original Protocol	Chicken Protocol	Amended Protocol
Malaysian Blue Flycatcher (*Cyornis turcosus*) (A14014)	1	1.505	4.750	7.161
	2	1.034	5.782	11.975
	3	1.203	5.369	11.351
	4	1.022	4.698	9.700
	5	1.101	4.871	7.831
Spotted Dove (*Streptopelia chinensis*) (E0246)	1	0.822	1.701	5.372
	2	0.276	1.711	5.242
	3	0.612	2.178	4.812
	4	1.013	1.617	6.154
	5	1.227	1.588	5.100
Crow-billed Drongo (*Dicrurus annectens*) (B4204)	1	3.476	5.607	8.810
	2	4.202	5.795	8.650
	3	3.945	6.793	11.032
	4	4.756	5.179	10.509
	5	4.674	4.892	8.632
Oriental Reed Warbler (*Acrocephalus orientalis*) (A10514)	1	3.762	6.577	8.816
	2	3.199	5.229	7.794
	3	2.738	7.650	6.743
	4	4.480	5.574	9.742
	5	3.982	7.013	9.058
Eurasian Tree Sparrow (*Passer montanus*) (A10515)	1	1.002	6.118	5.988
	2	0.281	6.204	7.303
	3	0.992	5.777	7.010
	4	0.877	5.404	6.282
	5	0.644	5.121	6.771
Yellow-vented Bulbul (*Pycnonotus goiavier*) (B4213)	1	5.786	6.529	11.032
	2	4.889	6.915	8.650
	3	5.131	6.088	10.509
	4	5.888	6.906	9.255
	5	4.407	6.333	12.644
Oriental Magpie-robin (*Copsychus sauLaris*) (B4214)	1	1.443	5.351	7.951
	2	0.195	4.862	9.131
	3	0.782	6.011	7.738
	4	1.200	4.991	8.254
	5	0.972	5.744	8.856

refine the protocol based on the DNA extraction outcomes using the original and the chicken protocols.

During the sample preparation (Step 1) of the modified protocol, any residual ethanol was completely evaporated before proceeding with DNA extraction, as ethanol residue can interfere with DNA binding to the column. As is known, this ethanol evaporation step was only performed in the amended protocol. Before the commencement of the evaporation step, an aliquot of the homogenised sample was transferred to a new tube. As most of the faeces samples were in liquid form, homogenisation was easier than that of the hard-form samples. After the evaporation step, PBS solution was added to a tube containing 100 mg to 150 mg of faeces sample before adding 1,000 uL lysis buffer ST1.

There are two reasons why PBS solution needs to be added to a tube containing the faeces sample. Firstly, adding only lysis buffer ST1 caused the faeces samples to absorb the buffer and swell, complicating mechanical disruption. The second reason is that adding water before the lysis buffer ST1 leads to inconsistent results. Therefore, PBS solution was added before adding the lysis buffer ST1. Current trials demonstrated that PBS buffer produced better and more consistent results. Moreover, the PBS buffer helps prevent unnecessary cell rupture by maintaining osmotic balance (Fukushima *et al*., 2005). Besides, this preserved the DNA integrity before adding lysis buffer ST1, as most DNA had already been released due to cell lysis caused by storage in molecular-grade ethanol.

In Step 2 of the modified protocol, the BeadBugÔ Benchtop Homogenizer machine was used for mechanical disruption to achieve better homogenisation. Meanwhile in Step 3, incubation after adding the lysis buffer ST2 was carried out in the freezer (–20°C), rather than on ice. The samples were left to incubate for 30 minutes. By implementing these revised procedures, the samples exhibited a noticeably clearer appearance following centrifugation. The clarity of the supernatant made it much simpler to pipette, significantly reducing the presence of any floating contaminants that could interfere with the results. In Step 9, the elution process was carried out twice, utilising a warm elution buffer designated as SE, which enhanced the efficiency of the extraction. DNA eluate from the first elution was passed through the same column membrane during the second elution to maximise yield.

ANOVA (Table 3) showed a significant relationship between the samples and protocol types, with a value of $2.31x10^{-9}$ (p-value < 0.05, which is significant). This proved that the modified protocol significantly improved the DNA extraction yield from bird faeces. Through troubleshooting the faeces extraction kit, a method to improve DNA yields from the faeces of wild birds has been achieved.

These findings showed that adjustments could improve DNA yield from faeces samples, but these adjustments need to fully address the challenges of DNA extraction from the faeces samples. Even with the modified protocol, most results still did not meet the Illumina sequencing platform's standard, requiring a minimum concentration of 10 ng/uL and a volume of

Table 3: ANOVA: Two-factors with replication for each species to determine the p-value

Source of Variation	SS	df	MS	F	p-value	F crit
Species	219.328397	6	36.55473279	52.89392814	$1.77x10^{-26}$	2.208554
Protocols	620.510939	2	310.2554695	448.9331273	$1.42x10^{-45}$	3.105157
Interaction	63.3520502	12	5.279337513	7.639090145	$2.31x1^{0-9}$	1.869289
Within	58.0519856	84	0.691095067			
Total	**961.243372**	**104**				

at least 30 uL. While the samples could be sequenced for NGS, there are concerns about the accuracy of the results. Although a previous study suggested that DNA concentration and quality might be less critical after successful DNA amplification (Herbet *et al*., 2010), the standard concentration is still required for a more accurate result, raising concerns about potential biases.

Apart from the issues of the DNA extraction methods, factors such as the storage duration of the faeces samples can also affect the DNA extraction yield. In this study, all the samples were stored in 99.8% molecular grade ethanol, as it was the most stable storage option during the two-week sampling period. A higher percentage of ethanol was preferred as a cheaper preservation medium as it can minimise the changes in the community composition of microbes in the faeces samples even at ambient temperature for weeks (Song *et al*., 2016). This is because ethanol at higher concentrations between 95% to 100% facilitates faster penetration of cellular membranes and deactivation of DNAses (King & Porter, 2004). Since a freezer was not available to store the faeces samples preserved in ethanol, the samples were kept in a cool box with ice packs that were continuously replaced to maintain a cold temperature. After returning from the sampling site, the faeces samples were stored in the lab freezer at -80°C and ready to be used for DNA extraction.

Another important factor is the uric acid found in bird faeces. It is known that uric acid and faecal matter are expelled simultaneously through the bird's cloaca, numerous studies suggest that this mixture affects the DNA extraction process (Eriksson *et al*., 2017). However, the issue can be minimised by adding Proteinase K to the faeces samples during sample lysis. Apart from uric acid, other secondary metabolites and some chemicals present in the cloacal canal can also interfere with DNA extraction, potentially leading to low yield and poor quality of DNA (Vargas-Pellicer *et al*., 2019). Moreover, a bird's diet can influence the consistency of its faeces ranging from liquid, soft, or hard matter. If certain food materials are not properly digested, they can introduce secondary metabolites in addition to uric acid that inhibit the DNA extraction process (Hou *et al*., 2021). Nevertheless, uric acid presence is a significant factor that affects DNA extraction efficiency.

Conclusions

This research aimed to enhance DNA extraction from wild bird faeces by comparing three methods. Testing samples from seven wild bird species, the revised protocol gave higher concentration readings than the chicken and original methods. This enhancement underscores the importance of adapting kits to bird faeces characteristics. Thus, our institute optimises DNA extraction from wild bird faeces and aids in developing standardised methods for microbiota and metagenomic analysis.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Appendix

Table A1: Count, sum, mean, and average of each species that will be used for ANOVA

Summary	Original Protocol	Chicken Protocol	Amended Protocol	Total
Malaysian Blue Flycatcher (*Cyornis turcosus*)				
Count	5	5	5	15
Sum	5.865	25.47	48.018	79.353
Mean	1.173	5.094	9.6036	5.2902
Variance	0.0396075	0.2184625	4.4486608	14.05736946
Spotted Dove (*Streptopelia chinensis*)				
Count	5	5	5	15
Sum	3.95	8.795	26.68	39.425
Mean	0.79	1.759	5.336	2.628333333
Variance	0.1344005	0.0576585	0.252382	4.222221952
Crow-billed Drongo (*Dicrurus annectans*)				
Count	5	5	5	15
Sum	21.053	28.266	47.633	96.952
Mean	4.2106	5.6532	9.5266	6.463466667
Variance	0.2806138	0.5314192	1.3283978	6.009668124
Oriental Reed Warbler (*Acrocephalus orientalis*)				
Count	5	5	5	15
Sum	18.161	32.043	42.153	92.357
Mean	3.6322	6.4086	8.4306	6.157133333
Variance	0.4613072	1.0056863	1.3787968	4.95850241
Eurasian Tree Sparrow (*Passer montanus*)				
Count	5	5	5	15
Sum	3.796	28.624	33.354	65.774
Mean	0.7592	5.7248	6.6708	4.384933333
Variance	0.0922427	0.2136127	0.2855387	7.37124521
Yellow-vented Bulbul (*Pycnonotus goiavier*)				
Count	5	5	5	15
Sum	26.101	32.771	52.09	110.962
Mean	5.2202	6.5542	10.418	7.397466667
Variance	0.3862577	0.1302117	2.4546865	6.054333981
Oriental Magpie-robin (*Copsychus sauLaris*)				
Count	5	5	5	15
Sum	4.592	26.959	41.93	73.481
Mean	0.9184	5.3918	8.386	4.898733333
Variance	0.2248223	0.2376117	0.3506195	10.32058564

Table A2: Count, sum, mean, and average of all seven species that will be used for ANOVA

Total (All Seven Species)	Original Protocol	Chicken Protocol	Amended Protocol
Count	35	35	35
Sum	83.518	182.928	291.858
Mean	2.386228571	5.226514286	8.3388
Variance	3.386644593	2.584513257	4.050384282