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EFFICIENCY OF DNA EXTRACTIONS METHODS FROM PIGEONS AND COCKATIELS FEATHERS

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Abstract: This study aimed to compare different DNA extraction methods to achieve higher amounts and purity levels from molted feathers of pigeons (*Columba livia* f. *domestica*) and cockatiels (*Nymphicus hollandicus*). We evaluated 226 animals consisting of 202 pigeons and 24 cockatiels for these purposes. We performed three commercially available DNA extraction kits to isolate DNA from the feather samples. These kits were compared regarding DNA yield and quality depending on the different applications made during the isolation. DNA concentration (ng/mL) and absorbance ratio (260/280) were measured using a Nanodrop spectrophotometer. Kruskal-Wallis test with the Dunn's post hoc comparison was performed for the statistical comparisons. The mean DNA concentration was the highest in isolation with the kit C. Among three commercial kits, statistically significant differences were observed concerning nucleic acid concentration (ng/µL) ($P<0.001$). Also, the best 260/280 nm ratio absorbance was obtained with the kit B, while the lowest purity was obtained from kit C. Moreover, the concentration and purity of DNA were detected as higher in cockatiels than in pigeons, and the significant differences were determined between birds based on spectrometric measurements ($P<0.001$). In conclusion, the reported findings in this study may be helpful for the DNA extraction from the feather samples collected non-invasively in the field for genetic analysis in birds.

Keywords: Feather, DNA extraction, Pigeon, Cockatiel, DNA quality, Spectrophotometer

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1. Introduction

In avian study, the accurate extraction of DNA is vital for a wide range of studies, including population genetics, phylogenetics, and conservation biology. Reliable DNA extraction methods enable researchers to obtain high-quality genetic material that can be used for various downstream applications. The choice of appropriate extraction protocols is particularly critical when comparing different bird species, as variations in feather structure and composition can affect the efficiency and quality of DNA extraction (Taberlet and Bouvet, 1991; Bello et al., 2001; Freedman et al., 2008; Adam, Scharff and

Honarmand, 2014). Traditionally, obtaining genetic information required invasive methods, such as capturing birds and collecting blood or tissue samples. However, these methods can be stressful for birds and time-consuming for researchers. Non-invasive sampling methods have emerged as a promising alternative, allowing scientists to extract DNA without physically handling the birds (Ellegren, 1991; Sacchi et al., 2004; Horváth et al., 2005; Presti et al., 2013; Zemanova, 2021). Pigeons (*Columba livia*) and cockatiels (*Nymphicus hollandicus*) are two avian species commonly studied due to their diverse genetic backgrounds and population dynamics of bird species. However, there is a lack of standardized protocols for extracting DNA from feathers in these species. Hence, it is imperative to assess and contrast different DNA extraction techniques designed specifically for pigeons and cockatiels (Yılmaz and Boz., 2012; Grindol, 1998). Pigeons and cockatiels, in particular, have long been favored for their unique qualities. Pigeon breeding is an ancient practice that has been refined over generations. The domestic pigeon has been bred for different purposes for 6,000 years or more. More than 800 breeds have been described since it was domesticated. They are bred for their beauty in appearance, ability to fly and navigation, and meat. Pigeons can be grouped as diver, tumbler, reeler, spinner, fleet flyer, high flyer, mail, ornamental and passerine according to their breeding purposes (Yılmaz and Boz, 2012). By selecting pigeons with desired traits such as feather patterns or flight capabilities, breeders have been able to create a wide variety of pigeon breeds. Cockatiels, on the other hand, are beloved for their intelligence and ability to mimic human speech. Breeding parrots requires a deep understanding of their complex social structures and behavioral patterns. By pairing parrots with compatible personalities and ensuring optimal living conditions, breeders aim to produce healthy and well-adjusted offspring. This not only enhances the welfare of the birds but also contributes to the

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conservation efforts of endangered parrot species (Grindol, 1998; Banaszewska et al., 2015).

Commercial DNA extraction kits provide reagents and spin column filters to isolate DNA from feather samples. Kits often use lysis buffers and Proteinase K to break down feather material, then DNA is bound to a silica membrane spin column and washed. Kits can be more expensive but convenient, avoiding toxic chemicals and providing high-quality, concentrated DNA. Kits designed specifically for isolating DNA from hair, feathers, or other keratinized materials tend to work best for feather samples (Şentürk et al., 2023).

Although the use of shed feathers is preferred because it is difficult to take blood and tissue samples in birds, the keratin structure of the feathers complicates the process. However, determining the practical method of DNA isolation is essential for the continuity of genetic analysis. In this context, this study aims to compare the effectiveness of different DNA isolation methods from the molted feathers of birds, including pigeons and cockatiels.

2. Materials and Methods

2.1. Sample Collection

In this study, naturally fallen feathers in a cage from the wing and tail parts of the birds were used, and they were placed in tubes with the help of ethanol-sterilized forceps for DNA analysis. Pigeon feathers typically contain an expected range of 10-30 nanograms of DNA per feather, while cockatiel feathers contain approximately 5-15 nanograms. Thus, at least 3-5 feathers per bird were used to obtain enough high-quality DNA for the designed study. Samples in each tube were labeled with the bird's identity, feather type, and collection date and stored at +4°C until DNA isolation. The critical point to note here is that the earlier the DNA is isolated, the better results can be obtained since the yield and quality of DNA may decrease over time.

2.2. DNA Extraction

Feather samples were obtained from individual breeders to isolate genomic DNA. Two hundred twenty-six birds consisting of 202 pigeons and 24 cockatiels were chosen randomly and used in this study. Under sterile conditions, feathers are placed on the petri dish to cut each sample. Before applying commercial isolation kits, the DNA-containing part of two different areas for feathers, which are the basal tip of the calamus and blood clot from the superior umbilicus (Figure 1), were cut with the help of a scalpel and divided into small pieces (Horváth et al., 2005). Three commercial DNA extraction kits were used to isolate DNA from feather samples, following each kit protocol based on manufacturer instructions.



Figure 1. Different sampling areas for feathers are shown: (A) barbs; (B) blood clot from the superior umbilicus; (C) calamus and basal tip of the calamus.

2.3. Statistical Analysis

Statistical analysis was performed by GraphPad Prism 9 (Graph-Pad Software, La Jolla, USA). The Anderson-Darling test was utilized to assess the normality of the data. Kruskal-Wallis with Dunn's post hoc multiple comparison tests were performed to determine differences between groups, with $P<0.05$ considered statistically significant.

3. Results and Discussion

The use of noninvasive techniques has increased greatly over the past decades as the development of molecular methods has facilitated the use of noninvasive tissues to sample genetic material from natural populations. DNA sexing in birds can be done on a variety of easily accessible non-invasive samples, such as feces, feathers, or buccal swabs. Due to the feather shafts' ability to shield DNA molecules from damaging factors like UV rays, hydrolysis, frequent freezing and thawing, and bacteria, molted feathers are potentially a valuable source of DNA. The relationship between the selected extraction kits with regard to nucleic acid concentration

was presented in Table 1 respectively. The mean DNA concentration was the highest in isolation with the kit C, followed by kit A and kit B results. The difference between these three kits was found to be statistically significant for the amount (Figure 2A) and purity (Figure 2B). Means, standard errors, minimum and maximum values for nucleic acid concentration (ng/µL) based on isolation with different commercial kits are presented in Table 1.

Table 1. Means, standard errors (SE), minimum (min) and maximum (max) values for nucleic acid concentration (ng/µL) based on the isolation with the different commercial kits

Isolation kit	Mean±SE	CV	Min	Max
Kit A	16.81± 3.33[b]	201,30	1.00	331.80
Kit B	5,74±0.95[c]	159,16	0.40	58.10
Kit C	24.01± 5.76[a]	131,37	1.30	108.50

[a,b,c]Means with different superscripts are different (P<0.001). CV refers to coefficient of variation.

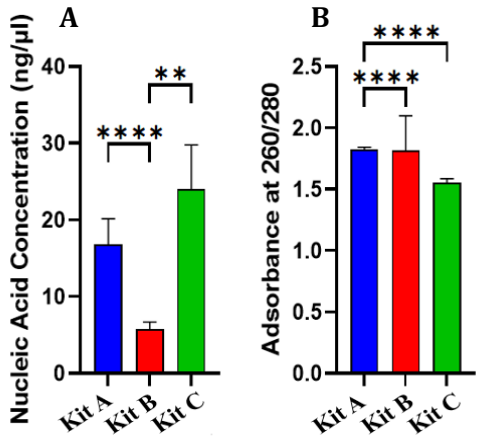


Figure 2. The comparison among three commercially available DNA isolation kits regarding amount and purity of the DNA samples. (A) The analysis on nucleic acid concentration, ng/µL. (B) The analysis on purity of the samples, absorbance at 260/280. The statistical analysis was performed using Kruskal Wallis with the Dunn's post hoc comparison. *P<0.05; **P<0.01; ***P<0.001; ****P<0.00001.

The ratio between the absorbance of the sample at the wavelength of 260 and 280 nm is used to assess DNA purity and integrity. A ratio of about 1.8 is generally accepted as "pure" for DNA. If the ratio is lower than 1.6, it may indicate the presence of phenol or other contaminants that absorb strongly at or near 280 nm. Higher ratios can indicate that DNA has contaminated isolated proteins (William et al., 1997). The impacts of the selected extraction kits, bird species, anatomic region, and incubation alteration on the absorbance ratio of 260/280 nm were presented in Tables 2, 3, 4, 5, 6, 7 and 8, respectively. In this study, significant differences in DNA purity were observed among the studied three kits (Figure 2B). The ideal 260/280 ratio value was obtained with the kit B, while the lowest value was obtained from kit C. On the other hand, DNA concentration was the highest in isolation with the kit C, followed by kit A and kit B results. The difference between these three kits was found to be statistically significant (P<0.001). Table 2 shows the means, standard errors, minimum and maximum values for the absorbance ratio of 260/280 nm based on the isolation with the different commercial kits.

Table 2. Means, standard errors (SE), minimum (min) and maximum (max) values for the absorbance ratio of 260/280 nm based on the isolation with the different commercial kits

Isolation kit	Mean±SE	CV	Min	Max
Kit A	1.82±0.02[b]	11,62	1.24	2.32
Kit B	1.82±0.28[a]	148,46	0.10	20.85
Kit C	1.55±0.03[c]	12,09	1.14	1.93

[a,b,c]Means with different superscripts are different (P<0.001). CV refers to coefficient of variation.

Table 3. Means, standard errors (SE), minimum (min) and maximum (max) values for nucleic acid concentration (ng/µL) based on bird species

Species	Mean±SE	CV	Min	Max
Pigeon	11.04±1.20[b]	152.61	0.40	108.50
Cockatiel	31.5 ±13.3[a]	206.64	6.5	331.8

[a,b]Means with different superscripts are different (P<0.001). CV refers to coefficient of variation.

Table 4. Means, standard errors (SE), minimum (min) and maximum (max) values for the absorbance ratio of 260/280 nm based on bird species

Species	Mean±SE	CV	Min	Max
Pigeon	1.78±0.13[b]	103.29	0.10	20.85
Cockatiel	1.85±0.02[a]	6.23	1.49	2.000

[a,b]Means with different superscripts are different (P<0.001). CV refers to coefficient of variation.

Table 5. Means, standard errors (SE), minimum (min) and maximum (max) values for nucleic acid concentration (ng/µL) based on anatomic region

Anatomic Region	Mean±SE	CV	Min	Max
Only the calamus	12.34±2.04[b]	219,68	0.4	331.80
With barbs	16,26±3.73[a]	162,25	0.5	108.50

[a,b]Means with different superscripts are different (P<0.001). CV refers to coefficient of variation.

Table 6. Means, standard errors (SE), minimum (min) and maximum (max) values for the absorbance ratio of 260/280 nm based on anatomic region

Anatomic Region	Mean±SE	CV	Min	Max
Only the calamus	1.782±0.098	72,68	0.1	15.970
With barbs	1.795±0.396	155,98	0.1	20.850

CV refers to coefficient of variation.

Table 7. Means, standard errors (SE), minimum (min) and maximum (max) values for the nucleic acid concentration (ng/µL) based on incubation time

Incubation Time	Mean	CV	Min	Max
1 Hour	13.64 ±1.9[c]	39.69	5.30	22.50
2 Hour	18.66 ±4.56[b]	208.87	2.30	331.80
3 Hour	10.48±1.74[d]	182.47	0.40	108.50
4 Hour	22.8±10.5[a]	122.01	8.3	85.3
Overnight	5.11±1.29[e]	104.04	1.40	22.30

[a,b,c,d,e]Means with different superscripts are different (P<0.001). CV refers to coefficient of variation.

Table 8. Means, standard errors (SE), minimum (min) and maximum (max) values for the absorbance ratio of 260/280 nm based on incubation time

Incubation Time	Mean	CV	Min	Max
1 Hour	1.943±0.048[a]	6.93	1.69	2.12
2 Hour	1.791±0.019[e]	9.15	1.37	2.20
3 Hour	1.763±0.215[c]	134.30	0.10	20.85
4 Hour	1.879±0.067[b]	9.75	1.51	2.10
Overnight	1.802±0.099[d]	22.59	1.07	2.42

[a,b,c,d,e]Means with different superscripts are different (P<0.001). CV refers to coefficient of variation.

When studying large bird species, using feathers instead of blood as a source for genomic DNA reduces stress on the bird and makes sampling easier. In the study by Bello et al. in 2001, it was emphasized that lysis temperature and incubation times differ depending on feather size. In this study, regardless of the size of the cockatiel and pigeon feathers examined, the calamus parts were cut and the incubation time and lysis temperature appropriate to the procedures were applied. The study noted a substantial influence of cockatiel feathers being longer than pigeon feathers on concentration and purity (Figure 3A). In addition, when the 260/280 nm absorbance ratio was compared according to bird species, it was found that DNA purity was better in cockatiels than in pigeons (Figure 3B). The difference between birds was also statistically significant (P<0.001). Means, standard errors, coefficient of variation, minimum and maximum values for nucleic acid concentration (ng/µL) and absorbance ratio of 260/280 nm based on bird species are presented Table 3 and Table 4.

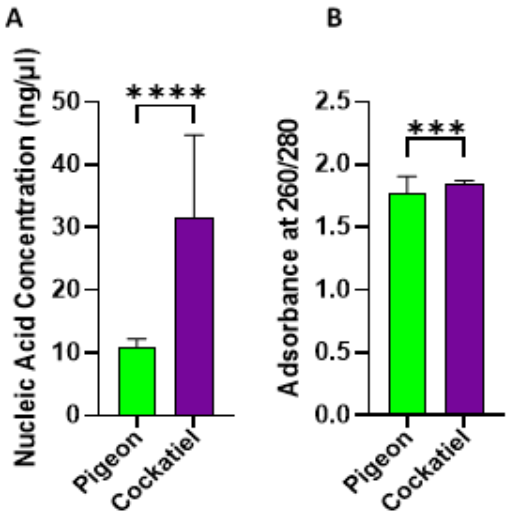


Figure 3. Comparison between the sampled species regarding amount and purity of the DNA samples. (A) The analysis on nucleic acid concentration, ng/µL. (B) The analysis on purity of the samples, absorbance at 260/280. The statistical analysis was performed using Mann-Whitney U test. **P<0.001; P<0.0001.

Several studies reported that the quality and quantity of DNA obtained from non-invasive samples can vary significantly, requiring optimization of extraction techniques and the development of standardized protocols (Avanus and Koenhemsi, 2018; Şentürk et al., 2023). In the study conducted by De Volo et al. in 2008, the effect of feather size on DNA yield was examined and as a result, it was stated that large feathers had higher DNA yield than small feathers, but no significant difference was observed between feather size and DNA amplification. In addition, it has been determined that the use of the superior umbilicus part of the bird feathers in addition to the calamus parts will increase the DNA concentration obtained by approximately two times. In this study, we observed that there is a significant relationship with DNA concentration when we included the superior umbilicus part of both cockatiel feathers and pigeon feathers, in addition to the calamus parts ($P<0.01$) (Figure 4A). However, no significant relationship was detected with the 260/280 nm absorbance ratio ($P>0.05$) (Figure 4B).

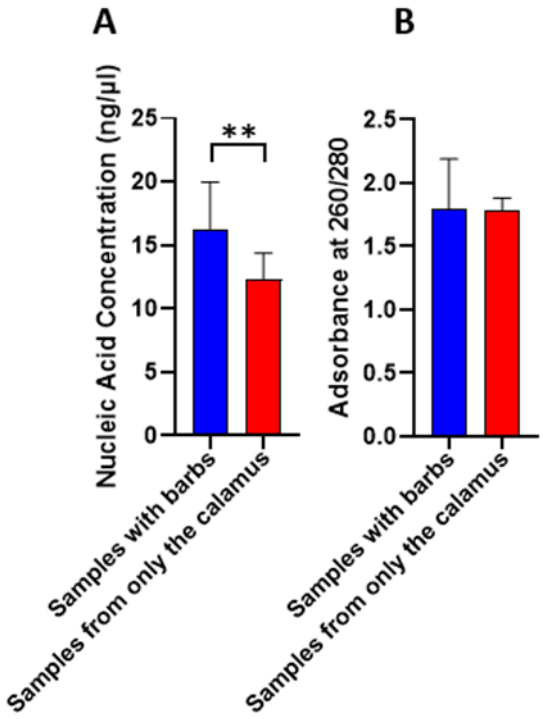


Figure 4. Comparison of structural features of sampled feathers regarding amount and purity of the DNA samples. (A) The analysis on nucleic acid concentration, ng/µL. (B) The analysis on purity of the samples, absorbance at 260/280. The statistical analysis was performed using Mann-Whitney U test. **P<0.01.

For nucleic acid content (ng/µL) and absorbance ratio of 260/280 nm based on anatomic region, means, standard errors, coefficients of variation, minimum and maximum values are shown in Tables 5 and 6. Furthermore, this study investigated the impact of variations in incubation times during the kit procedures on nucleic acid concentration and the 260/280 absorbance value. Means, standard errors, coefficient of variation, minimum and maximum values for the DNA concentration and absorbance ratio of 260/280 nm based on incubation time are investigated table 7 and table 8. In this context, significant results were observed for both DNA concentration and purity. The highest purified DNA amount was observed for 4h incubation (Figure 5A). The present results indicated that the most desirable 260/280 absorbance values were observed after a 2-hour incubation period (Figure 5B).

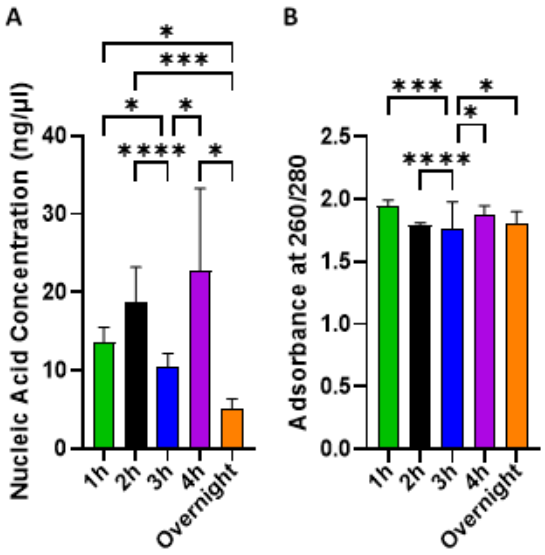


Figure 5. The comparison among various incubation applications regarding amount and purity of the DNA samples. (A) The analysis on nucleic acid concentration, ng/µL. (B) The analysis on purity of the samples, absorbance at 260/280. The statistical analysis was performed using Kruskal Wallis with the Dunn's post hoc comparison. *P<0.05; ***P<0.001; ****P<0.00001.

In DNA samples, particularly in routine studies, purity takes precedence over quantity. Hence, DNA isolation kits are more suitable for routine applications, where speedy results are desired. DNA purity is a critical factor regardless of the scenario, as samples with impurities outside the desired range pose challenges for subsequent processing and lead to lower success rates. Various factors, including the amount of proteinase K used in the procedure, the degree of agitation during incubation, and the temperature and duration of incubation, can influence the results. Therefore, it is essential to gather data on these factors to optimize the methodology. In this study, we found that a 2-hour incubation period yields the desired level of purity. It is worth noting that differences may arise due to individual practices, application specifics, and sample characteristics. The feather structures of birds, sourced from diverse regions and displaying distinct characteristics, can exhibit variations not only between species but also among individual birds (Figure 6). Nevertheless, this study can serve as a valuable reference for future research.



Figure 6. Various feather structures of birds from distinct body regions in the present study, each possessing its unique characteristics.

When comparing the results of this study with the commonly used standard phenol-chloroform isolation method, it becomes evident that although the standard method is cost-effective and provides higher genomic DNA yields, it is a complex and time-consuming process. Commercial DNA isolation kits are designed to provide a standardized and reproducible DNA isolation protocol and consistent results between experiments. This eliminates the need for manual optimization and reduces the possibility of human error (Silva et al., 2020; Şentürk et al., 2023; Sakyi et al., 2023). This study delves into the commonly employed methods and their adaptations for achieving the desired levels of DNA yield and purity during the isolation process from bird feathers. Methodological investigations hold significant value in genetic research, serving to optimize techniques and address challenges that may arise during their implementation, offering potential solutions. These findings are particularly invaluable for small-sized bird species prone to stress.

4. Conclusion

DNA isolation is the most essential step in genetic analysis. High quantity and quality DNA samples are indispensable for successful genetic analysis. Therefore, the determination of DNA isolation methods is of great importance. The structure of the tissue to be isolated also affects the quality of the isolated DNA sample. Some methods need to be modified in keratin-rich tissues, such as feathers. This study details the effects of three different methods and the effects of modifications on DNA quality and quantity based on commercially available DNA isolation kits. As a result, it may be thought that the results obtained will shed light on a wide range of studies in the fields of bird molecular genetics, population genetics, and phylogenetics.

Author Contributions

The percentage of the author contributions is presented below. The author reviewed and approved the final version of the manuscript.

	Ö.Ç	N.Ş.	S.A.
C	50		50
D	50		50
S	40	20	40
DCP	40	30	30
DAI	20	40	40
L	30	40	30
W	40	30	30
CR	30	30	40
SR	30	30	40

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision

Conflict of Interest

The author declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on live animals or humans.

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