ISSN: 1220-3173; E-ISSN: 2457-7618

AN EXPERIMENTAL STUDY OF COLLECTION AND PRESERVATION OF BIOLOGICAL SAMPLES FOR MOLECULAR SEXING IN COMPANION BIRDS

UN STUDIU EXPERIMENTAL PRIVIND RECOLTAREA ȘI CONSERVAREA PROBELOR BIOLOGICE ÎN VEDEREA SEXĂRII MOLECULARE A PĂSĂRILOR DE COMPANIE

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ABSTRACT | REZUMAT

The sex determination of many companion birds is challenging due to the absence of clear sexual dimorphic traits. Early sex determination in monomorphic birds holds various practical applications. Molecular genetic sexing is recognized as one of the most accurate methods for determining the sex of monomorphic birds. Samples, whether collected by qualified personnel or owners, require processing in a specialized laboratory. This study aimed to assess the integrity of samples kept at room temperature (20–22 °C) in order to optimize sample transport and storage while comparing DNA concentrations in various sample types collected in companion birds. Oral swabs and whole blood, along with fresh and heparinized blood collected on absorbent filter paper (DBS - dried blood spots), were simultaneously sampled from three companion bird species (*Columba livia domestica, Psittacula krameri,* and *Psittacus erithacus*). These samples were stored at room temperature for 30 days, and molecular sexing was performed on days 0, 1, 2, 4, 7, 14, and 30. PCR using P2 and NP primers, was conducted to amplify the CHD1W and CHD1Z genes in females and the CHD1Z gene in males. After 30 days at room temperature, the dried blood spots (DBS) and oral swabs tested in this study proved to be reliable for genetic sex determination.

Keywords: pet birds; genetic sexing; PCR; blood; DBS; oral swabs; room temperature

Determinarea sexului reprezintă o provocare la multe păsări de companie, datorită absenței trăsăturilor dimorfice sexuale. Identificarea precoce a sexului la păsările monomorfe are diverse aplicații practice. Sexarea genetică moleculară este recunoscută ca una dintre cele mai precise metode pentru determinarea sexului la păsările monomorfe. Probele, fie ele colectate de personal calificat sau de către proprietari, necesită procesare într-un laborator specializat. Acest studiu își propune să evalueze integritatea probelor păstrate la temperatura camerei (20–22 °C) în scopul optimizării condițiilor de transport și a stocării acestora, comparând în același timp concentrațiile de ADN în diverse tipuri de probe recoltate de la păsări de companie. Probe de tampoane orale, probe de sânge integral colectate pe hârtie de filtru absorbantă (DBS - pete uscate de sânge) și probe de sânge proaspăt heparinizat au fost recoltate simultan de la trei specii de păsări de companie (*Columba livia domestica, Psittacula krameri* și *Psittacus erithacus*). Probele au fost depozitate la temperatura camerei 30 de zile, iar sexarea moleculară a fost efectuată în zilele 0, 1, 2, 4, 7, 14 și 30. PCR cu primerii P2 și NP a fost utilizat pentru amplificarea genelor CHD1W și CHD1Z la femele și a genei CHD1Z la masculi. După 30 zile la temperatura camerei, ”DBS” și tampoanele orale s-au dovedit a fi fiabile pentru determinarea genetică a sexului păsărilor, în timp ce probele de sânge integral s-au deshidratat.

Cuvinte cheie: păsări de companie; sexare genetică; PCR; DBS; tampoane orale; temperatura camerei

More than half of the avian species on Earth lack recognizable sexual dimorphic characteristics (16). Sexual dimorphism may manifest in certain parrot species, such as African Grey Parrots (*Psittacus erithacus*), upon reaching sexual maturity (7). African Grey Parrots, generally considered monomorphic, attain sexual maturity between 3 and 5 years without exhibiting sexual dimorphism during this period (7; 16).

In the case of Rose-ringed Parakeets (*Psittacula krameri*), sexual dimorphism is apparent only in adult males, distinguished by the presence of a black ring around their necks; females and sexually immature individuals of both sexes lack this feature. Sexual maturity and associated sexual dimorphism in Rose-ringed Parakeets typically occur after the age of 3 years (7; 16). Domestic pigeons (*Columba livia domestica*), considered monomorphic, are additionally perceived as monogamous (23). Traditional methods for sexing pigeons involve observing secondary sexual traits like neck plumage, body size, and beak dimen-

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sions, as well as monitoring behaviour, including distinctive songs and dances during mating rituals—an approach that, while belated, has 100% accuracy in identifying females (16;23).

Various disciplines, including behavioural medicine, conservation biology, wildlife management, evolutionary studies through avian species breeding, and forensic medicine, have widely applied genetic sexing methods to birds. Molecular sexing, as highlighted by Cerit and Avanus (2007), Fritz and Janák (2022), Ito et al. (2003), and Morinha et al. (2012), not only plays a crucial role in these fields but also contributes significantly to poultry reproduction strategies and the enhancement of captive bird breeding programs. The genetic sexing of birds is particularly relevant in the context of highly social pet birds that are often kept in pairs, as early sex identification is proven to be valuable for bird owners. Peng and Broom (2021) underline the positive impact of pairing parrots on their welfare. The sex determination system in birds is characterized by ZW sex chromosomes in females and ZZ in males, contrary to the XY system in mammals. The CHD (chromodomain helicase DNA-binding) gene, situated on the sex chromosomes of birds, functions as a crucial marker for determining gender through amplification. Male birds carry only the CHD1Z gene, while females possess both the CHD1W and CHD1Z genes (8, 10). Although PCR-based sexing stands out as one of the most accurate methods, its application requires adjustments to laboratory protocols for each avian species (12). Genetic sexing in birds categorizes sample collection into invasive (blood samples, biopsies), moderately invasive (plucked feathers, oral swabs), and non-invasive (moulted feathers, eggshell membranes, faeces) methods. Additionally, from carcasses, various organs such as blood, liver, brain, gonads, intestines, kidneys, skin, bone, and tissues in different stages of autolysis can be used for genetic sexing (4; 20; 22). Given that blood samples exhibit a higher concentration of DNA compared to oral swabs or feather samples, utilizing blood samples for molecular sexing has yielded the most accurate outcomes (11;12;24;25). Blood serves as a sample type from which DNA of satisfactory quality and quantity can be extracted using relatively straightforward techniques. However, the primary drawback of blood collection is its invasive nature, necessitating careful handling of birds, particularly those of smaller sizes (15). An alternative approach for bird blood sample collection involves sampling dried blood spots (DBS) on filter paper, offering advantages such as cost reduction and solving issues related to sample collection, transportation, and processing (21). For sexing very young chicks, small-sized adult birds, or species where blood collection is considered excessively intrusive, oral swabs have been offered as moderately invasive samples (4; 11; 12; 24). These samples are often collected by owners or trained personnel and subsequently transported to specialized laboratories. To enhance the transport and storage conditions of blood and oral swab samples, we have conducted an experimental study to assess the integrity of these biological samples at various time intervals.

The main objective of this study was to determine whether the DNA present in oral swabs, blood samples collected on filter paper (as DBS - "dried blood spots"), and whole blood samples undergo changes when stored at room temperature (20-22 °C) for 30 days.

The aim of this study is to facilitate the transport and storage conditions of samples until processing. As a secondary goal, the study aimed to compare DNA concentrations in blood samples collected with anticoagulants and dried blood spots on filter paper (DBS) with the intention of finding a more effective alternative for the transportation, storage, and processing of blood samples for sex determination in companion birds. Furthermore, the research involves a comparison of DNA quantities derived from fresh blood drops on filter paper with standard 20 µl drops of anticoagulated blood on filter paper from the same individuals. Moreover, the study evaluates oral swabs as a minimally invasive alternative for collecting samples for molecular bird sexing in three species of companion birds (*Columba livia domestica, Psittacula krameri* and *Psittacus erithacus*).

MATERIALS AND METHODS

Sample Collection

This study includes both monomorphic and dimorphic companion birds that have undergone sexing through various methods. Live adult birds, whose sex had already been determined using diverse methods, were included in the research. Owners provided all relevant information about the birds, such as species, age, sex, and the previous sexing method, through a questionnaire (Table 1). All procedures conducted on the birds received written consent from the owners.

All samples were collected individually from each bird on the same day. Each bird included in the study underwent the collection of two oral swabs, approximately 0.2-0.3 ml of blood with anticoagulant, and a minimum of 3 filter papers each containing a blood drop (Table 1). Oral swab samples followed the protocol outlined by Handel et al. (2006), utilizing sterile cotton swabs (Prima, Taizhou Honod Medical Co., Ltd., Zhejiang, China). Blood samples were obtained via vein puncture of the metatarsal veins, with 0.2-0.3 ml collected and stored in anticoagulant-containing tubes. Following blood collection, prior to hemostasis, drops of various sizes of fresh blood were collected on a minimum of 3 filter papers (Fig. 1) from the same

Table 1

Samples collected from the 3 companion bird species *(Columba livia domestica, Psittacula krameri and Psittacus erithacus)* included in this study

Species	Sex	Previous sexing methods	Age	Oral swabs	Freshly sampled DBS	Whole blood
Columba livia domestica	F	traditional methods - egg laying	4 years	2	minimum 3	0.2-0.5 ml
	M	traditional methods - mating dance	3 years	2	minimum 3	0.5-0.7 ml
Psittacus erithacus	F	molecular sexing in another laboratory	16 years	2	minimum 3	0.2-0.3 ml
Psittacula krameri	F	sexual dimorphism	11 years	2	minimum 3	0.2-0.3 ml

vein. Moreover, the filter paper used in this study underwent prior sterilization under UV light.

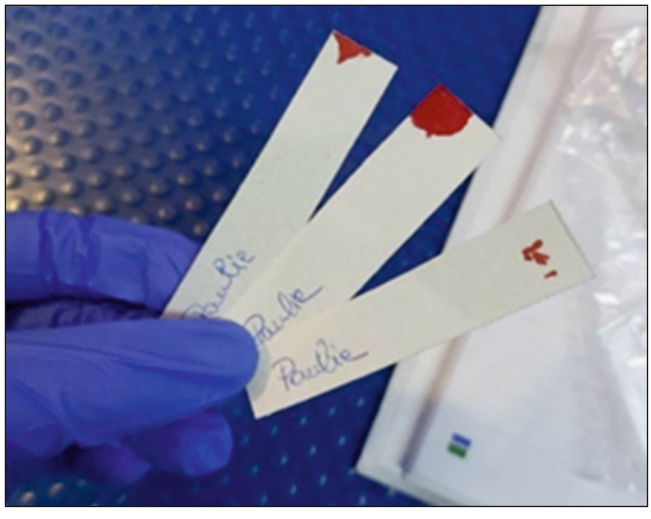

Fig. 1. Freshly sampled DBS

Working protocol

In the laboratory, 20 µl of blood with anticoagulant was distributed onto a minimum of 3 filter papers from the collected 0.2-0.3 ml of blood in tubes. All samples (oral swabs, whole blood tubes, fresh blood filter papers, and anticoagulated blood filter papers) were stored at room temperature (20-22 °C) from February to March 2023. DNA extraction was performed on days 0, 1, 2, 4, 7, 14, and 30. On each day, oral swabs, fresh blood on filter paper, and DBS of 20 µl drops of anticoagulated blood were tested. Additionally, on day 0, DNA was directly extracted from 20 µl of whole blood with an anticoagulant to serve as a control.

DNA Extraction and PCR

DNA extraction was performed using a commercial kit (The DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer`s protocol. Oral swabs and filter papers were cut with sterile scissors and placed in Eppendorf tubes. Samples were processed as "tissue" for oral swabs and filter paper with blood and as "nucleated blood" for whole blood. Before extraction, tissue samples were vortexed with a prelysis solution and proteinase K, and left for 3 hours at 56°C for efficient cell wall degradation. DNA quantification from tissue samples collected from birds was performed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). PCR was conducted using the previously described protocol by Ito et al. in 2003 with primers P2 (5'-TCTGCATCGCTAAATCCTTT-3') and NP (5'-GAGAAACTGTGCAAAACAG-3') (Generi-Biotech, Hradec Králové, Czech Republic). The 25 µl reaction mix included 12.5 µl Mastermix (MyTaq™ Red Mix, Bioline,Meridian Bioscience, Cincinnati, Ohio),1 µl of each primer, 100 ng DNA template, and ultrapure water. Amplification was performed in a C1000TM Thermal Cycler (Bio-Rad Laboratories, Hercules, California). The amplification steps were as follows: 5 sec at 95°C for initial denaturation, followed by 45 sec at 52 °C, 45 sec at 72 °C, and 30 sec at 95 °C (x 35 cycles); 1 min at 50 °C, and for the final extension, 5 min at 72 °C. Subsequently, the amplicons were subjected to electrophoresis on a 3% agarose gel.

RESULTS AND DISCUTION

Results of DNA quantification of samples collected from the three species included in this study are detailed in Table 2. The highest quantity of DNA was identified in whole blood samples of 20 µl. The concentration of DNA resulting from freshly collected blood drops directly on filter paper was lower than the amount of DNA resulting from blood with anticoagulant placed as a standard 20 µl drop on filter paper, while the lowest quantity of DNA was identified in the oral swab samples (Table 2).

The PCR results and electrophoresis identified a single band corresponding to the CHD1Z gene in males, while females showed two bands corresponding to CHD1W and CHD1Z (Fig. 2, Table 3). Moreover, the resulting bands had the same intensity on day 0 as on day 30. Within this study, a male and fe-

Table 2

DNA concentration of the 38 samples collected from the 3 bird species included in the study (Domestic pigeon, Rose-ringed Parakeet, and African Gray Parrot)

Sample type	DNA Concentration (ng/µl)		Mean DNA Concentration (ng/µl)
	MIN.	MAX.	
Oral swab	8.5 ng/µl	17.1 ng/µl	12.52 ± 3.07 ng/µl
Freshly sampled DBS	8.9 ng/µl	29.7 ng/µl	16.21 ± 5.40 ng/µl
DBS of 20 µl drops of anticoagulated blood	12.9 ng/µl	29.7 ng/µl	25.8 ± 5.02 ng/µl
Whole blood with anticoagulant (20 µl)	44.7 ng/µl	45.4 ng/µl	45.05 ± 0.49 ng/µl

male pair of domestic pigeons were identified, and their sexes concurred previous determinations made through traditional behavioural monitoring methods. Additionally, two females were identified, one Rose-ringed Parakeet and the other an African Grey Parrot, whose sexes matched with earlier determinations based on the observation of phenotypic characteristics and a prior molecular test conducted in a different laboratory. Variations in band migration have been observed, particularly among females, displaying distinct patterns based on species. In female birds, the migration of the two bands associated with their gender varies depending on the species. Specifically, the bands in female domestic pigeons migrated within the range of approximately 300-350 bp (Fig. 2, row 9), contrasting with the migration of bands in *Psittacine* females (Rose-ringed Parakeet and African Grey Parrot), which occurred between approximately 350-400 bp (Fig. 2, rows 7 and 8). The success rate of the PCR reaction for sex determination was 100% for whole blood samples, dried blood spots, and oral swabs collected from the three bird species included in this study (Domestic pigeon, Rose-ringed Parakeet, and African Grey Parrot).

The highest DNA quantity was observed in 20 µl whole blood samples, while the concentration of DNA from freshly collected blood drops directly on filter paper was lower than that from blood with anticoagulant placed as a standard 20 µl drop on filter paper.

This discrepancy is attributed to the smaller size of the drops collected on the filter paper, resulting in a lower DNA concentration for freshly collected drops. Notably, the drops of freshly collected blood on filter paper from the male domestic pigeon were an exception, as they were very large, leading to a higher DNA quantity (29.7 ng/µl) compared to the average of standard 20 µl drops of blood with anticoagulant (25.8 ± 5.02 ng/µl). Oral swab samples exhibited the lowest DNA amount; however, they offer the advantage of being minimally invasive and causing minimal stress to the bird, unlike blood collection (14).

Despite the lower DNA quantity, oral swab samples yielded readable and clear bands in PCR and gel electrophoresis.

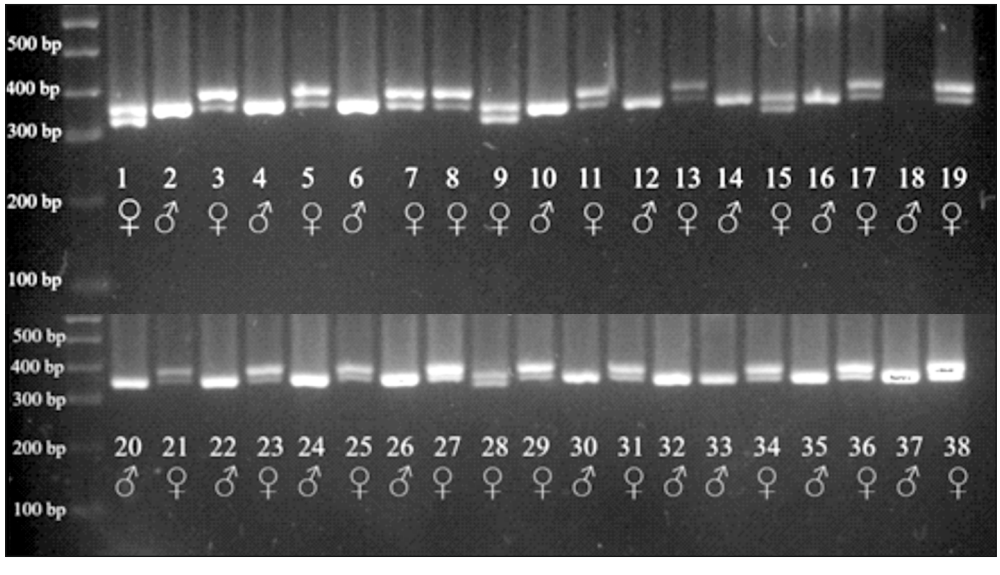

Fig. 2. PCR results of the 38 samples included in the present study identified a single band in males and two bands in females

Table 3

DNA concentrations obtained from various types of samples collected from the 3 bird species included in the study

No.	Sample type	Species	Sex	Day	Concentration (ng/ul)
1	Oral swab	Domestic pigeon (*Columba livia domestica*)	F	0	14.3
2	Freshly sampled DBS	Domestic pigeon (*Columba livia domestica*)	M	0	16.6
3	Freshly sampled DBS	Rose-ringed parakeet (*Psittacula krameri*)	F	0	19.4
4	DBS of 20 µl drops of anticoagulated blood	Domestic pigeon (*Columba livia domestica*)	M	0	13.2
5	DBS of 20 µl drops of anticoagulated blood	African grey parrot (*Psittacus erithacus*)	F	0	12.9
6	Whole blood with anticoagulant (20 µl)	Domestic pigeon (*Columba livia domestica*)	M	0	44.7
7	Whole blood with anticoagulant (20 µl)	African grey parrot (*Psittacus erithacus*)	F	0	45.4
8	Oral swab	Rose-ringed parakeet (*Psittacula krameri*)	F	1	8.5
9	Freshly sampled DBS	Domestic pigeon (*Columba livia domestica*)	F	1	10.5
10	Freshly sampled DBS	Domestic pigeon (*Columba livia domestica*)	M	1	17.3
11	DBS of 20 µl drops of anticoagulated blood	African grey parrot (*Psittacus erithacus*)	F	1	26.1
12	DBS of 20 µl drops of anticoagulated blood	Domestic pigeon (*Columba livia domestica*)	M	1	21.3
13	Oral swab	African grey parrot (*Psittacus erithacus*)	F	2	9.3
14	Freshly sampled DBS	Domestic pigeon (*Columba livia domestica*)	M	2	29.7
15	Freshly sampled DBS	Domestic pigeon (*Columba livia domestica*)	F	2	20.9
16	DBS of 20 µl drops of anticoagulated blood	Domestic pigeon (*Columba livia domestica*)	M	2	15.5
17	DBS of 20 µl drops of anticoagulated blood	African grey parrot (*Psittacus erithacus*)	F	2	25.0
18	Oral swab	Domestic pigeon (*Columba livia domestica*)	M	4	11.8
19	Freshly sampled DBS	African grey parrot (*Psittacus erithacus*)	F	4	16.6
20	Freshly sampled DBS	Domestic pigeon (*Columba livia domestica*)	M	4	18.2
21	DBS of 20 µl drops of anticoagulated blood	African grey parrot (*Psittacus erithacus*)	F	4	32.1
22	DBS of 20 µl drops of anticoagulated blood	Domestic pigeon (*Columba livia domestica*)	M	4	32.8
23	Oral swab	Rose-ringed parakeet (*Psittacula krameri*)	F	7	10.5
24	Freshly sampled DBS	Domestic pigeon (*Columba livia domestica*)	M	7	18.5
25	Freshly sampled DBS	African grey parrot (*Psittacus erithacus*)	F	7	8.9
26	DBS of 20 µl drops of anticoagulated blood	Domestic pigeon (*Columba livia domestica*)	M	7	26
27	DBS of 20 µl drops of anticoagulated blood	African grey parrot (*Psittacus erithacus*)	F	7	21.4
28	Oral swab	Domestic pigeon (*Columba livia domestica*)	F	14	15.8
29	Freshly sampled DBS	Rose-ringed parakeet (*Psittacula krameri*)	F	14	9.4
30	Freshly sampled DBS	Domestic pigeon (*Columba livia domestica*)	M	14	14.8
31	DBS of 20 µl drops of anticoagulated blood	African grey parrot (*Psittacus erithacus*)	F	14	22.1
32	DBS of 20 µl drops of anticoagulated blood	Domestic pigeon (*Columba livia domestica*)	M	14	41.0
33	Oral swab	Domestic pigeon (*Columba livia domestica*)	M	30	17.1
34	Oral swab	African grey parrot (*Psittacus erithacus*)	F	30	12.9
35	Freshly sampled DBS	Domestic pigeon (*Columba livia domestica*)	M	30	13.9
36	Freshly sampled DBS	African grey parrot (*Psittacus erithacus*)	F	30	12.3
37	DBS of 20 µl drops of anticoagulated blood	Domestic pigeon (*Columba livia domestica*)	M	30	48.1
38	DBS of 20 µl drops of anticoagulated blood	African grey parrot (*Psittacus erithacus*)	F	30	23.7

In 2013, Morinha et al. (15) conducted a study quantifying DNA extracted from blood, feathers, and oral swab samples across various bird species. The samples with the highest DNA concentration were derived from blood, followed by oral swabs, and then feathers. Similar findings were reported previously (14; 24; 25; 26). The current study aligns with these results, as depicted in Table 2. Prioritizing tissues that can be minimally collected from live birds, and aiming for both qualitative and quantitative DNA, oral swab samples are recommended (24).

No sex-related differences were identified in DNA quantification from samples collected from female birds compared to males. Similarly, there were no discernible differences in DNA quantification based on the tested bird species. However, it's worth mentio-

ning that the study included a relatively small sample size, limiting the ability to make meaningful species-wise comparisons.

The success rate of the PCR reaction for sex determination was 100% for all samples tested. Similar to the present study, oral swabs have been compared to dried blood spots (DBS) to assess the effectiveness of different DNA sources for bird sexing using PCR (2). They achieved the highest success rate of PCR sexing from dried blood spots, reaching 100%, while it was 74% for oral swabs. Additionally, they compared success rates between buccal swabs (74%) and cloacal swabs (75.47%) without identifying a statistically significant difference. Moreover, both methods are minimally invasive. They concluded that the minimally invasive method using oral or cloacal swabs could be the suitable choice for a DNA source for bird sexing via PCR (2). Similar results were obtained by other authors (1; 2; 12; 28; 29).

In the present study, oral swab samples, fresh blood drops on filter paper, filter paper with a standard 20 µl drop of anticoagulant-treated blood, and liquid whole blood samples were tested for DNA quantification and amplification, stored at room temperature at 20-22 °C. This was done to compare the working technique, the amount of DNA, and the accuracy of the results on days 0, 1, 2, 4, 7, 14, and 30. The resulting bands had the same intensity on day 0 as on day 30. No differences were identified between whole blood, filter paper with freshly collected blood, and filter paper with anticoagulant-treated blood regarding the appearance and clarity of the obtained bands. Even though oral swab samples had the lowest DNA concentration, the resulting bands from their amplification were clear and easily interpretable, except for the oral swab sample collected from a male domestic pigeon that regurgitated during the oral swab collection (Fig. 2, row 18) and was later retested. Therefore, related to our previous studies (24;25), oral swabs are not recommended in birds diagnosed with ingluvitis and symptoms of regurgitation.

The isolation of genomic DNA is a fundamental necessity for many molecular biology analytical procedures. To achieve successful DNA analysis, great attention must be paid to both sample collection and their preservation in a manner consistent with the primary goal of obtaining the purest DNA possible (19). Therefore, it is recommended to use fresh tissues and/or properly stored samples, in a state of low degradation (15). Whole blood samples should be collected in tubes containing anticoagulant (e.g., EDTA or heparin) and then centrifuged at 350 g for 15 minutes at room temperature. The isolated plasma and blood elements can be stored and frozen at -20°C (15). Freshly collected non-heparinized blood samples should be immediately transferred to a buffer solution and can be stored at room temperature for 1-8 months before processing (11).

For bird sexing purposes, blood can also be collected using the dried blood spots (DBS) method, where blood drops are collected on sterile filter paper. Collecting blood on filter paper can help reduce costs and address issues related to the transportation, storage, and processing of blood samples (21). For this method, Suriyaphol and colleagues (2014) recommend using Whatman grade 1 filter paper, and for optimizing laboratory reagent costs, they suggest a combination of methanol fixation and boiling. Additionally, Suriyaphol and colleagues (2014) demonstrated that using the same pair of scissors for multiple DBS samples did not affect PCR results. Furthermore, samples stored in liquid preservatives or on a physical support (e.g., paper) offer advantages over freezing, as they do not require massive storage space and can be easily transported and shipped, thus reducing the cost of long-term storage (21).

A global questionnaire study conducted in 2022 assessed the effectiveness of commonly used methods by researchers for field collection and long-term storage of blood samples and DNA extracts from wild birds (6). Ethanol, lysis buffer, direct freezing, and filter paper were the preferred methods for blood sample storage, in descending order. Regarding storage temperatures, blood samples were mostly kept at -20°C, followed by room temperature, -80°C, and +4°C (6). In human medicine, short-term blood storage for up to 60 days at 4°C has been considered an alternative strategy for research laboratories conducting molecular genetic tests on clinical samples (Rahila et al., 2017).Recently, Aslam et al. (2023) tested three types of samples, feathers, dried blood spots (DBS), and whole blood collected from chicken chicks, which they stored at three different temperatures: freezer, refrigerator, and room temperature for up to 5 years. They tested the samples on days 1, 7, 15, 30, then at 3 months, 6 months, 1 year, 3 years, and 5 years, obtaining high-quality PCR results for feathers and blood samples collected on filter paper-DBS stored at room temperature (~25 °C) (3).

Oral swabs containing epithelial cells can also be stored at -20°C (15). Freshly collected oral swabs can be air-dried in the field (summer) or in the laboratory (winter) for 10–15 minutes, in order to prevent molding, then placed in individual plastic collection tubes and stored in the laboratory at room temperature for 1–18 months before processing (11). Collecting oral swabs in birds may have certain limitations due to the type of material from which the oral swab shaft is made. Large-sized parrots with a strong beak tend to destroy the oral swab during the collection of oral epithelial cells, and it may turn into a swallowed foreign body. Therefore, for parrots, we recommend using oral

swabs with a plastic shaft instead of wooden ones, as they exhibit greater resistance.

The types of samples included in this study (oral swabs, filter paper with freshly collected blood, and filter paper with a standard 20 µl blood drop with anticoagulant) did not degrade at room temperature (~20-22 °C) and produced reliable results even after 30 days of storage on the laboratory bench for all three tested species of companion birds in this study (*Columba livia domestica, Psittacula krameri*, and *Psittacus erithacus*). For the ease of blood sample storage, we recommend filter paper and storing samples in the form of dried blood spot (DBS) drops as an alternative to anticoagulant blood tubes. Blood collected on filter paper remains stable at room temperature for a minimum of 1 month. However, blood sample collection is an invasive method of genetic material collection that exposes birds to the risk of manipulation and stress (14). We recommend oral swabs as a minimally invasive alternative for collecting samples for molecular bird sexing.

CONCLUSIONS

The current study compared samples of oral swabs, whole blood and DBS tested simultaneously from the same individuals for molecular bird sexing in three species of companion birds (*Columba livia domestica, Psittacula krameri* and *Psittacus erithacus*). The highest DNA concentration was observed in whole blood samples, succeeded by blood samples with anticoagulant placed as a standard 20 µl drop on filter paper, followed by fresh blood samples collected as a drop directly on filter paper, and finally, the lowest DNA concentration was detected in oral swab samples. The PCR reaction success rate for bird sex determination was 100% for all tested samples.

The current study demonstrates that transport and storage of DBS - "dried blood drops on filter paper" and oral swabs at room temperature for 30 days does not compromise the quality of DNA required for genetic sexing in birds. For ease of storage and sample preservation, it is recommended to use filter paper and store the samples in the form of dried blood spots (DBS) as a viable alternative to anticoagulant tubes. Moreover, as a less stressing alternative for blood sampling in birds, collecting oral swabs is a minimally invasive technique used for molecular bird sexing in juvenile and newly hatched birds.

Funding

This work was funded by the New Companion Animals Veterinary Clinic of Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca and by an internal grant of UASVMCN (no. 23293/14.10.2021).

Institutional Review Board Statement

The animal study protocol was approved by Ethics Committee of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca (protocol no. 351/06.12.2022), according to the national law 43/2014 and EU Directive 2010/63/EU and the owners.

Acknowledgments

We owe a debt of gratitude to all anonymous reviewers for their comments and suggestions, and to all bird owners through which the sampling of companion birds was made in the same timeframe. A special thanks to Iulia Bejan, Maria Moraru, Octavia Fărcaș and Patricia-Maria Laczko.

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