Molecular Sexing of Wild and Companion Birds Using Samples Collected by Minimally Invasive Methods

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Article

Molecular Sexing of Wild and Companion Birds Using Samples Collected by Minimally Invasive Methods

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Simple Summary: Over 50% of birds are monomorphic, showing no sexually dimorphic traits and in nestling the percentage is higher. Early sex determination can be of great value in management of wild birds, breeding of different bird species, improvement of breeding programs of captive birds, evolutionary studies fields, but also for bird owners. The purpose of this study was molecular sexing of wild and companion birds from various orders, such as: *Falconiformes, Accipitriformes, Galliformes, Anseriformes, Passeriformes, Psittaciformes*. Samples of oral swabs, feathers and blood were collected from 43 birds. Conventional PCR was applied in order to amplified the CHD1-Z and CHD1-W genes. The obtained results show that all types of samples can be used for molecular sexing of studied species of birds. In conclusion, instead of using blood samples, our recommendation is to use minimally invasive samples (swab-feathers) and test both types of samples on each bird.

Abstract: Birds are highly social and need pairing in order to increase their welfare. Most bird species are monomorphic, therefore, molecular sexing helps to provide appropriate welfare for birds. Moreover, early sex determination can be of great value for bird owners. The aim of this study was to demonstrate that sex identification in birds by molecular methods, using samples collected by minimally invasive methods is fast, efficient and accurate. A total of 100 samples (29 paired samples of feathers - oral swab and 14 tripled samples of feathers - oral swab - blood) from 43 birds were taken into study, as follows: wild birds (*Falconiformes*, *Accipitriformes*, landfowl – *Galliformes*, waterfowl– *Anseriformes*), companion birds (*Passeriformes*, *Psittaciformes* – large, medium and small size parrots). Amplification of CHD1-Z and CHD1-W genes was performed by conventional PCR. The results obtained from feathers were compared to those obtained from oral swabs and with those obtained from blood samples, where was the case. The obtained results show that all types of samples can be used for molecular sexing of all studied species of birds. According to our knowledge, the present study reports for the first time the molecular sex identification in: Red Siskin (*Carduelis cucullata*) and Goldfinch (*Carduelis carduelis major*). For higher accuracy, our recommendation is to use minimally invasive samples (swab-feathers) and to test both types of samples, for each bird, instead of blood samples.

Keywords: birds; molecular sexing; minimally invasive methods

1. Introduction

The origin of the present birds dates back to about 66 million years ago, when their ancestors survived a mass extinction event. They evolved and developed into a very large number of highly diverse species (more than 10.000) which now are spread all over the world. Despite their great

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phenotypic diversity, a large number of birds are monomorphic, the sexual dimorphism being absent (especially in chickens and juveniles) or hardly observable, even in some adults [1].

The sexual determinism in birds is chromosomal. Female birds are heterogametic, they have two distinct sexual chromosomes Z and W (ZW), while male birds are homogametic, presenting only Z chromosome (ZZ). The chromo-helicase-DNA binding protein (CHD1) gene, which is well conserved and present in both sex chromosomes of all birds allowed the sex identification in the majority of avian species [2]. More specifically, sex identification is determined by PCR reaction markers used to amplify the homologous regions of the two genes CHD1-Z and CHD1-W. The differences between these two genes is given by the length polymorphism of introns. The amplifications products are presented as a single copy of gene for males (being two Z chromosomes the gene copy is usually of identical length) and in females two copies are present due to the different length polymorphism of genes located on Z and W chromosomes [3]. Molecular sexing in birds, based on distinctive characteristics of birds sex chromosomes Z and W, is a non-invasive method, compared to the classical sexing methods, that has many advantages, most important being accuracy and precision. DNA sexing also has economic advantage, given the reduced costs of sample analysis. Molecular sexing is considered a safe method, as collecting samples does not endanger the birds' lives or expose them to risk of infection. Being able to collect feather samples even moulted feathers from the nest area is an advantage for wild birds, thus avoiding the stress of handling wild birds. Birds of all ages can be safely sexed, especially juveniles or newly-hatched birds using oral swab-samples [4–8].

After fish, cats and dogs, birds are the fourth most common pet in the US [9,10], while in the EU, it ranks third as the most popular pet [11,12]. In birds bred for more generations in captivity, such as Canaries (*Serinus canaria*), Budgerigars (*Melopsittacus undulatus*), Zebra Finches (*Taeniopygia guttata*), Lovebirds (*Agapornis* sp), Cockatiels (*Nymphicus hollandicus*) etc, their behavior and physiology varies little from that of wild individuals [13].

In the present paper, we used PCR primers (P2/NP) located inside the CHD1 gene, in order to determine their efficiency in sex identification in wild and companion birds belonging to six different orders, such as: *Falconiformes, Accipitriformes, Galliformes, Anseriformes, Passeriformes, Psittaciformes*.

Psittaciformes, often known as parrots, comprise roughly 400 species and are the most popular pet birds [14,15]. Most species of parrots are monomorphic, some species showing dysmorphic characters only after reaching sexual maturity. For instance, male *Melopsittacus undulatus* parakeets have predominantly blue nostrils, whereas females have pinkish-brown nostrils. Unfortunately, these sexually dimorphic traits don't appear until parakeets are 6–8 months old, when they achieve sexual maturity [15,16].

Passeriformes represent the group of birds with the greatest diversity of species, over 6500 in total, and many of them are monomorphic [17]. Passerines are among the most well-known of all birds due to their diversity, abundance, and global distribution [18]. They have also played a significant role in human culture and science. Passerines are most closely linked to parrots (*Psittaciformes*), which are most closely connected to falcons (*Falconiformes*), according to DNA-sequence research [18].

The order *Accipitriformes* includes 225 species of birds of prey, mainly diurnal. They were initially classified in the order *Falconiformes*, but after the new genetic researches they were reclassified. Both in *Falconiformes* and in *Accipitriformes* males are known to be smaller than females [19], and in rare cases plumage is a sexually differentiating feature. Sexual dimorphism does not occur in *Falco subbuteo* at an early age, so it will be easier to identify the sex using a molecular method [20].

Genetic sexing of birds has many applications in various fields such as behavioral medicine, conservation medicine, management of wild birds, breeding of different bird species, improvement of breeding programs of captive birds, analysis of breeding strategies of poultry, evolutionary studies and forensic medicine [3].

The main purpose of this study was the sex identification by PCR techniques (using P2, NP and MP primers) of the above mentioned wild and companion monomorphic birds (orders: *Falconiformes, Accipitriformes, Galliformes, Anseriformes, Passeriformes, Psittaciformes*), using moderately invasive collected samples (oral swabs and feathers) and invasively collected samples (whole blood) where

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the owners agreed to the procedure. Birds are highly social and need pairing in order to increase their welfare. Molecular sexing helps provide welfare elements for birds by early pairing. Mating parrots has been shown to increase their welfare, and therefore early sex determination can be of great value to bird owners [21].

2. Materials and Methods

2.1. Sample Collection

During January-June 2023, samples of feathers, oral swabs and blood were randomly collected from 43 wild and companion birds as follows: wild birds (*Falconiformes* – *Falco subuteo*, *Accipitriformes* – *Buteo buteo*, *Galliformes* (landfowl) – *Phasianus colchicus*, *Anseriformes* (waterfowl) – *Cygnus cygnus*) and companion birds (*Passeriformes* and *Psittaciformes* – large, medium and small size parrots) (Table 1). A total of 100 samples: 29 paired samples of feathers - oral swab and 14 tripled samples of feathers - oral swab - blood were taken into de study. All the samples were collected during routine check-ups of live birds (*Galliformes, Anseriformes, Passeriformes, Psittaciformes)* or cadavers *(Falconiformes, Accipitriformes*) admitted to the New Companion Animals veterinary clinic of Faculty of Veterinary Medicine, UASMV Cluj-Napoca, Romania.

Table 1. Oral swabs, feathers and blood samples collected from wild and companion birds.

Order	Species	No*	Oral swabs	Feathers	Blood
Falconiformes	*Falco subbuteo*	1	1	1	1
Accipitriformes	*Buteo buteo*	3	3	3	3
Galliformes	*Phasianus colchicus*	2	2	2	-
Anseriformes	*Cygnus cygnus*	5	5	5	3
Passeriformes	*Taeniopygia castanotis*	2	2	2	-
	Chloebia gouldiae	2	2	2	-
	Carduelis cucullata	2	2	2	-
	Carduelis carduelis major	2	2	2	-
	Serinus canaria forma domestica	2	2	2	-
Psittaciformes	*Ara macao*	4	4	4	3
	Psittacus erithacus	4	4	4	2
	Cacatua alba	1	1	1	1
	Psittacula krameri	2	2	2	1
	Psephotus haematonotus	2	2	2	-
	Nymphicus hollandicus	3	3	3	-
	Agapornis fischeri	4	4	4	-
	Melopsittacus undulatus	2	2	2	-
TOTAL		**43**	**43**	**43**	**14**

Legend: * - No. of tested individuals.

Two contour feathers with intact calamuses were sampled from the wings or abdominal region of each bird. Oral swabs were collected using sterile cotton swabs (Prima, Taizhou Honod Medical Co., Ltd., Zhejiang, China) [22]. Blood samples were collected from 10 live birds from the orders *Anseriformes* and *Psittaciformes* by phlebocentesis of the metatarsal veins using heparinized syringes. A total quantity of 0.2-0.3 ml of blood was collected and stored in heparin tubes. Blood samples collected from deceased birds (n=4) from the order *Falconiformes* and *Accipitriformes* were sampled directly from the heart, being identified post mortem in the form of blood clots. The breeders gave consent for these procedures. All samples were collected using surgical gloves, labeled individually and stored at −20 °C until processing.

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2.2. DNA Extraction and PCR

Genomic DNA was extracted from all samples (n=100; feathers, oral swabs and blood) collected from birds. For all type of samples we used the same protocol. The DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol. The feather's calamus was sectioned in small pieces and then subjected to mechanical destruction by high-speed shaking with steel beads using TissueLyserII (Qiagen, US). Oral swabs were transferred to 1.5 mL Eppendorf tubes using sterile scissors. For DNA extraction were used 25 mg of feathers/blood and the entire oral swab. The DNA concentration was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

DNA was tested for the presence of specific genes CHD1-W and CHD1-Z by conventional PCR, according to the protocol described by Ito et al. [23]. The CHD1 gene was amplified using the primers P2 (5′-TCT GCA TCG CTA AAT CCT TT-3′) and NP (5′-GAG AAA CTG TGC AAA ACA G-3′) (Generi-Biotech, Hradec Králove, Czech Republic). PCR amplification was carried out in a 25 µl reaction mixture consisting of 12.5 µl of MyTaq Red HS Mix (Meridian Bioscience, USA) and 25 pM of each primer. Between 50 and 270 ng of DNA was used as a template. The lowest DNA concentration was recorded from feathers. The amplification was performed in Bio-Rad C1000TM Thermal Cycler (Bio-Rad Laboratories, Hercules, California). Cycling conditions were: 95 °C for 5 min; followed by 35 cycles consisted of: 52 °C for 45 sec, 72 °C for 45 sec, 95 °C for 30 sec. The amplification was finished with 1 min at 50 °C and a final elongation for 5 min at 72 °C. For sex identification in *Falconiformes* and *Accipitriformes* was used a set of three primers: P2, NP and MP (5′-AGT CAC TAT CAG ATC CGG AA-3′) [23]. PCR amplification was carried out in the same condition as the previous one.

Aliquots of each PCR product were electrophoresed on 3% agarose gel stained with RedSafe Nucleic Acid Staining Solution 20.000× (iNtRON Biotechnology), and examined under UV light (Bio-Rad BioDoc-ItTM Imagine System). The fragment size of DNA was compared with 100 bp DNA ladder (Fermentas; Thermo Fisher Scientific, Waltham, Massachusetts) and assigned sex by counting the visible bands in each lane. Females are characterized by obtaining two bands corresponding to the CHD1-W and CHD1-Z genes, while males present only one band corresponding to the CHD1-Z gene.

3. Results

All the samples collected from the birds (oral swabs, feathers and blood) provided a good DNA template for molecular sex identification. Identical results were obtained from all types of samples collected from the same bird. Molecular amplification of the CHD1 gene allowed the identification of the 24 males and 19 females (Table 2). The gel electrophoresis of the obtained results are presented in Figures 1 and 2.

Table 2. Results of molecular sexing of wild and companion birds included in this study.

Order	Species	No*	Males	Females
Falconiformes	*Falco subbuteo*	1	-	1
Accipitriformes	*Buteo buteo*	3	1	2
Galliformes	*Phasianus colchicus*	2	2	-
Anseriformes	*Cygnus cygnus*	5	3	2
Passeriformes	*Taeniopygia castanotis*	2	1	1
	Chloebia gouldiae	2	1	1
	Carduelis cucullata	2	1	1
	Carduelis carduelis major	2	1	1
	Serinus canaria forma domestica	2	1	1
Psittaciformes	*Ara macao*	4	4	-
	Psittacus erithacus	4	1	3
	Cacatua alba	1	1	-

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	Psittacula krameri	2	1	1
	Psephotus haematonotus	2	1	1
	Nymphicus hollandicus	3	2	1
	Agapornis fischeri	4	2	2
	Melopsittacus undulatus	2	1	1
TOTAL		**43**	**24**	**19**

Legend: * - No. of tested individuals.

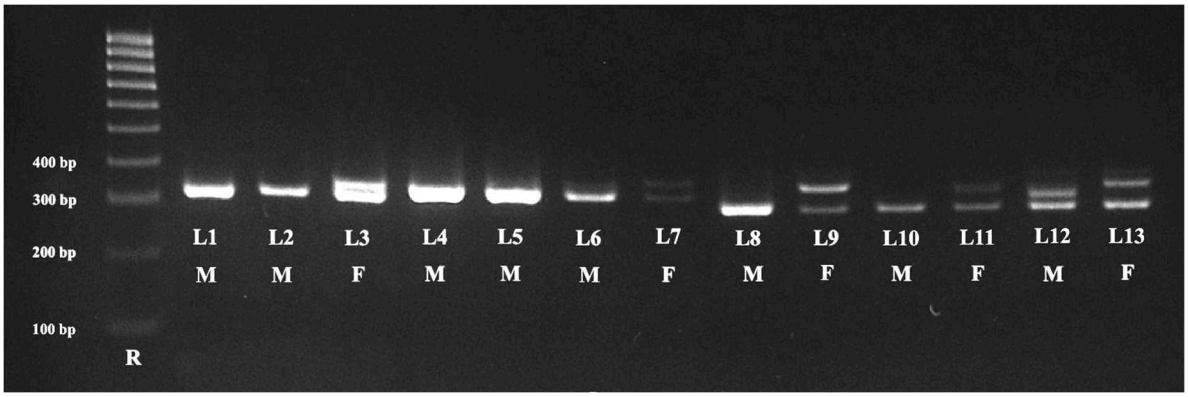

Figure 1. Molecular sex identification in *Galliformes* (L1), *Anseriformes* (L2, L3) and *Passeriformes* (L4-L13). **Legend:** L1 - *Phasianus colchicus*; L2, L3 - *Cygnus cygnus*; L4, L5 - *Taeniopygia castanotis*; L6, L7 - *Chloebia gouldiae*; L8, L9 - *Carduelis cucullata*; L10, L11 - *Carduelis carduelis major*; L12, L13 - *Serinus canaria forma domestica*. The females (F) presented two bands (L3, L7, L9, L11, L13), while the males (M) presented a single band (L1, l2, L4-L6, L8, L10, L12).

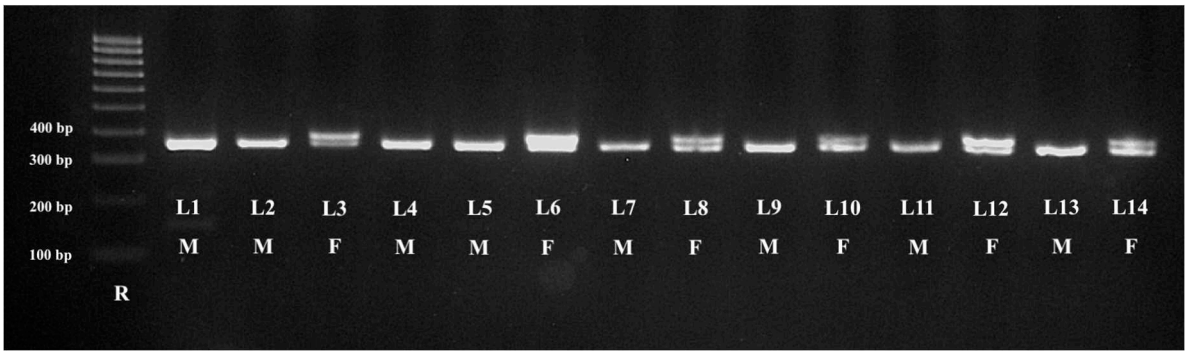

Figure 2. Molecular sex identification in *Psittaciformes*. **Legend:** *Psittaciformes* large size: L1 - *Ara macao*; L2, L3 - *Psittacus erithacus*; L4 - *Cacatua alba*; *Psittaciformes* medium size: L5, L6 - *Psittacula krameri*; L7, L8 - *Psephotus haematonotus*; L9, L10 - *Nymphicus hollandicus*; L11, L12 - *Agapornis fischeri*; *Psittaciformes* small size: L12, L13 - *Melopsittacus undulatus*. The females (F) presented two bands (L3, L6, L8, L10, L12, L14), while the males (M) presented a single band (L1, l2, L4, L5, L7, L9, L11, L13).

Since in the samples collected from *Buteo buteo* (*Accipitriformes*) and *Falco subbuteo* (*Falconiformes*) we failed to identify the sex with the help of the P2 and NP primers (in both males and females only one band appeared), we retested the samples using three primers P2, NP and MP, according to the recommendations of Ito et al. [23]. The results obtained in *Accipitriformes*/*Falconiformes* are shown in Figure 3.

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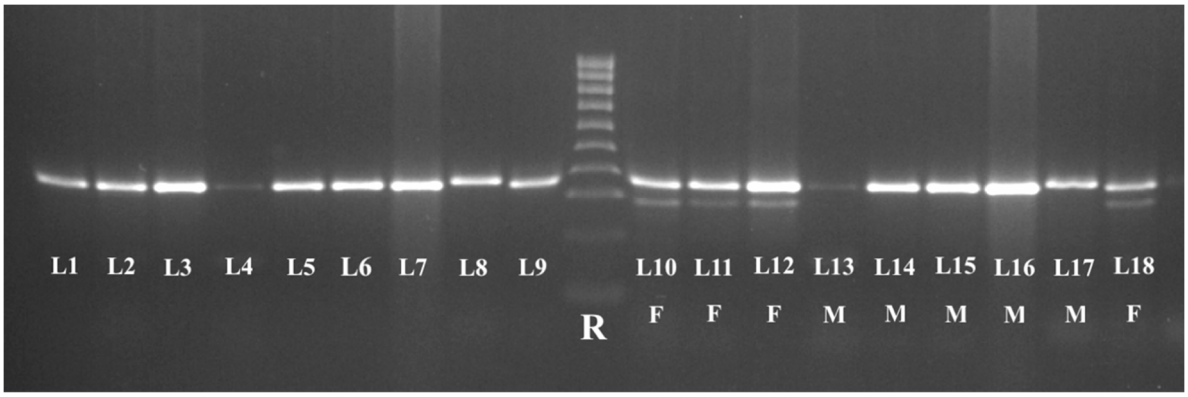

Figure 3. Molecular sex identification in raptors *Accipitriformes* (L1-L8; L10-L17) and *Falconiformes* (L9; L18). **Legend:** L1-L2 – P2/NP primer pair was used. A single band was obtained in both sexes. L10-L18 – P2/NP/MP primers were used. The females (F) presented two bands (L10-L12, L18), while the males (M) presented a single band (L13-L17).

4. Discussion

In an attempt to find the universal method, several molecular genetic techniques have been tested for identifying the sex of birds, as well as many PCR markers based on CHD1 [2,24,25], ATP synthase α-subunit (ATP5A1) [26], W-linked gene for the altered form of protein kinase C-interacting protein (Wpkci) [27], Nipped-B homolog (NIPBL) [28], Spindlin (SPIN) [29] or RAS p21 protein activator 1 (RASA1) genes [30]. These genes are used on the purpose to identify differences between the homologous regions of the two Z and W chromosomes, based on the variations in the length polymorphism of introns located in these regions [31].

In the present study we tested the efficiency of CHD1 gene amplification using the P2/NP primer pairs [23] for sex identification in birds from different orders, such as: *Accipitriformes, Falconiformes, Galliformes, Anseriformes, Passeriformes and Psittaciformes.* With the exception of *Accipitriformes* and *Falconiformes*, the obtained results showed a percentage of 100% sex identification in wild and companion birds by the molecular method based on the intronic length polymorphism. Similar DNA templates were provided for the molecular sexing reactions by all types of samples, including feathers, oral swabs and blood.

In a previous study, using the pair of primers P2/P8, the PCR success rate of sex identification in birds classified in *Columbiformes* and *Psittaciformes* orders was 94.06% from oral swabs and 82.43% from feathers [32]. According to the results obtained in the present study, and also by Ito et al. [23], the P2/NP primer pairs may be able to identify the sex in more species than the P2/P8 primer pair, since the P8 primer site is less conserved than the NP primer site.

All the *Buteo buteo* (n=3) and *Falco subbuteo* (n=1) birds included in the present study were cadavers, therefore sex could be determined by gonad identification. We were unable to determine the sex using the P2/NP primers because only a single band appeared in both males and females, probably due to the small difference in size between CHD1-Z and CHD1-W [23]. According to Nesje and Røed [20], there is just a one base difference between the two bands of female *Falco subbuteo*. Thus, we retested the samples using multiplex PCR with an additional primer (P2/NP/MP), in accordance with the recommendations of Ito et al. [23]. MP is a 3'-terminal mismatch primer which allowed the detection of a fragment situated only on W chromosome [33]. Female-specific CHD1-W was detected by NP/MP primers, whereas CHD1-Z was amplified by NP/P2 [23]. These primers successfully allowed the identification of the sexes of *Accipitriformes* and *Falconiformes* included in the present study. In both species, the females have two bands, compared to the males' single band.

Sex can be also distinguished in birds using a molecular method, based on the amplification of a unique sequence located on the W chromosome, regardless of intronic size variation [34]. Therefore, a multiplex PCR, which uses in addition to the P2/P8 primer pair [2] a new P0 primer specific for CHD1-W was developed [34]. With the help of this method, several species of birds from 12 avian

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orders could be sexed, such as: *Accipitriformes*, *Galliformes*, *Anseriformes*, *Passeriformes* and *Psittaciformes* [34].

A new approach to sex determination in birds may be quantitative real-time PCR (qPCR) based on copy number variation of genes associated only with the Z chromosome (CHRNA6, DDX4, LPAR1, TMEM161B, VPS13A for neognath species, and DOCK8, FUT10, PIGG and PSD3 for paleognath species) and absent from the W chromosome [35]. This method has been applied to 73 species with great success, and it has been shown to be a reliable molecular sex identification tool for birds [35].

Until now, even if remarkable research has been done in the field, no truly universally valid marker or method has been found for the sexing of all bird species. The use of multiple markers is advised for the efficacy of molecular sexing in birds [36], as well as the simultaneous testing of at least two types of minimally invasive samples (feathers, buccal swab).

5. Conclusions

The present study demonstrates the applicability of all sample types (feathers, oral swab, blood) for molecular sexing of all examined bird species. Instead of using blood samples, we advise using minimally invasive samples as feathers and oral swabs, and testing both types of samples for each bird for accuracy. Molecular sex identification in Red Siskin (*Carduelis cucullata*) and Goldfinch (*Carduelis carduelis major*) has never been reported before, as far as we are aware. Because only a small number of each species' individuals were examined, our study was constrained. Due to the lack of sexual dimorphism in many species and in all nestlings, as well as the absence of universal marker that can be used across all bird species, accurate and effective sexing of birds continues to be challenging.

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Institutional Review Board Statement: The animal study protocol was approved by Ethics Com- mittee 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.

Data Availability Statement: All the results of the study are presented within the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Brusatte, S.L.; O'Connor, J.K.; Jarvis, E.D. The origin and diversification of Birds. *Curr. Biol.* **2015**, *25*, R888–R898.
2. Griffiths, R.; Double, M.C.; Orr, K.; Dawson, R.J. A DNA test to sex most birds. *Mol. Ecol.* **1998**, 7, 1071–1075.
3. Morinha, F.; Cabral, J.A.; Bastos, E. Molecular sexing of birds: A comparative review of polymerase chain reaction (PCR)-based methods. *Theriogenology*. **2012**, *78*, 703–714.
4. Griffiths, R. Sex identification in birds. In *Seminars in Avian and Exotic Pet Medicine*; WB Saunders: Philadelphia, PA, USA, **2000**; pp. 14–26.
5. Bermúdez-Humarán, L.G.; García-García, A.; Leal-Garza, C.H.; Riojas-Valdes, V.M.; Jaramillo-Rangel, G.; Montes-de-Oca-Luna, R. Molecular sexing of monomorphic endangered *Ara* birds. *Journal of Experimental Zoology* **2002a**, *292*, 677-680.
6. Bermúdez-Humarán, L. G.; Chávez-Zamarripa, P.; Guzmán-Velasco, A.; Leal-Garza, C. H.; Montes de Oca-Luna, R. Loss of restriction site DdeI, used for avian molecular sexing, in *Oreophasis derbianus*. *Reproduction in domestic animals* **2002b**, *37*, 321-323.
7. Cerit, H.; Avanus, K. Sex identification in avian species using DNA typing methods. *World's Poultry Science Journal* 2007, 63, pp. 91-100.

8. Vučićević, M.; Stevanović, J.; Simeunović, P.; Vučićević, I.; Đelić, N.; Stanimirović, Z.; Stojić, V. Analysis of the CHD gene for sex determination of protected bird species. In Proceedings of the *International symposium on hunting, "Modern aspects of sustainable management of game population"*, **2012**, *24*, 83-86.
9. Kidd, A.H.; Kidd, R.M. Problems and benefits of bird ownership. *Psychol. Rep.* **1998**, *83*, 131–138.
10. Meyers, N.M. Perspectives on pet bird welfare from the pet industry. *J. Am. Vet. Med Assoc.***1998**, *212*, 1238–1242.
11. Davis, C. Appreciating avian intelligence: The importance of a proper domestic environment. *J. Am. Vet. Med. Assoc.* **1998**, *212*, 1220–1222.
12. Graham, D.L. Pet birds: Historical and modern perspectives on the keeper and the kept. *J. Am. Vet. Med. Assoc.* **1998**, *212*, 1216–1219.
13. Wyndham, E. Diurnal cycle, behaviour and social organization of the budgerigar *Melopsittacus undulatus*. *Emu* **1980**, *80*, 25–33.
14. Curro, T.G. Anesthesia of pet birds. In *Seminars in Avian and Exotic Pet Medicine;* WB Saunders: Philadelphia, PA, USA, **1998**; *7*, pp. 10–21.
15. Forshaw, J. M. Parrots of the World. Princeton, NJ: Princeton University Press, **2010**; *70*.
16. O'Malley, B. *Clinical Anatomy and Physiology of Exotic Species*. Elsevier Saunders: Philadelphia, PA, USA, **2005**; pp. 138–142.
17. Ellrich, H.; Salewski, V.; Fiedler W. Morphological sexing of passerines: not valid over larger geographical scales. *J Ornithol*. **2010**, *151*, 449–458.
18. Schmitt, C.J.; Edwards, V.S. Passerine birds. *Curr. Biol*. **2022**, *32*, R1149-R1154.
19. Hill, N.P. Sexual dimorphism in the *Falconiformes*. *Auk*. **1944**, *61*, 228-234.
20. Nesje, M.; Røed, K.H. Sex identification in falcons using microsatellite DNA markers. *Hereditas*. **2000**, *132*, 261-3.
21. Peng, S.; Broom, D.M. The sustainability of keeping birds as pets: Should any be kept? *Animals (Basel).* **2021**, *11*, 582.
22. Handel, C.M.; Pajot, L.M.; Talbot, S.L.; Sage, G.K. Use of buccal swabs for sampling DNA from nestling and adult birds. *Wildlife Society Bulletin* **2006**, *34*, 1094-1100.
23. Ito, H.; Sudo-Yamaji, A.; Abe, M.; Murase, T.; Tsubota, T. Sex identification by alternative polymerase chain reaction methods in Falconiformes. *Zool. Sci.* **2003**, *20*, 339–344.
24. Fridolfsson, A.K.; Ellegren, H. A simple and universal method for molecular sexing of non-ratite birds. *J. Avian Biol.* **1999**, *30*, 116–121.
25. Lee, J.C.; Tsai, L.C.; Hwa, P.Y.; Chan, C.L.; Huang, A.; Chin, S,C.; Wang L.C.; Lin, J.T.; Linacre, A.; Hsieh, H.M. A novel strategy for avian species and gender identification using the CHD gene. *Mol Cell Probes.* **2010**, 24, 27–31.
26. Fridolfsson, A.K.; Cheng, H.; Copeland, N.G.; Jenkins, N.A; Liu, H.C.; Raudsepp, T.; Woodage, T.; Chowdhary, B.; Halverson, J.; Ellegren, H. Evolution of the avian sex chromosomes from an ances- tral pair of autosomes. *Proc Natl Acad Sci USA*. **1998**, *95*, 8147–8152.
27. O'Neill, M.; Binder, M.; Smith, C.; Andrews, J.; Reed, K.; Smith, M.; Millar, C.; Lambert, D.; Sinclair, A. ASW: a gene with conserved avian W-linkage and female specific expression in chick embry- onic gonad. *Dev Genes Evol*. **2000**, *210*, 243–249.
28. Suh, A.; Kriegs, J.O.; Brosius, J.; Schmitz, J. Retroposon insertions and the chronology of avian sex chromosome evolution. *Mol. Biol. Evol.* **2011**, *28*, 2993–2997.
29. de Kloet, R.S.; de Kloet, S.R. Evolution of the spindlin gene in birds: Independent cessation of the recombination of sex chromosomes at the spindlin locus in neognathous birds and tinamous, a palaeognathous avian family. *Genetica* **2003**, *119*, 333–342.
30. Li, W.; Xue, F.; Li, L.; Li, X.; Yue, B.; Li, J. A triple-primer PCR approach for the sex identification of endangered Phasianidae birds. *Eur. J. Wildl. Res.* **2012**, *58*, 289–294.
31. Kroczak, A.; Wierzbicki, H.; Urantówka, A.D. In Silico Analysis of Seven PCR Markers Developed from the *CHD1*, *NIPBL* and *SPIN* Genes Followed by Laboratory Testing Shows How to Reliably Determine the Sex of Musophagiformes Species. *Genes* **2022**,*13*,932.
32. Turcu, M.C.; Paștiu, A.I.; Bel, L.V.; Pusta, D.L. A comparison of feathers and oral swab samples as DNA sources for molecular sexing in companion birds. *Animals (Basel)* **2023**, *13*, 525.
33. Newton, C.R.; Graham, A.; Heptinstall, L.E.; Powell, S.J.; Summers, C.; Kalsheker, N.; Smith, J.C.; Markham, A.F. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucletic Acids Res* **1989**, *17*, 2503–2516.
34. Han, J.I.; Kim, J.H.; Kim, S.; Park, S.R.; Na, K.J. A Simple and improved DNA test for avian sex determination. *The Auk* **2009**, *126*, 779–783.
35. Mazzoleni, S.; Němec, P.; Albrecht, T.; Lymberakis, P.; Kratochvíl, L.; Rovatsos, M. Long-term stability of sex chromosome gene content allows accurate qPCR-based molecular sexing across birds. *Mol Ecol Resour*. **2021**, *21*, 2013– 2021.

 doi:10.20944/preprints202310.0327.v1

36. Kroczak, A.; Wołoszyńska, M.; Wierzbicki, H.; Kurkowski, M.; Grabowski, K.A.; Piasecki, T.; Urantówka, A.D. New Bird sexing strategy developed in the order Psittaciformes involves multiple markers to avoid sex misidentification: Debunked myth of the Universal DNA marker. *Genes* **2021**, *12*, 878.
37. Peng, S.; Broom, D.M. The sustainability of keeping birds as pets: Should any be kept? *Animals (Basel).* **2021**, *11*, 582.