PhD THESIS SUMMARY

Comparative sexing techniques in birds that do not display sexual dimorphism

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I. Introduction

Determining the sex of birds can be done through several methods, these being represented by traditional methods, surgical methods, genetic methods, hormonal methods (determination of steroid hormones) and ultrasound methods (of the genital tract) (Fig. 1) (O'Malley, 2005; Stanford, 2010).

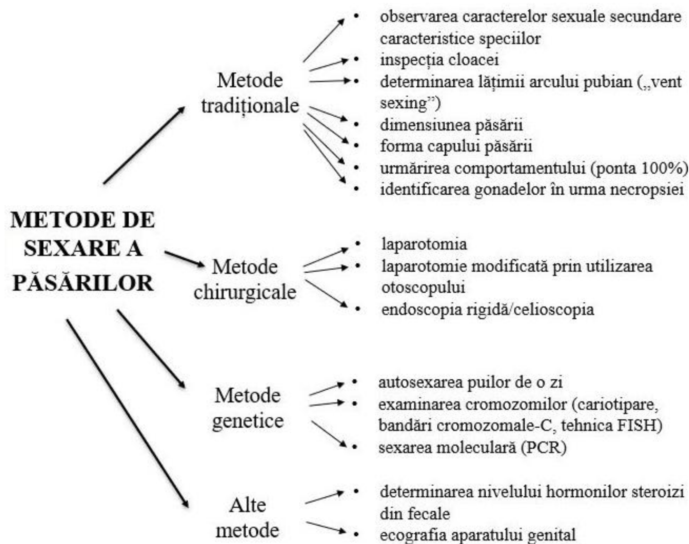

Fig. 1 Scheme of sexing methods in birds (original)

The **working hypothesis** was based on the fact that the majority of birds do not exhibit sexual dimorphism, and the accurate determination of sex has numerous applications in behavioral medicine, conservation medicine, wild bird management, breeding of various bird species, improvement of captive bird breeding programs, analysis of poultry breeding strategies, evolutionary studies, and forensic medicine.

The research **objectives** were represented by bird sexing through surgical methods (endoscopic sexing), genetic methods (molecular sexing), and traditional methods (identification of gonads following necropsy), as well as the comparison of these techniques.

Endoscopic sexing was performed using surgical methods. The study was conducted on carcasses of Domestic Pigeon (*Columba livia domestica*), with the objective of identifying the gonads and comparing the results with those obtained through necropsy in order to assess the accuracy of these techniques.

The main objective of the genetic studies was the identification of sex in birds using molecular genetic techniques (PCR). Genetic methods for bird sexing were based on the identification of the CHD (chromodomain-helicase-DNA-binding protein) gene, which is well conserved in the sex chromosomes of birds (CHD1Z and CHD1W). For

molecular sexing in birds, several types of samples collected by invasive (blood), minimally invasive (oral swab, plucked feathers) and non-invasive (molted feathers) methods were compared. Moreover, several DNA pre-lysis and extraction techniques were compared, as well as several DNA amplification protocols by PCR reaction in different species of non-ratites species (wild and companion birds).

Ultimately, comparative studies were carried out regarding the three techniques used, endoscopic, molecular and necropsy sexing in Domestic Pigeon (*Columba livia domestica*). Addionally, endoscopic sexing was compared to molecular sexing in the live Lovebirds (*Agapornis spp*.).

II. Personal contributions

The chapter "Personal contribution" from the thesis entitled "**Comparative sexing techniques in birds that do not display sexual dimorphism**" includes 9 studies whose main purpose was to identify the sex of birds through surgical, molecular and necropsy techniques.

1. Endoscopic sexing in Domestic Pigeon (*Columba livia domestica*)

The main objective of this study was to compare the endoscopic sexing technique with the necropsy technique in the Domestic Pigeon (*Columba livia domestica*) for gonad identification, in order to evaluate the accuracy of these techniques.

This study included a total of 21 pigeons. The materials required for the endoscopy method included the Karl Storz® Veterinary Endoscopy America Inc. laparoscopic equipment, xenon light source, laparoscopy tower camera, monitor, 2.7 mm telescope, and surgical instruments.

Conclusions:

1. Of the two sexing methods, necropsy (81%) was found to be more effective than endoscopic sexing (71%) in Domestic Pigeon (*Columba livia domestica*).
2. Endoscopic sexing had lower efficiency due to reduced visibility in the coelomic cavity (caused by intracoelomic hemorrhage or coelomitis with adhesions).
3. Experience of the examiner plays an important role in successful sexing of birds using celioscopy through the left lateral approach.
4. Juveniles could not be sexed by either of the two methods because they display small, poorly differentiated gonads.

2. A comparative study regarding invasive, minimally invasive and non-invasive sample collection for molecular bird sexing

The main objective of this study was to identify the CHD (chromodomain helicase DNA binding protein) genes from the sexual chromosomes of birds (Z and W), by PCR technique, using various tissue samples collected from two species of pet birds (carcasses of *Melopsittacus undulatus* and live *Gallus gallus domesticus*) exhibiting sexual

dimorphism. The secondary objective was to determine the types of samples, collected by invasive, minimally invasive and non-invasive methods, that lend themselves most effectively to DNA extraction and molecular sexing.

The materials required for molecular sexing were categorized into materials for sample collection, materials for sample processing, and equipment from the Molecular Genetics Laboratory. Molecular genetic sexing of birds through PCR technique encompassed the following steps: sample collection, sample storage, DNA extraction using commercial kits, DNA quantification using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, ME), DNA amplification using the C1000TM Thermal Cycler (Bio-Rad Laboratories, Hercules, California), agarose gel electrophoresis, examination of DNA fragments using the BioDoc-It imaging system transilluminator (Bio-Rad Laboratories), and interpretation of the results.

In this study, two pairs of birds (4 individuals) from the species *Melopsittacus undulatus* and *Gallus gallus domesticus* were included. The Isolate II Genomic DNA kit (Meridian Bioscience, London, UK) was used for DNA extraction, and a classic PCR protocol described by Griffiths et al. (1998) was implemented, using the P2/P8 primers.

In Budgerigar (*Melopsittacus undulatus*) a 100% concordance was obtained between the results obtained through molecular sexing and those obtained following necropsy examination. All tissue samples taken from Budgerigars (blood, feathers, oral swabs, skin, intestine, myocardium, testis) provided qualitative and quantitative DNA to be able to identify the sex of the birds. In Chicken (*Gallus gallus domesticus*), we obtained conclusive results only in the male individual, while in the female the sex could not be confirmed by molecular methods (Fig. 2).

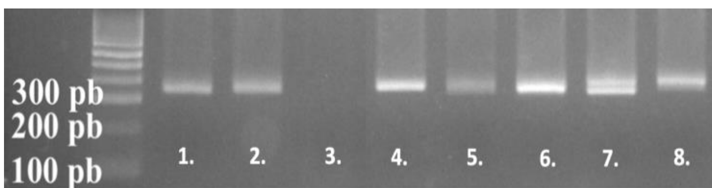

Fig. 2 DNA sexing by PCR method using primers P2 and P8 (original)

Legend: 1., 2. *Gallus gallus domesticus*, male, feathers; 3. *G. g. domesticus*, female, feathers; 4. *Melopsittacus undulatus*, male, blood; 5. *M. undulatus*, male, oral swab; 6. *M. undulatus*, male, liver; 7. *M. undulatus*, female, oral swab; 8. *M.s undulatus*, male, feathers.

Conclusions:

1. The concentration of DNA obtained from cadavers was highest in liver (181.1 ng/µl), testis (163.9 ng/µl) intestine (96 ng/µl), followed by samples of blood (21.3 ng/µl), oral swabs (19.9 ng/µl) and feathers (4.4 ng/µl).
2. Prior denaturation of the feathers using the TissueLyserII, before the actual DNA extraction, resulted in a higher DNA concentration.

3. DNA amplification from feather samples could not be successfully performed in all species of pet birds tested in this study (*Gallus gallus domesticus* and *Melopsittacus undulatus*), therefore we recommend DNA amplification from paired feather-oral swab samples from each bird.

3. A comparison of feathers and oral swab samples as DNA sources for molecular sexing in companion birds

The main objective of this study was to compare the results of molecular sexing by PCR from paired samples of feathers (processed by mechanical shock using the TissueLyserII) and oral swabs, collected from four species of pet birds: *Psittacula krameri, Neophema splendida, Agapornis spp.* and *Columba livia domestica*. The secondary objective was to find a minimally invasive sampling alternative that could be used for early bird sexing.

In this study, 101 companion birds belonging to the orders *Columbiformes (Columba livia domestica)* and *Psittaciformes (Psittacula krameri, Neophema splendida, and Agapornis spp.)* were included. The Isolate II Genomic DNA kit from Meridian Bioscience, Newtown, OH, USA, was used for DNA extraction, along with a classic PCR protocol described by Griffiths et al. (1998), using the P2/P8 primers.

The overall PCR success rate for sexing was significantly higher from oral swabs (94.06%) than from feathers processed with the TissueLyserII (82.43%) in *Psittacula krameri, Neophema splendida, Agapornis spp.* and *Columba livia domestica*. For the molecular sexing of Domestic Pigeon carcasses, the most reliable results were obtained from blood clot samples (100%), then from feathers (95.34%) and finally from oral swabs (93.02%). When using feather samples, the results were low in the case of contour feathers, small in size with poorly developed calamus, which present a low amount of DNA. On the other hand, reliable results were obtained in the present study even from down feather samples in the case of a pigeon aged approximately 7 days. A similarity between the molecular sexing results from feather samples and oral swab samples (Fig. 3) was obtained in 78.38% of the tested birds.

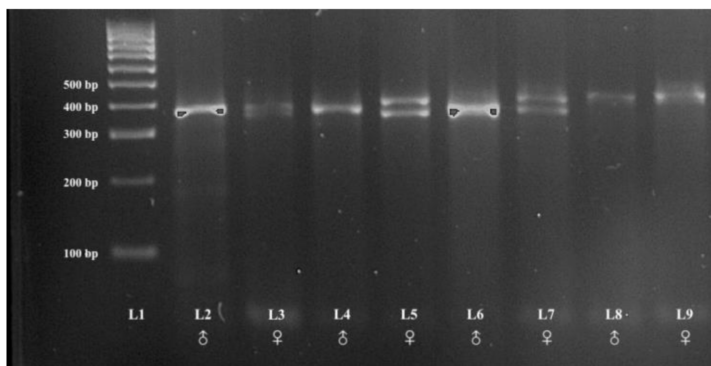

Fig. 3 PCR result from oral swab samples (original)

Legend: L1 - Size standard (100-bp DNA ladder); ♂ - male; ♀ - female; L2 and L3 *Columba livia domestica*; L4 and L5 *Neophema Splendida*; L6 and L7 *Psittacula krameri*; L8 and L9 *Agapornis spp.*

Conclusions:

1. The success rate of the PCR reaction for molecular sexing was significantly higher from oral swab samples (94.06%) than from feather samples (82.43%) in the test species: *Psittacula krameri, Neophema splendida, Agapornis spp.* and *Columba livia domestica*.
2. For the molecular sexing of Domestic Pigeon carcasses, the most reliable results were obtained from blood clot samples (100%), then from feathers (95.34%) and lastly from oral swabs (93.02%).
3. The success rate of the PCR reaction was lower in juvenile birds (72.09%) in the case of contour feathers, small in size with poorly developed calamus, that contain a reduced amount of DNA.
4. In the present study sexing from down feather samples was succesfully performed in the case of a 7 days old pigeon.
5. Oral swab samples were effective for sexing newly hatched featherless chicks, but in the case of carcasses displaying stomatitis lesions and pseudomembrane deposits in the oral cavity, DNA could not be isolated, because the samples had a reduced number of epithelial cells and implicitly, of DNA.

4. An experimental study of collection and preservation of biological samples for molecular sexing in birds

The main objective of this study was to determine whether DNA present in oral swabs and dried blood spots (DBS) can be preserved if stored for 30 days at room temperature (20-22 °C). The purpose of this study is to facilitate the conditions of transport and storage of samples until processing. The secondary objective was to compare the DNA concentration of blood collected on anticoagulant with dried blood drops on filter paper (DBS), to find a more efficient alternative for blood transport, storage and processing for sexing of pet birds. The present study also compares the amounts of DNA obtained from drops of fresh blood collected on filter paper with standard 20 µl drops of anticoagulant blood collected on filter paper.

In this study, 4 individuals from three companion bird species (*Columba livia domestica, Psittacula krameri* and *Psittacus erithacus*) were included. The DNeasy Blood & Tissue kit from Qiagen was used for DNA extraction, along with a classic PCR protocol described by Ito et al. (2003), using the P2/NP primers.

DNA extraction from oral swab and DBS samples stored at room temperature was performed on days 0, 1, 2, 4, 7, 14 and 30. The resulting bands had the same intensity on day 0 as on day 30 (Fig. 4).

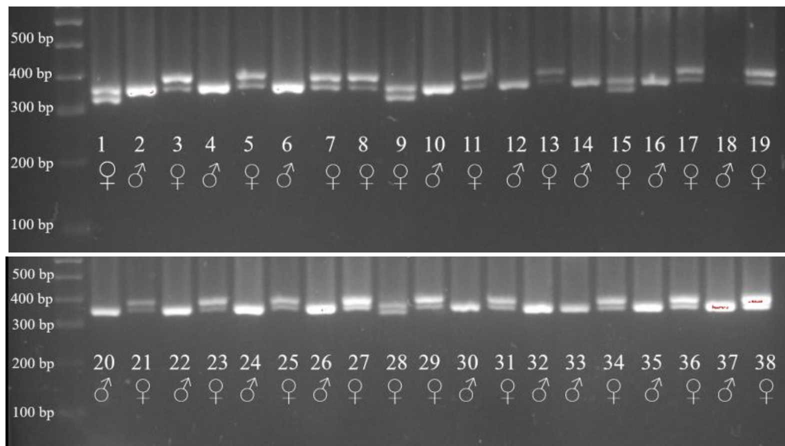

Fig. 4 PCR results of the 38 samples included in the present study identified a single band in males and two bands in females (original)

Conclusions:

1. Following quantification, the highest amount of DNA was identified in whole blood samples (45.05 ± 0.49 ng/µl), followed by blood samples with anticoagulant placed in the form of a standard drop of 20 µl on filter paper (25.8 ± 5.02 ng/µl), then by fresh blood samples collected as a drop directly on filter paper (16.21 ± 5.40 ng/µl), and finally, the lowest amount of DNA was identified in oral swabs (12.52 ± 3.07 ng/µl).
2. DNA concentration resulting from blood samples collected as a drop directly on filter paper was depended on the size of the collected drops (10.81-21.61 ng/µl).
3. DNA quantification proved to be essential in the optimization of PCR protocol, such that 100 ng/µl of DNA is required for a successful PCR reaction.
4. PCR success rate for molecular sexing was 100% from whole blood, dried blood drops and oral swabs.
5. Species-specific differences in band migration were identified in females. Bands from female Domestic Pigeons migrated between 300-350 bp, compared to those from female *Psittacine* (*Psittacula krameri* and *Psittacus erithacus*) that migrated between 350-400 bp.
6. The present study proves that transportation and storage of DBS - "dried blood drops" on filter paper and oral swabs at room temperature for 30 days does not affect the quality of DNA required for genetic sexing in birds.

5. A comparative study for DNA sample extraction techniques in bird sexing

The main objective of this study was molecular sexing of companion birds from various tissue samples (blood, feathers, oral swabs, liver, skin, intestine, myocardium, testis) collected from Chicken (*Gallus gallus domesticus*), African Gray Parrot (*Psittacus erithacus*), Yellow-breasted Macaw (*Ara ararauna*) and Budgerigar carcasses

(*Melopsittacus undulatus*). The secondary objectives of this study were to establish a working protocol for pre-lysis/denaturation of feather samples in order to obtain an optimal quantity and quality of DNA for PCR amplification, as well as to test two commercial DNA extraction kits.

Feather calamus was cut into small pieces of 2–4 mm and then processed by different techniques before the extraction protocol: 1) scalpel-cut feathers; 2) feathers cut and then crushed with a mortar; and 3) feathers cut and then subjected to mechanical shock by high-speed shaking togheter with stainless steel beads using the TissueLyserII (Qiagen, USA). DNA was extracted according to the manufacturer using two commercial kits (Isolate Genomic DNA kit; Bioline Reagents Limited, London, U.K.; Patho Gene-spin™ DNA/RNA Extraction Kit, Intron). NanoDrop ND-1000 was used for DNA spectrophotometer quantification.

In this study, 6 individuals from four companion bird species (*Gallus gallus domesticus, Melopsittacus undulatus, Psittacus erithacus* and *Ara ararauna*) were included. A classic PCR protocol described by Griffiths et al. (1998) was implemented, using the P2/P8 primers (Fig. 5).

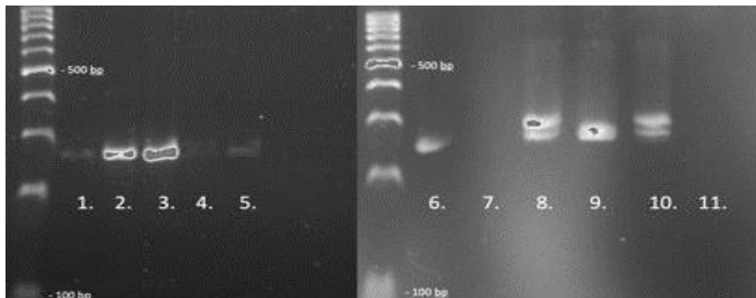

Fig. 5 Molecular sexing through PCR using P2 and P8 primers (original)

Legend*:* 1. *Melopsittacus undulatus*, male, blood; 2. *M. undulatus*, male, oral swab; 3. *M. undulatus*, male, liver; 4. *M. undulatus*, female, oral swab; 5. *M. undulatus*, male, feathers; 6. *Gallus gallus domesticus*, male, feathers; 7. *G. g. domesticus*, female, feathers; 8. *Psittacus erithacus*, female, feathers; 9. *Ara ararauna*, male, feathers; 10. *P. erithacus*, female, oral swab; 11. *A. ararauna*, male, blood

Conclusions:

1. All tissue samples studied, collected both from dead birds (blood, feathers, skin, intestine, heart, testis) and from live birds (blood, feathers, oral swab) can be used to identify the sex of monomorphic birds.
2. For DNA extraction from tissue samples from birds we recommend the use of Isolate II Genomic DNA kit, Bioline, due to the efficiency proven in the present study.
3. For feathers, the highest DNA concentration was obtained by mechanical denaturation using the TissueLyserII, followed by denaturation by mortar and the least efficient extraction method was sectioned with a scalpel.

4. DNA amplification was successfully performed from blood, oral swab, feather and liver samples in *Melopsittacus undulatus*; from oral swab and feather samples in *Psittacus erithacus*; and from feather samples in *Ara ararauna* and male *Gallus gallus domesticus*. It was not possible to amplify DNA from blood samples in *Ara ararauna* (autolyzed corpses) and from feather samples in female *Gallus gallus domesticus*, (small calamus feathers), therefore we recommend DNA amplification from oral swab samples.

6. PCR protocols for molecular sexing in monomorphic birds

The objective of this study was to compare three DNA amplification techniques and establish an optimal DNA amplification protocol for sexing companion birds. Feather, oral swab and blood samples (DBS) were collected from birds belonging to the orders *Psittaciformes (Ara ararauna, Psittacus erithacus* and *Psittacula krameri*) and *Columbiformes (Columba livia domestica*). Two of the protocols tested were conventional PCR, using primers P2/P8 (Griffiths et al., 1998) and P2/NP (Ito et al., 2003), while the third protocol was multiplex PCR using primers P0, P2 and P8 (Han et al., 2009).

In this study, 8 individuals belonging to the orders *Psittaciformes (Ara ararauna, Psittacus erithacus* and *Psittacula krameri)* and *Columbiformes (Columba livia domestica*) were included. DNA extraction was performed using the Isolate II Genomic DNA kit from Meridian Bioscience, USA.

The present study demonstrated that all three PCR protocols can be used for the molecular sexing of the orders *Psittaciformes (Ara ararauna, Psittacus erithacus* and *Psittacula krameri*) and *Columbiformes (Columba livia domestica*). The PCR products showed two bands in females (corresponding to the CHD1-W and CHD1-Z genes) and a single band in males (corresponding to the CHD1-Z gene), in all three PCR protocols tested (Fig. 6).

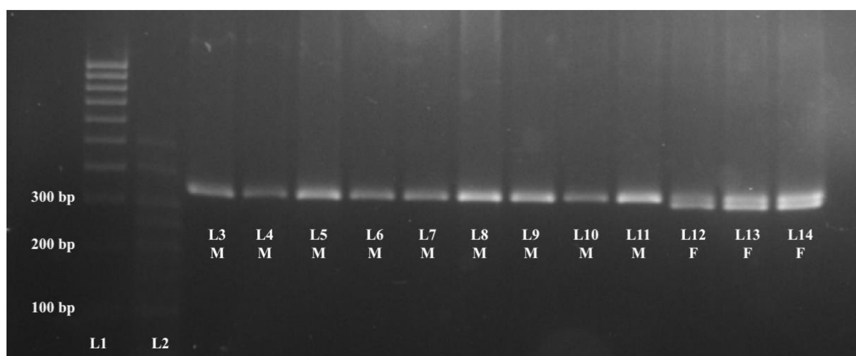

Fig. 6 PCR gel from *Columbiformes* using protocol 2 (original)

Legend: L1 - Size standard (100-bp DNA ladder); L2 - Size standard (25-bp DNA ladder); L3, L6, L9, L12 - oral swab; L4, L7, L10, L13 - feathers; L5, L8, L11, L14 - dried blood spots; M - male; F- female

In the present study an additional band was identified in the female *Psittacula krameri*, this being the product of the amplification of the primer pair P0/P2 (Fig. 7).

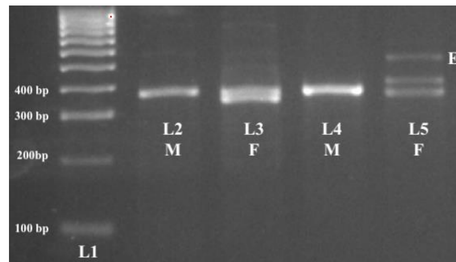

Fig. 7 PCR gel from *Columbiformes* and *Psittaciformes* using protocol 3 (original)

Legend: L1 - Size standard (100-bp DNA ladder); L2, L3 - *Columba livia domestica*; L4, L5 - *Psittacula krameri*; M - male; F - female; E - additional band

Conclusions:

1. PCR sexing of the monomorphic birds included in this study was successfully performed using feather, oral swab and DBS samples.
2. All three PCR protocols can be used for molecular sexing of the orders *Psittaciformes (Ara ararauna, Psittacus erithacus* and *Psittacula krameri*) and *Columbiformes (Columba livia domestica*).
3. We recommend the protocol using the P2/NP primer pair described by Ito et al. (2003), which provided the clearest bands in all species tested *(Ara ararauna, Psittacus erithacus, Psittacula krameri* and *Columba livia domestica*).

7. Molecular sexing of wild and companion birds using samples collected by minimally invasive methods

The main objective of this study was to use molecular genetic techniques in order to sex wild and companion birds belonging to the orders *Falconiformes, Accipitriformes, Galliformes, Anseriformes, Passeriformes, Psittaciformes*, using minimally invasive sample types (oral swabs and feathers) and samples collected invasively (whole blood).

In this study, 43 individuals belonging to the orders *Falconiformes, Accipitriformes, Galliformes, Anseriformes, Passeriformes* and *Psittaciformes* were included. DNA extraction was performed using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany).

The present study demonstrates the applicability of all sample types (feathers, oral swab and blood) for molecular sexing of the bird orders examined. P2/NP primers (Ito et al., 2003) were used for sexing birds from the orders *Galliformes, Anseriformes, Passeriformes* (Fig. 8), and *Psittaciformes* (Fig. 9). Since the samples collected from *Buteo buteo (Accipitriformes*) and *Falco subbuteo (Falconiformes*) failed to identify the sex with the help of P2/NP primers (only one band appeared in both males and females), we

retested the samples using three primers P2, NP, and MP, as recommended by Ito et al. (2003) (Fig. 10).

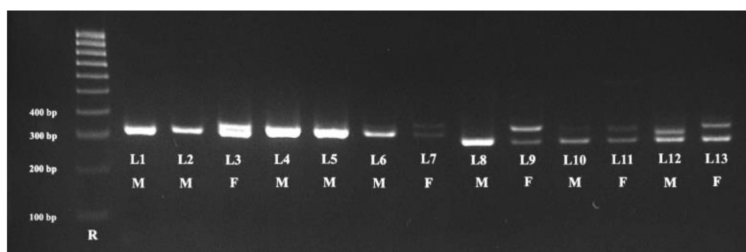

Fig. 8 Molecular sex identification in *Galliformes* (L1), *Anseriformes* (L2, L3) and *Passeriformes* (L4-L13) (original)

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*. Females (F) showed two bands (L3, L7, L9, L11, L13), while males (M) showed a single band (L1, l2, L4-L6, L8, L10, L12)

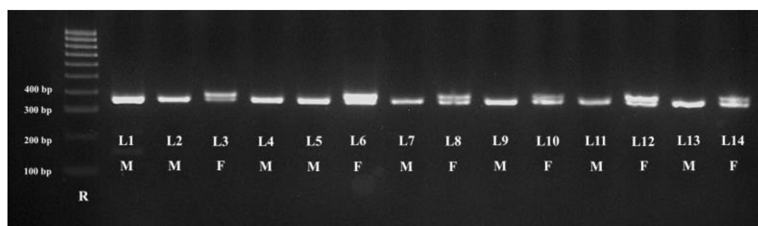

Fig. 9 Molecular sex identification in *Psittaciformes* (original)

Legend: Large sizes *Psittaciformes*: L1 - *Ara macao*; L2, L3 - *Psittacus erithacus*; L4 -*Cacatua alba*; Medium size *Psittaciformes*: L5, L6 - *Psittacula krameri*; L7, L8 - *Psephotus haematonotus*; L9, L10 - *Nymphicus hollandicus*; L11, L12 - *Agapornis fischeri*; Small size *Psittaciformes*: L12, L13 - *Melopsittacus undulatus*. Females (F) showed two bands (L3, L6, L8, L10, L12, L14), while males (M) showed a single band (L1, l2, L4, L5, L7, L9, L11, L13)

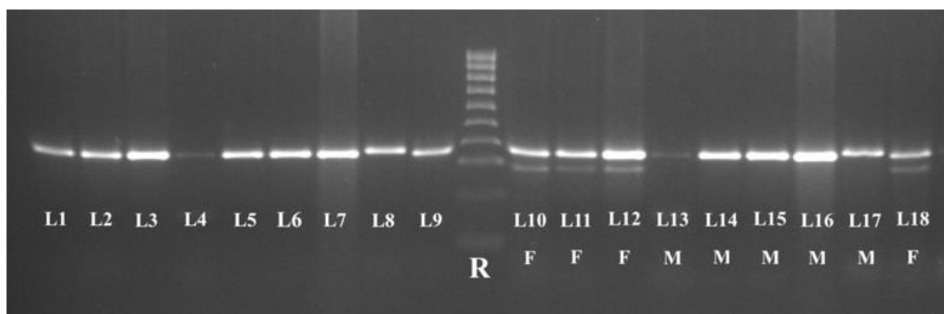

Fig. 10 Molecular sex identification in raptors *Accipitriformes* (L1-L8; L10-L17) and *Falconiformes* (L9; L18) (original)

Legend: Primer pair P2/NP – L1-L2 was used. A single band was obtained in both sexes. The primer pair P2/NP/MP – L10-L18 was used. Females (F) showed two bands (L10-L12, L18), while males (M) showed a single band (L13-L17)

Conclusions:

1. The present study demonstrates the applicability of all sample types (feathers, oral swab and blood) for molecular sexing of the bird orders examined (*Galliformes, Anseriformes, Passeriformes, Psittaciformes, Accipitriformes* and *Falconiformes*).
2. P2/NP primers were used for sexing birds from the orders *Galliformes, Anseriformes, Passeriformes* and *Psittaciformes*.
3. Molecular sexing of the orders *Accipitriformes (Buteo buteo*) and *Falconiformes (Falco subbuteo*) could be achieved using an additional primer, MP, alongside the P2/NP pair.
4. The molecular identification of the sex in Carduelis cucullata and Carduelis carduelis major has not been reported in literature to date.
5. We recommend using samples collected through minimally invasive techniques such as feathers and oral swabs, instead of using blood samples. We also recommend testing both types of samples for each bird species.

8. A comparative study in sexing Domestic Pigeon (*Columba livia domestica*) carcasses using endoscopic and molecular sexing techniques

The main objective of this study was to compare endoscopic sexing techniques with molecular sexing techniques and to analyze the accuracy of these techniques. The secondary objectives of this study were endoscopic sexing of 25 Domestic Pigeon (Columba livia domestica) carcasses, confirmation of results through necropsy, molecular sexing through testing of oral swab, feather, and blood clot samples, comparison of two DNA extraction techniques (Isolate II Genomic DNA kit, Bioline and DNeasy Blood & Tissue kit, Qiagen), and two PCR amplification protocols (classic PCR), one using the P2 and P8 primers (Griffiths et al., 1998), and the other using the P2 and NP primers (Ito et al., 2003), along with result comparison.

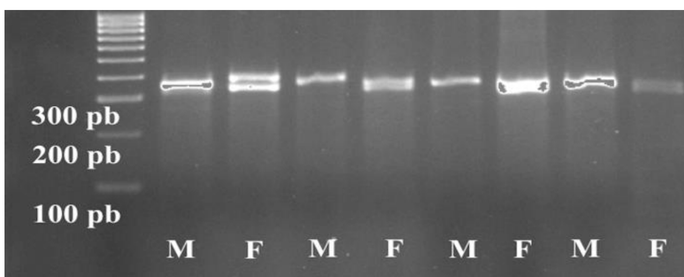

Fig. 11 Results of DNA sexing of Domestic Pigeons identifying in males (M) a single band corresponding to the CHD1Z gene, and in females (F) two bands corresponding to CHD1W and CHD1Z, from samples of oral swabs, feathers and blood clots (original)

Conclusions:

1. The most effective sexing method in Domestic Pigeon (*Columba livia domestica*) was molecular sexing (100%), followed by necropsy (84%) and finally endoscopic sexing (72%).
2. Endoscopic sexing had the lowest efficiency due to reduced visibility of the coelomic cavity (caused by intracoelomic hemorrhage or coelomitis with adhesions) and examiner experience.
3. Juveniles with small, poorly differentiated gonads could only be sexed by the molecular sexing method.
4. For molecular sexing, the most reliable results were obtained from blood clot samples (100%), then oral swabs (88%) and finally feathers (80%).
5. Oral swab samples collected from pigeons displaying stomatitis and pseudomembrane deposits could not be amplified.
6. Even though blood tests have proven to be the most accurate for pigeon sexing, oral swabs are the minimally invasive samples recommended by the authors. This sampling technique can be applied even to newly hatched chicks and can be a very useful tool for bird breeders.

9. Comparative evaluation of two techniques of sex determination in Lovebirds (*Agapornis spp*.)

The objectives of this study were to determine the sex of Lovebirds (*Agapornis spp.*) by two methods, surgical sexing by celioscopy and sexing by molecular testing from blood samples, as well as to compare the results of the two methods in order to evaluate their accuracy.

In this study, a total of 42 individuals were sexed surgically via celioscopy and genetically sexed through molecular testing from blood samples.

Conclusions:

1. Following the two methods of sexing the 42 Lovebirds, 26 males and 16 females were identified by the endoscopy technique and 25 males and 17 females by molecular sexing.
2. Considering the accuracy of molecular sexing in birds, proven in previous studies, we argue that molecular sexing is more accurate than celioscopy in sexing Lovebirds (*Agapornis spp*.).

III. Final conclusions and recommendations

Our research aimed to follow three main directions represented by surgical methods for endoscopic sexing, genetic methods for molecular sexing and traditional methods regarding the identification of gonads following necropsy, as well as comparing these techniques, organized in several studies that generated the following conclusions:

1. The technique of endoscopic sexing in Domestic Pigeon (*Columba livia domestica*) had an accuracy of 71% for carcasses compared to 81% in the case of necropsy. Juveniles showing poorly differentiated gonads could not be sexed by either method. Following sexing in live Lovebirds, different results were obtained in the case of one individual in endoscopic sexing, where it was identified as male, compared to molecular sexing, where it was identified as female.
2. Genetic methods of bird sexing were based on the identification of the CHD gene (CHD1Z and CHD1W) by various PCR techniques for the following non-ratites species of birds: *Agapornis spp. (ex: Agapornis fischeri), Ara spp. (*2 subspecies*: Ara ararauna* și *Ara macao), Buteo buteo, Cacatua alba, Carduelis spp. (*2 subspecies*: Carduelis carduelis major* și *Carduelis cucullata), Chloebia gouldiae, Columba livia domestica, Cygnus cygnus, Falco subbuteo, Gallus gallus domesticus, Melopsittacus undulatus, Neophema splendida, Nymphicus hollandicus, Phasianus colchicus, Psephotus haematonotus, Psittacula krameri, Psittacus erithacus, Serinus canaria forma domestica* și *Taeniopygia castanotis*.
3. The experimental study proves that transportation and storage of DBS (dried blood drops on filter paper) and oral swabs at room temperature for 30 days does not cause alterations in the quality of DNA required for genetic sexing in birds.
4. The highest concentration of DNA from feathers was obtained after processing by mechanical shock with the TissueLyserII apparatus (Qiagen).
5. Quantification of DNA has proven to be essential in the optimization of the PCR protocol, such that 100 ng/µl of DNA is required for a successful PCR reaction.
6. Four PCR protocols were tested for bird sexing, as follows:
   - Classic PCR with primers P2 and P8 (Griffiths et al., 1998) in *Agapornis spp., Ara ararauna*, *Columba livia domestica, Gallus gallus domesticus, Melopsittacus undulatus, Neophema splendida, Psittacula krameri* and *Psittacus erithacus;*
   - Classic PCR with primers P2 and NP (Ito et al., 2003) in *Agapornis fischeri, Ara spp. (*2 subspecii*: Ara ararauna* și *Ara macao), Carduelis spp. (*2 subspecii*: Carduelis carduelis major* și *Carduelis cucullata), Chloebia gouldiae, Columba livia domestica, Cygnus cygnus, Melopsittacus undulatus, Nymphicus hollandicus, Phasianus colchicus, Psephotus haematonotus, Psittacula krameri, Psittacus erithacus, Serinus canaria forma domestica* and *Taeniopygia castanotis;*
   - Multiplex PCR with primers P0, P2 and P8 (Han et al., 2009) in *Ara ararauna*, *Columba livia domestica, Psittacus erithacus* and *Psittacula krameri;*
   - Multiplex PCR with primers P2, NP and MP (Ito et al., 2003) in *Buteo buteo* and *Falco subbuteo.*
7. The overall success rate of the PCR reaction in birds sexing was significantly higher from oral swabs (94.06%), followed by that from feathers processed using TissueLyserII (82.43%).
8. Different amplicon migration lengths were highlighted depending on the species. In female Domestic Pigeons they migrated between 300-350 bp, compared to

those in female *Psittacines (Psittacula krameri* and *Psittacus erithacus*) that migrated between 350-400 bp.

9. Molecular sex identification of *Carduelis cucullata* and *Carduelis carduelis major* thas never been reported before in the literature.
10. The comparative study of sexing Domestic Pigeon (*Columba livia domestica*) demonstrated that the most effective method was the molecular one (100%), followed by necropsy (84%) and finally endoscopic sexing (72%).

Recommendations:

1. For molecular sexing in birds, we recommend the use of oral swabs, as it is a minimally invasive method and is suitable for all age categories, including newly hatched featherless chicks.
2. We do not recommend the collection of oral swabs in the case of birds diagnosed with ingluvistis and regurgitation symptoms, as well as the presence of pseudomembrane deposits in the oral cavity, stomatitis or mucosal lesions.
3. In case of *Psittacines*, we recommend using oral tampons with a plastic rod instead of wooden ones.
4. In the case of using feathers for molecular sexing, we recommend mechanically processing with TissueLyserII at least two freshly plucked feathers, harvested from the wings or abdomen, of large sizes, with intact calamus, properly stored, as well as avoiding the use of moulted feathers.
5. To facilitate the storage of blood samples, we recommend filter paper and the storage of samples in the form of dried blood drops (DBS) as an alternative to anticoagulant blood tubes and their transportation at room temperature (20-22°C).

IV. Originality and innovative contributions

1. The present thesis includes an original and complex study that combines all three techniques of sexing birds that do not display sexual dimorphism (endoscopic, molecular and necropsy) as well as comparative studies between them.
2. To the best of our knowledge, molecular sex identification in *Carduelis cucullata* and *Carduelis carduelis major* was reported for the first time in the specialized literature.
3. The current study reports for the first-time sex determinations using paired feather and oral swab samples in the species *Psittacula krameri, Neophema splendida* and *Agapornis spp*.
4. The thesis presents for the first time in literature the mechanical denaturation of feathers using the TissueLyserII and its maximum efficiency in terms of the amount of DNA obtained from feathers.

5. Different amplification lengths were identified in PCR product migration concerning bands in females, for example in female Domestic Pigeons, which migrated between 300-350 bp, compared to those in female *Psittacines* (*Psittacula krameri* and *Psittacus erithacus*) that had migrated between 350-400 bp.
6. Another innovative contribution is the immediate practical applicability of the research in this thesis for molecular sexing techniques in birds that do not display sexual dimorphism, within the Laboratory of Molecular Genetics, Genetics and Medical-Veterinary Eredopathology subject within FMV - USAMV Cluj- Napoca and the provision of cost-effective services to bird owners, breeders, and the Romanian Ornithological Society.