

## **Sexing as a tool for reinsertion of *Amazona aestiva* parrots to nature: use of less invasive technique**

**Sexagem como ferramenta para re inserção de papagaios *Amazona aestiva* à natureza: uso de técnica menos invasiva**

**El sexado como herramienta para la re inserción de los loros *Amazona aestiva* a la naturaleza: uso de técnica menos invasiva**

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### **Abstract**

The sexing birds is considered an important tool for behavioral studies and programs for the reintroduction of animals into the wild. Several techniques are used for this purpose, such as laparoscopy, magnetic resonance and molecular sexing. The first are considered more invasive and stressful for the animal, and the last is considered the most accurate. According to it, the aim of this study was to compare the effectiveness of using three sets of primers in the molecular sexing process of true parrots (*Amazona aestiva*). Blood samples from 10 animals were collected at a Wildlife Screening Center (CETAS) in Bahia, Brazil. The DNA was extracted and the molecular markers amplified by Polymerase Chain Reaction (PCR) using primer pairs P2/P8, 1237L/1272H and 2250F/2718R. The amplified material was visualized with electrophoresis performed at 2% agarose and 12.5% polyacrylamide gels. Among the primer sets used, the 2250F/2718R pair showed the best results for the sexing process, including visualization of the amplified products on an agarose gel. Agarose gel electrophoresis is considered to be faster and cheaper. The results revealed a sample composition of 5 males (0.5) and 5 females (0.5).

**Keywords:** CHD-Z/W; PCR; Molecular sexing; Primer 2250F/2718R.

### **Resumo**

A sexagem das aves é considerada uma importante ferramenta para estudos comportamentais e programas de reintrodução de animais na natureza. Diversas técnicas são utilizadas para esse fim, como laparoscopia, ressonância magnética e sexagem molecular. Os primeiros são considerados mais invasivos e estressantes para o animal, e o último

é considerado o mais preciso. Tendo isto em vista, o objetivo deste estudo foi comparar a eficácia do uso de três conjuntos de *primers* no processo de sexagem molecular de papagaios verdadeiros (*Amazona aestiva*). Amostras de sangue de 10 animais foram coletadas em um Centro de Triagem de Animais Selvagens (CETAS) na Bahia, Brasil. O DNA foi extraído e os marcadores moleculares amplificados por Reação em Cadeia da Polimerase (PCR) utilizando os iniciadores P2/P8, 1237L/1272H e 2250F/2718R. O material amplificado foi visualizado com eletroforese, realizada em géis de agarose 2% e poliacrilamida 12,5%. Dentre os conjuntos de primers utilizados, o par 2250F/2718R apresentou os melhores resultados para o processo de sexagem, incluindo a visualização dos produtos amplificados em gel de agarose. A eletroforese em gel de agarose é considerada mais rápida e barata. Os resultados revelaram uma composição amostral de 5 machos (0,5) e 5 fêmeas (0,5).

**Palavras-chave:** CHD-Z/W; PCR; Sexagem molecular; Iniciadores 2550F/2718R.

### Resumen

El sexaje de aves es considerado una herramienta importante para estudios de comportamiento y programas de reintroducción de animales en el medio natural. Diversas técnicas son utilizadas para este fin, como la laparoscopia, la resonancia magnética y el sexaje molecular. Las primeras son consideradas más invasivas y estresantes para el animal y la última es considerada la más precisa. Ante el expuesto, el presente trabajo tuvo el objetivo de comparar la eficiencia del uso de tres conjuntos de primers en el proceso de sexaje molecular de loros verdaderos (*Amazona aestiva*). Así, se recogieron muestras sanguíneas de diez animales, en el Centro de Triage de Animales Silvestres (CETAS) del estado de Bahía, Brasil. El ADN fue extraído y los marcadores moleculares amplificados por Reacción en Cadena de la Polimerasa (PCR), utilizando los pares de cebadores: P2/ P8, 1237/1272H y 2250F/2718R. Para la visualización del material amplificado se realizó electroforesis en geles de agarosa 2% y de poliacrilamida 12,5%. Entre los conjuntos de cebadores utilizados, el par 2250F/2718R presentó los mejores resultados para el sexaje, incluyendo la visualización de los productos amplificados en gel de agarosa. La electroforesis en gel de agarosa es considerada más rápida y menos costosa. Los resultados revelaron una composición muestral de 5 machos (0,5) y 5 hembras (0,5).

**Palabras clave:** CHD-Z/W; PCR; Sexaje molecular; Cebadores 2550F/2718R.

## 1. Introduction

The biodiversity of birds in South America has a significant prominence. According to the publication of the Brazilian Society of Ornithology (2015), much of this emphasis is attributed to Colombia and Brazil, the countries with the greatest biodiversity of birds in the world. Also, Brazil holds the biggest parrot biodiversity, with 87 species described (Piacentini *et al.*, 2015).

Brazil also stands out for its important wildlife trafficking routes (Renctas, 2016). The Wildlife Screening Centers (CETAS) are involved in the management of wildlife in Brazil. These are units authorized by the Brazilian Institute of the Environment (IBAMA) to receive animals from seizure of traffic, inspection actions and voluntary deliveries (Brasil, 2015). In CETAS, the animals are identified (species level), rehabilitated, and when possible, released in the natural habitat through reintroduction programs. On the specific case of an animal that cannot be reintroduced into its natural habitat, this one is conducted to zoos, scientific breeders or maintainers (Ibama, 2016).

From the seizures of the trafficking of wild animals, most of the specimens belong to the family Psittacidae (Freitas *et al.*, 2015). *Amazona brasiliensis* (Linnaeus, 1758) - purple parrot; *Anodorhynchus hyacinthinus* (Latham, 1790) - Great Blue Macaw and *Aratinga cactorum* - jandaia-gangarra are two of these species. Inside genus *Amazona*, the true parrot (*Amazona aestiva*, Linnaeus, 1758) characteristically it is easy to tame, it has exuberance of color and ability to reproduce human speeches (Ribeiro & Silva, 2007). These factors make the species in question one of the most found in Brazilian zoos, popular as a pet and, along with the passerines, one of the most apprehended of the traffic (Silva *et al.*, 2015).

Preferably, in the release programs of wild animals, the option is to release the animal's couple, however, this procedure is complicated by the fact that most birds do not present sexual dimorphism (Allgayer & Cziulik, 2007). In this context, sexing becomes necessary. This methodology has an impact on several activities, such as: (i) behavioral studies, management and conservation of avian fauna (Pereira *et al.*, 2021), (ii) improvement of *ex situ* captive breeding programs (Morinha *et al.*, 2012) and (iii) studies the evolution of sex chromosomes (Shizuka & Lyon, 2008).

Actually, there are several techniques for sexing birds. Among them: surgical methods, laparoscopy and molecular analysis. However, the first two cited can be life threatening to species. This is because involves the administration of anesthetics and incisions; can causing internal injuries (Vieira *et al.*, 2012). Molecular genotyping, based on the polymerase chain reaction (PCR), analyzes regions of the genome of the birds responsible for differences between males and females (Gonçalves, 2013). PCR is a technique considered fast, non-invasive and accurate, and it is possible to use different biological samples (such as blood and feather bulb) (Vieira *et al.*, 2012).

In birds, the sex is determined by genes placed on the sex chromosomes Z and W (Ellegren, 1996). The first findings on the use of genetic markers in the sexual identification of birds occurred in the early 1990s. Initially, the methods were not suitable for large numbers of species (Dos Remedios *et al.*, 2010). This scenario began to change after the publication of the studies by Griffiths *et al.* (1996), Miyaki *et al.* (1998) and Ellegren (1996). These studies investigated the existence of a specific gene for molecular sexing of carinatus (non-ratite) birds: *CHD-1* (Vieira, 2009; Vucicevic *et al.*, 2012). The allelic versions of this gene are different (i) in females (heterogametes), whose *CHD-Z* and *W* alleles are allocated in chromosomes Z and W and (ii) in males (homogametes), whose *CHD-Z* allele is allocated in the two chromosomes Z (Fridolfsson & Ellegren, 1999).

The *CHD-1* gene is considered the most reliable gene for sexing carinata birds (Vucicevic *et al.*, 2012). In ratites (ostrich and emu) the Z and W sex chromosomes are morphologically similar to the autosomal ones (Ellegren, 1996; Vieira, 2009). Molecular sexing studies, mainly, report the use of three sets (pairs) of primers for carinata birds: (i) P2/P8 (Griffiths *et al.*, 1998), (ii) 1237L/1272H (Khan *et al.*, 1998) and (iii) 2550F/2718R (Fridolfsson & Ellegren, 1999). Other pairs of primers, such as the W1/K7 pair (Dash *et al.*, 2013), are used for the sexing of rat birds.

Regardless of the pair of primers used for the sexing of carinata birds, there may be interspecies variations in the size of the bands generated; which is the result of the interspecies variations in the size of the introns. Therefore, for the sexing of birds, it is recommended to test different sets of primers in order to choose the best one for each species (Jensen *et al.*, 2003).

Based on above explanation, the aim of this study was to evaluate which of the three protocols described in the literature (P2/P8, 1237H/1272L and 2550F/2718R) is the most suitable for the sexing of *A. aestiva* parrots. This aim was sought in parallel with the use of techniques considered more appropriate and less costly for the sexing of animals, whose samples are collected in the field.

## 2. Methodology

Blood samples were collected from 10 true parrots (*A. aestiva*), pups, kept at the Wild Animals Screening Center (CETAS), located in Vitória da Conquista, Bahia, Brazil. The research project was reviewed and approved by the Ethics Committee on Animal Use (CEUA) of the Multidisciplinary Health Institute of the Federal University of Bahia (IMS-UFBA), protocol number 029/2015. This is descriptive quantitative research (Dalfovo *et al.*, 2008; Pereira *et al.*, 2018). Samples were obtained, in duplicates, from the nail cut of the birds, using specific pliers. The material was collected on strips (cut strips of 1.0 x 0.5 cm) of sterile filter paper (JProLab®). The samples were placed in sterile microtubes and immediately transported at room temperature to the sample-processing site (Cellular and Molecular Biology Laboratory, IMS-UFBA).

DNA extraction from blood was approved through adaptations of the protocol described in the Sample Preparation Manual for use in FTA - GE Healthcare Life Sciences®. This adapted protocol was defined by work presented and denominated Alternative Protocol of Extraction (PAE). As part of the adaptation, the FTA card was replaced by a 110 mm paper filter (JProLab®). The filter paper was cut into small tapes using sterilized laminar flow scissors with ultraviolet light (UV). Once the blood was collected on filter paper and transported in microtubes to the laboratory, it was transferred to petri dishes (properly sterilized and capped), which were dried at room temperature for at least 1 hour.

As for the DNA extraction protocol, the samples (paper) were first washed in 100 mM Tris-0.1% SDS solution, tender and continuous agitation for 30 minutes. Thereafter, a further wash with 500 µL of ultrapure water was performed under the same stirring conditions. This procedure was repeated three times (in order to eliminate cellular debris and possible PCR inhibitors). Then, after discarding the water used in the wash, 50 µL of ultrapure water was added to the samples (on paper), and the material (without agitation) was kept in water bath at 90°C for 20 minutes. Finally, DNA samples eluted in ultrapure water (the filter paper was not removed from the microtube) were stored at -20°C (freezer). The extracted DNA was used in the PCRs. Amplifications were performed either (i) immediately after extraction (within 3 days), (ii) or within 7 months, (iii) or within 12 months.

Concentration and purity readings of the samples were performed on NanoDrop 2000 (Thermo Scientific, MA, USA). These readings were performed using 1 µL of each sample at three times after DNA extraction: (i) immediately, (ii) seven months after extraction and (iii) twelve months after extraction. The values of the DNA concentration of the samples were obtained directly in the equipment, in unit ng/µL. For the analysis of DNA purity, the following reference values were used: (i) for DNA purity in relation to organic contaminants (ratio 260 nm/230 nm): if pure, values between 2.0 and 2.2 are expected; - (ii) relative to protein purity (ratio 260 nm/280 nm): if pure, values between 1.7 and 2.0 are expected (Desjardins & Conklin, 2010). DNA conservation analysis (non-degradation) was performed by visualizing the bands of interest in the electrophoretic runs (of the amplified samples).

Molecular sexing was performed by amplification (and when necessary reamplification) of the DNA by Polymerase Chain Reaction (PCR), using Taq DNA Polymerase (Invitrogen®, SP, Brazil) in MyGenie96 Thermal Block thermocycler (Bionner, Korea). When samples were reamplified, 4 µL of the product of the first reaction was added as the template DNA for the second reaction. Three pairs of allele-specific primers, numbered as follows (i) Pair of primers 1 (P2/P8), (ii) Pair of primers 2 (1237L/1272H) and (iii) Pair of primers 3 (2250F/2718R). Such primers are characterized in Table 1. The reaction and thermocycling conditions used, together with each of the set of primers, are described below as Protocols P1, P2 and P3. Such conditions were optimized from modifications of the protocols described by Griffiths *et al.* (1998), Jensen *et al.* (2003) and Vucicevic *et al.* (2012).

**Table 1.** Set of primers used for sexing *A. aestiva*.

Nomenclature used for primers	Original nomenclature of primers	Sequence of primers	Protocol base to the PCR	Protocol used in PCR
Set of primers 1	P2/P8*	P2(5'-TCTGCATCGCTAAATCCTTT-3') P8(5'-CTCCCAAGGATGAGRAAYTG-3')	Griffiths <i>et al.</i> , 1998	P1
Set of primers 2	1237L/1272H**	1237L(5'-GAGAAACTGTGCAAAACAG-3') 1272H(5'-TCCAGAATATCTTCTGCTCC-3')	Jensen <i>et al.</i> , 2003	P2
Set of primers 3	2250F/2718R***	2250F(5'-GTTACTGATTCGTCTACGAGA-3') 2718R(5'-ATTGAAATGATCCAGTGCTTG-3')	Vucicevic <i>et al.</i> , 2012	P3

Primers initially described by \*Griffiths *et al.* (1998), \*\*Khan *et al.* (1998), \*\*\*Fridolfsson and Ellegren (1999).  
 Source: Survey done by the authors.

Table 1 shows the specifications of the primers used in the three different protocols followed in this work, including their respective bibliographic references.

Protocol 1 (P1) proceeded as follows. Considering the use of set of primers 1, the reaction conditions used in the present work were as follows: Buffer 1X, 3mM MgCl<sub>2</sub>, 0.2mM dNTP, 0.5uM of each primer (P2/P8), 0.17U/μL Taq DNA Polymerase, 4μL of extracted DNA, for final reaction volume of 16μL. The thermocycling conditions used were adapted from those described by Miyaki *et al.* (1998), namely: (i) Initial denaturation at 95°C for 5 minutes, (ii) followed by 36 denaturation cycles at 95°C for 1 minute, annealing at 41°C for 30 seconds and extension at 72°C for 30 seconds and (iii) Final extension at 72°C for 7 minutes.

Protocol 2 (P2) proceeded as follows. Considering the use of set of primers 2, the following reaction conditions were used: Buffer 1X, 4mM MgCl<sub>2</sub>, 0.2mM dNTP, 2uM of each primer (1237L / 1272H), 0.2U/μL Taq DNA Polymerase, 4μL of extracted DNA, To 18μL of final reaction volume. The amplifications, the results of which are described below, occurred under the following thermocycling conditions: (i) Initial denaturation at 95°C for 5 minutes, (ii) followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 59°C for 45 seconds and Extension at 72°C for 1 minute and (iii) Final extension at 72°C for 7 minutes. Such conditions were optimized from those described by Jensen *et al.* (2003), specifically the only modification was the elevation of 5°C at the annealing temperature (from 54°C to 59°C).

Protocol 3 (P3) proceeded as follows. Associated with the use of set of primers pair 3, the following reaction conditions were used: 1X Buffer, 1.5mM MgCl<sub>2</sub>, 0.1mM dNTP, 1.0 uM of each primer (2250F / 2718R), 0.05U/μL Taq DNA Polymerase, 4μL of extracted DNA and final reaction volume of 20μL. The reactions, whose data are show in the item results, occurred in the following thermocycling conditions: (i) Initial denaturation at 95°C for 4 minutes, (ii) Followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 45 seconds and (iii) Final extension at 72°C for 4 minutes. These conditions are the result of the optimization, performed from the conditions described by Vucicevic *et al.* (2012). After optimization it was determined only by the use of two degrees below the annealing temperature of the primers, compared to that described by the cited author (53°C, instead of 55°C) was used.

The products obtained by PCR were submitted to electrophoresis on agarose and polyacrylamide gels. 2% agarose gels stained with ethidium bromide (EtBr) were used. In this, the samples were visualized in UV light in L-PIX Photodocumentator (Loccus Biotecnologia®, SP, Brazil). The polyacrylamide gels employed had a concentration of 12.5%, DNA (visualized on the gel) stained with silver nitrate (AgNO<sub>3</sub>). The run on both gels occurred at 90 V and 120 mA. As for the average run time of the electrophoresis, (i) when in polyacrylamide gel this was 5 hours and 30 minutes and (ii) when in agarose 1 hour and 45 minutes.

The interpretation of the results for the sexing of the individuals occurred as follows. (I) When the amplification product was visualized as two bands (of expected sizes), the individuals were diagnosed as female. The two bands were interpreted as W and Z alleles, and the genotype of the individual as WZ; (ii) When only one band of expected size was visualized, the individual was identified as male. The mentioned fragment was interpreted as Z allele, and the individual genotyped as ZZ. The sizes of the bands equivalent to the W and Z alleles varied depending on the pair of primers used in the amplification.

For the research of the frequency of males and females in the sample of parrots analyzed, a simple frequency calculation was performed, dividing the number of individuals of each sex by the total sample number.

### 3. Results

The results that allowed us to arrive at the evaluation of the performance of the different protocols used in the sexing of true parrots. The mean time of execution of the adopted DNA extraction protocol (PAE) was approximately 1 hour and 45 minutes, with a final volume of 50 μL of DNA extracted (in solution) for the sample.

The results related to the concentration and purity of the analyzed DNA samples are reported in Table 2.

**Table 2.** Average concentration and purity of extracted DNA.

Type of analysis	1 <sup>st</sup> Analysis*	2 <sup>st</sup> Analysis**	3 <sup>st</sup> Analysis**	Average of the analyzes
<b>DNA concentration (ng/uL)</b>	230.86 (+/-59.37)	66.41 (+/- 19.5)	59.33 (+/- 17.76)	118.86 (+/-97.05)
<b>Purity</b>				
<b>DNA /Organic Contaminants</b>	1.65 (+/- 0.14)	1.79 (+/- 0.6)	0.95 (+/- 0.2)	1.79 (+/- 0.45)
<b>DNA/ Proteins</b>	1.80 (+/- 0.05)	1.80 (+/- 0.05)	1.71 (+/- 0.05)	1.77 (+/- 0.05)

\*Immediately after extraction. \*\*7 months post extraction. \*\*\*12 months post extraction.  
Source: Research data.

In Table 2, it is possible to observe the data distributed in three moments after extraction: (i) immediately, (ii) 7 months, and (iii) 12 months post extraction. Considering the same temporal order of reading, follow the details for each analysis. Each value represents the reading average of the 10 samples. Regarding the concentration of extracted DNA, the averages obtained were: (i) 230,86 (+/-59,37); 66,41 (+/- 19,55); 59,33 (+/- 17,76); final media: 118, 86 (+/- 97,05). The purity of the DNA for organic contaminants, presented the following averages: (i) 1,65 (+/- 0,14); (ii) 1,79 (+/- 0,6); (iii) 0,95 (+/- 0,20); final media 1,79 (+/- 0,45). The averages of DNA purity readings for proteins follow: (i) 1,80 (+/- 0,11); (ii) 1,80 (+/- 0,05); (iii) 1,71 (+/- 0,05); final media 1,77 (+/- 0,05).

The sizes of the fragments obtained in the PCRs (from the amplification of the genes used for sexing), considering each of the pairs of primers used, are described in Table 3. The data are cited in the following order, the marker related to the Z chromosome followed by the marker related to the W chromosome.

**Table 3.** Sizes of the molecular markers used for the sexing of *A. aestiva*.

Primers used	Molecular marker related to the Z chromosome (bp)	Molecular marker related to the W chromosome (bp)	Difference in size between markers (bp)
<b>Pair of primer 1</b>	396 *	412 *	16
<b>Pair of primer 2</b>	290 **	310 **	20
<b>Pair of primer 3</b>	610 ***	480***	130

Source: Research data.

In Table 3, it can be seen that amplifying with Pair of primers 1, fragments of approximately 396 bp and 412 bp were obtained. Using the pair of primer 2, fragments of about 290 bp and 310 bp were observed. Moreover, with the use of the pair of primers 3, bands of approximately 610 bp and 480 bp were found. The approximate differences between the markers obtained with the use of each pair of primers follow: pair of primers 1-16bp; pair of primers 2-20pb; and pair of primers 3-130bp. The size variation between the markers obtained considering the three pairs of primers, focusing on the molecular markers linked to each chromosome or allele, (i) for the W allele was observed variation from 310 to 480 bp, and (ii) For the Z allele variation was obtained from 290 bp to 610 bp.

The result of attempts of molecular sexing with the three pairs of primers are shown in figures 1 and 2. Figure 1 shows the results after agarose gel electrophoresis with each pair of primers obtained from the amplification (Fig. 1B) and



reamplification (Figs 1A and 1C) of the samples of the 10 parrots. The visualization of the fragments shows that the different pairs of primers have different effectiveness as the ability in the amplification.

As for the visualization of the products obtained with the use of Pair 1, it was not possible to observe amplified fragments, except for the sample 08; this was the only one that presented a visible band (in agarose gel). For amplification products with pair of primers 2, amplification products were visualized for most samples, except for samples 09 and 10. Visualization of the amplification products using pair of primers 3 demonstrated fragments amplified from all samples.

Considering the visualization of agarose gel amplification products, the performance of the primer pairs was shown as follows (mentioned from the most efficient to the least efficient): Pairs of primers 3, 2 and 1. In addition, Pair of primers 3, was the only one that generated allele-specific fragments with sizes different enough to (agarose gel) to be safely used for molecular sexing.

The figure 2 shows the molecular markers obtained from the reamplification (Fig. 2A) and amplification (Fig. 2B) of the samples of the 10 parrots with the Pair of primers 1 and 2 after polyacrylamide gel electrophoresis. The figure 2A shows the molecular markers generated by the use of Par of primer 1. In this, it can be observed that there was amplification of the fragments of interest, from all samples. Although, there was no clear separation of the Z and W allele bands in the female samples (samples 04, 07 and 08), it was possible to define the sex of the 10 individuals (using a separation pattern and band thickness).

In figure 2B it is possible to visualize the amplification products of the molecular markers generated with the use of primer pair 2. In this figure, we can see the expected allele-specific fragments, whose amplification occurred from all samples. The separation of the expected fragments by this pair of primers (pair of primers 2) occurred more efficiently (visible) than among the fragments generated by pair of primers 1. This allowed and facilitated the determination of the sex of the individuals, as follows: (i) individuals 01, 02, 03, 09 and 10 as males (these presented a single band, Z allele), (ii) as individuals 04, 05, 06, 07 and 08 were genotyped as females (presented with two bands, Z and W alleles). With this data it was possible to define that in the polyacrylamide gel between pairs of primers 1 and 2, pair of primers 2 (Fig. 2B) presented the best results for genotyping due to the sharpness and mainly the better separation between the equivalent bands to sex-specific alleles.

The data comparison between figures 1 and 2 shows that the amplification products with the primer pairs are displayed differently on the two types of gels employed, except for the products obtained with Primer pair 3 (2250R/2718F). Fragments generated by the use of primer pair 3 were only applied on agarose gel.

Considering the pair of primers 1 (P2/P8), while it was not possible to visualize the fragments generated by this in agarose gel (Fig. 1A), the application of the same fragments in polyacrylamide gel allowed (difficult) sexing (Fig. 2B). In relation to the pair of primers 2 (1237H/1272L), the correct visualization of the bands at the expected heights (290 bp for the Z allele and 310 bp for the W allele) was not possible in agarose gel (Fig. 1B). Specifically, although the alleged male subjects presented the only interest band; in the supposed female individuals it was not possible to visualize the two diagnostic bands. However, when the amplification products with this pair of primers were run on polyacrylamide gel, it was possible to sex the parrots. On those, the bands of expected sizes were visualized (Fig. 2B).

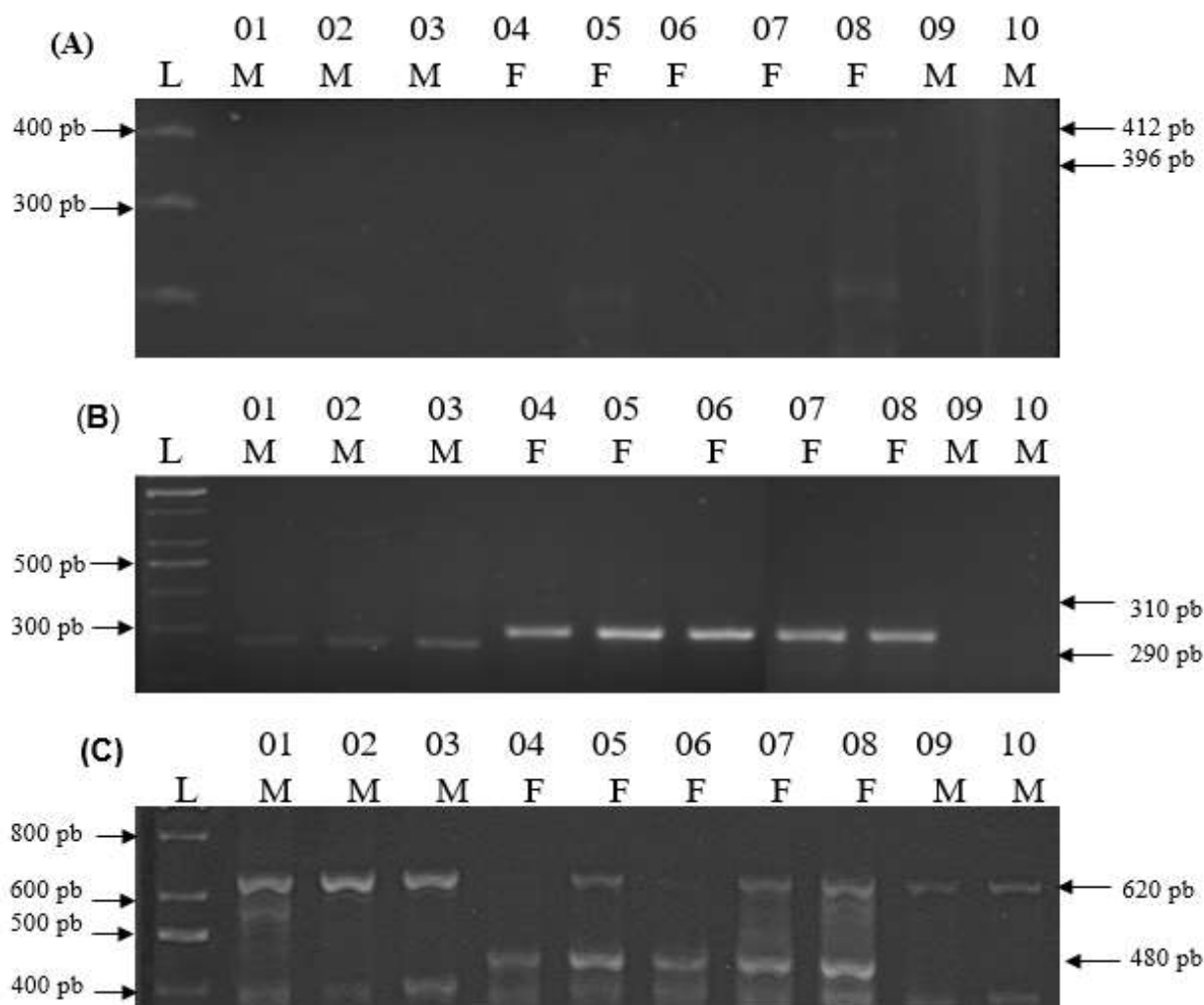
The small difference between the sizes of the diagnostic bands for the Z and W alleles with the use of Pair of primers 2 (20pb), justifies the performance difference of this primer pair (for genotyping) between the gels. Therefore, for this pair of primers, while the sexing interpretation was inconclusive on agarose gel, it was conclusive on polyacrylamide gel.

With the use of Pair of primers 3 it was possible to visualize the amplification products through 2% agarose gel electrophoresis (Fig. 1C), also allowing a clear distinction between allele-specific fragments. There is no difficulty in sexing in this case. Therefore, polyacrylamide electrophoresis was not required. The sex determination of true parrots was possible through

the usage of the 3 pairs of primers, even when they were used separately. However, sexing was facilitated when employing the pair of primers 3 (2550F/2718R).

When overlapping the completed genotypes with the use of the different pairs and primers, these were coincident with each other. As a result of the molecular sexing of the samples, of the 10 genotyped parrots, 5 were identified as males and 5 as females. Which is equivalent to the following relative frequencies - 0.5 males and 0.5 females.

**Figure 1.** Electrophoretic run, in agarose gel 2%, molecular markers used for the sexing of *A. aestiva* parrots.



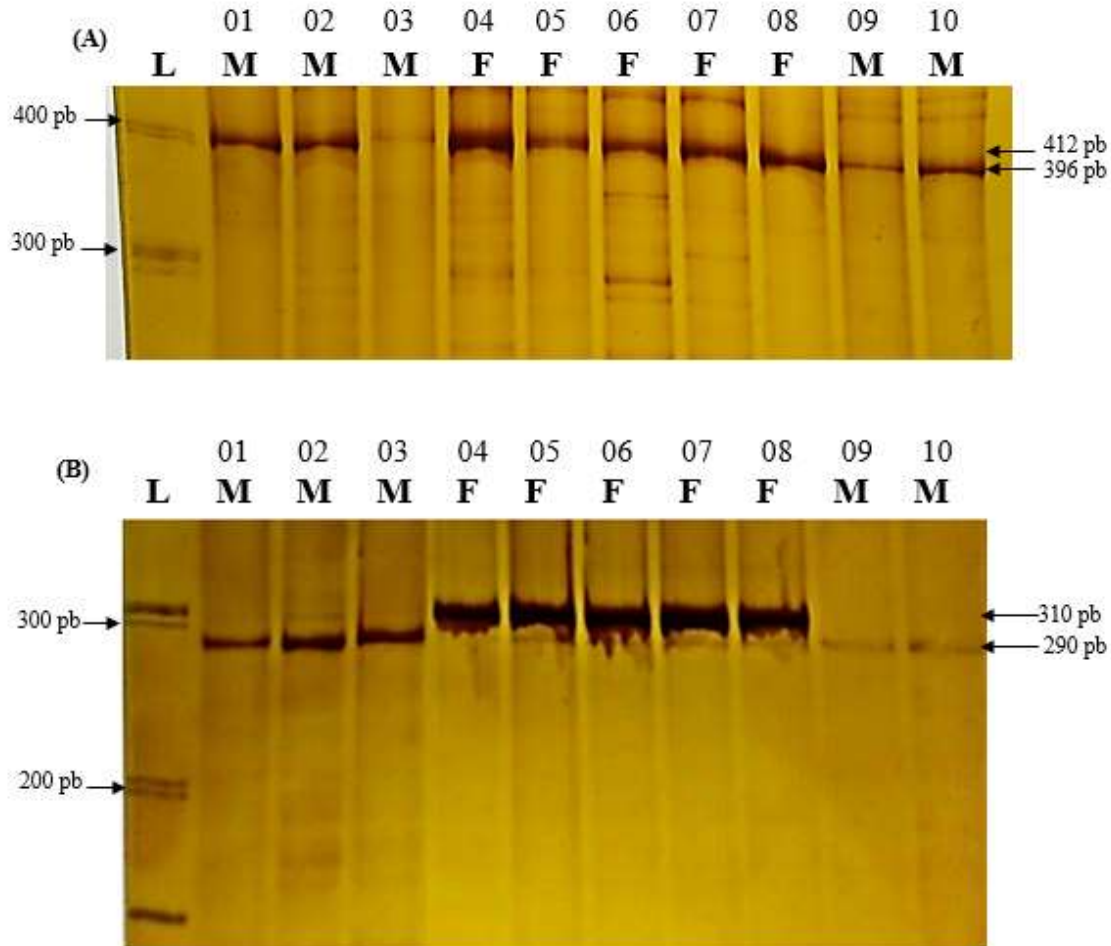
Source: Research data.

Figure 1 shows in (A) samples amplified with the Pair of primer 1 (P2/P8), expected fragments - 396 pb (interpretation - alleles Z) and 412 pb (interpretation - alleles W). In (B) shows samples amplified with the Pair of primer 2 (1237L/ 1272H); expected fragments - 290 pb (interpretation - alleles Z) and 310 pb (interpretation - alleles W). And in (C) shows samples amplified with the Pair of primer 3 (2250F/2718F); expected fragments - 620 pb (interpretation - alleles Z) and 450 pb (interpretation - alleles W). PCR products amplified from the DNA of 10 *A. aestiva* parrots; DNA stained with ethidium bromide. Electrophoresis at 90V, 120 mA, by 1 hour and 45 minutes. The same 10 samples amplified with each pair of primers were applied in the same order (from left to right) in all gels, samples identified in the figure from 01 to 10. Two fragments are visualized in the female individuals (ZW) and only one in the male individuals (ZZ). Interpretation of genotypes - ZZ: Male;



WZ: Female. F: Female Parrot; M: Male Parrot. L: Molecular weight standard, 100bp at 1Kb. The sizes (i) of the ladder bands are highlighted on the left side of the gels and (ii) the expected alleles highlighted on the right side of the gels.

**Figure 2.** Electrophoretic run, in polyacrylamide gel 12, 5 %, molecular markers used for the sexing of *A. aestiva* parrots.



Source: Research data.

Figure 2 shows in (A) samples amplified with the Pair of primer 1 (P2/P8), expected fragments - 396 pb (interpretation - alleles Z) and 412 pb (interpretation - alleles W). And in (B) shows samples amplified with the Pair of primer 2 (1237L/1272H); expected fragments - 290 pb (interpretation - alleles Z) and 310 pb (interpretation - alleles W). PCR products amplified from the DNA of 10 *A. aestiva* parrots; DNA stained with silver nitrate. Electrophoresis at 90V, 120 mA, by 5 hour and 30 minutes. The same 10 samples amplified with each pair of primers were applied in the same order (from left to right) in all gels, samples identified in the figure from 01 to 10. Two fragments are visualized in the female individuals (ZW) and only one in the male individuals (ZZ). Interpretation of genotypes - ZZ: Male; WZ: Female. F: Female Parrot; M: Male Parrot. L: Molecular weight standard, 100bp at 1Kb. The sizes (i) of the ladder bands are highlighted on the left side of the gels and (ii) the expected alleles highlighted on the right side of the gels.

#### 4. Discussion

The results obtained through this work were conclusive regarding the molecular sexing of *A. aestiva*. The methodology used basically included three molecular techniques, as follows: (i) DNA extraction, (ii) amplification of genetic markers for sexing, and (iii) electrophoresis.

The extraction of DNA, performed with Alternative Extraction Protocol (PAE), was efficient in relation to the concentration (and quantity) of the obtained DNA. As regards the purity of the extracted DNA, the average values of the (i) DNA/Proteins and (ii) DNA/Organic compounds showed purity within the recommended relative to the protein and not ideal relative to the organic components (although close in the first two analysis). Considering that this is an alternative protocol for DNA extraction, consisting essentially of the membrane lysis step (and not contemplating the steps of DNA purification, precipitation and rehydration), no ideal purity of the extracted DNA was expected. It is believed that the lack of purity of the extracted DNA did not strongly influence the amplification, since all samples were amplified using Primers Pair 3, what corroborates the research by Pereira *et al.* (2021).

Regarding the analysis of the conservation of extracted DNA, it was verified conservation for a period of at least 12 months after extraction. An alternative extraction protocol similar to the one reported here was also used by Vieira (2009). According to the author, in some respects the alternative protocol presented similar efficacy to the pre-established phenol/chloroform method, described by Sambrook *et al.* (1989). Among the advantages of the use of PAE, it is mentioned: (i) being less laborious, (ii) demand less time and less financial resources and (iii) do not generate toxic waste (does not use phenol).

Both the Vieira (2009) and the present study, obtained DNA amplification (post-extraction) for 7 months (or more, in the case of Vieira). In addition to the DNA conservation analysis, it should be noted that among the protocols used by Viera (2009) and the one used here, there was a difference in the DNA storage temperature. In the present work the samples were stored at -20°C, while Vieira (2009) kept them at room temperature. It was not possible to draw more parallels regarding the differences in the execution of the SAP protocols between the two protocols compared here, due to lack of data in the literature.

As for the amplification of the molecular markers, sometimes, besides the diagnostic bands of sexing, nonspecific bands were also observed. It is possible that such bands could be avoided by using DNA extraction protocol (i) that would generate purest genetic material, and/or (ii) that was specific for DNA extraction from eukaryotes. Specifically, because this could prevent the simultaneous extraction of DNAs from other organisms, such as pathogens (viruses and/or bacteria).

Jensen *et al.* (2003), using pair of primers 3, also obtained additional bands. As in the present work, the additional bands obtained in the mentioned article did not interfere in sexing. In relation to the amplification of additional bands, it is difficult to establish parallel with the literature because most of the articles do not mention if nonspecific bands in gel were observed. In addition, generally published images of electrophoresis gels are about photo cutouts. This provides a partial view of the bands obtained in the amplifications.

In the present research initially using the original protocols (whose authors were mentioned in the Methodology), (i) either the bands were not visualized, (ii) or they appeared very faintly. In general, optimization improvements were obtained in relation to the visualization of the bands of interest. This occurred (i) through changes in the thermocycling programs and (ii) by re-amplification of PCR products.

Regarding the modification of the thermocycling conditions, the annealing temperature of the pair of primers 2 was increased and the annealing temperature of the pair of primer 3 was increased. Several authors mention modifications in the thermocycling programs originally established for the sexing of birds. Jensen *et al.* (2003), changed the program to pair of primers 1 (P2/P8), originally described by Griffiths *et al.* (1998). Thus, Jensen *et al.* (2003) obtained the sexing of 47 species of birds, among them twelve species of the family Psittacidae (which the species *A. aestiva* belongs to). Ong and Vellayan (2008),

altered the thermocycling program initially used with Pair of primers 2; and thus sexing 32 species of birds, five of them belonging to the family Psittacidae. These describe variation in annealing temperature (1°C - 5°C), a feature used when the PCR product was poorly visible on gel. Vucicevic *et al.* (2012), modified the program originally described for Pair of primers 3; and thus obtained the sexing of 58 species of birds (among them, *A. aestiva*). As for PCR reamplification, the use of such a feature has already been mentioned in the literature, being used when the product of the first amplification was not visible, or weakly visible in gel (Vucicevic *et al.*, 2012).

Despite the multiple pairs of primers described for molecular sexing of carinata birds, studies have shown that they do not have the same efficacy for genotyping of all species. Malaitad *et al.* (2015) considered P2/P8 as the one that generates the best result for the sexing of swallows (*Hirundorustica*), when comparing the performance of the three pairs of primers described in the present study. Jensen *et al.* (2003) also described higher efficiency of Pair 1 primers for the sexing of passeriform birds when compared to non-passeriform birds. In the same study, the authors mention that the fragments corresponding to the Z and W alleles in passerine birds presented larger size than those generated from the other birds. This, according to Jensen *et al.* (2003), was possibly due to some not clearly defined evolutionary advantage.

Wang *et al.* (2007) published a sexing study using the *CHD* gene for *A. aestiva*, and another 79 species, using two of the pairs of primers used in this study (pairs of primers 2 and 3). In fact, sexing for species *A. aestiva* can be determined with the simultaneous use of two pairs of primers (Wang *et al.*, 2007). Also in this study, the authors obtained results that enabled the diagnosis of most birds (63 species - 78.75%) when using the pair of primers 2.

Vucicevic *et al.* (2012), also performed sexing of *A. aestiva*, however comparing the activity of primers pairs 1 and 3. In this, the authors point out better results with the use of pair of primers 3, which involved better visualization of the products obtained in gel of 2% agarose. In the same study, in only 8 of the 58 species studied, the use of set of primers 1 was more successful than the use of set of primers 3. In the present study, the amplification results of the 10 samples of *A. aestiva* were also interpreted faster and more securely when using the pair of primers 3.

According to Griffiths *et al.* (1996, 1998), pair of primers 1 presents considerable precision for the sexing of most birds. The specificity of this pair of primers is justified by the high proportion of cytosine and guanidine (C and G) in the composition of the primers involved (Griffiths *et al.*, 1996, 1998 in Vieira, 2009). However, polymorphisms were verified in intronic regions of the *CHD1* gene, generating difficulties in the molecular sexing of carinat birds.

Dawson *et al.* (2001), found in the species *Aethia pygmaea* polymorphisms in the Z allele; which sometimes led to the definition of males as females. This occurred because the Z allele presented two allelic forms, generating two fragments by PCR, one of them being similar in size to the W allele (found in females). Since pair of primers 2 acts in the same intronic region of the *CHD1* gene as pair of primers 1, their amplified products are also considered vulnerable to the occurrence of the same configuration variations related to pair of primers 1 (Dawson *et al.*, 2001). Although not common in the species studied by the authors, it has been reported.

According to Dawson *et al.* (2001) during PCR there may be preference of primer pairs for the amplification of alleles that originate smaller products. Primers pairs 1 and 2 show lower Z allele products than the W allele. Thus, in amplifications that produce poorly visible gel products, female individuals could occasionally be described as males (Casey *et al.*, 2009). What is warranted by both sexes has the Z allele, and this is preferably amplified. According to Miyaki *et al.* (1998), the pair of primers ideal for sexing non-ratites birds is one that, when there is a preference for amplification of a sex-specific allele, has a preference for ringing with the W allele since it differentiates the sexes (occurring exclusively in females).

Considering these recommendations, when comparing the three pairs used in the present study, the pair of primers most suitable for sexing ratites would be the pair of primers 3. In theory, unlike the first two pairs of primers cited, using primer pair

3 there is no problem of preferential annealing of the primers by the Z allele. This is because this pair of primers was designed in such a way that the amplified fragment from the W allele is smaller than that amplified from the Z allele (Fridolfsson & Ellegren, 1999). However, more studies are needed with the pair of primers 3, since comparing it with the other pairs, it was tested in fewer species (Dawson *et al.*, 2001).

Besides the products amplified with Pair of primers 3 can be visualized in agarose gel, when compared to the products of the other pairs, they present: (i) ease in the visualization in the gel, due to the sizes and yield in the amplification of the fragments; (ii) better band separation, due to the larger size difference between the amplified fragments. Furthermore, since the region amplified by this pair of primers is larger, although there is a predisposition to the occurrence of polymorphism, it probably exerts less influence on the occurrence of sexing error (Dawson *et al.*, 2001).

In the present study, it was not possible to define the sex of the birds through the use of agarose gels in the electrophoresis of the products amplified by pairs of primers 1 and 2. For these primers, when the amplification product was visualized (in agarose gel). It was not clear enough to allow interpretation of which allele it was referring to. To overcome this limitation, polyacrylamide gel electrophoresis was attempted. Vieira (2009), when studying the sexing of the species *A. aestiva* using pair of primers 1, described that it was only possible to visualize the product of the amplification from the realization of electrophoresis in polyacrylamide gel.

The results of the present study reveals that the agarose gel presents better performance when used for visualize amplified DNA with Pair of primers 3. This results are in agreement with previous studies that used this specific Pair of primers 3 (Ágh *et al.*, 2018; Del Puerto *et al.*, 2017; Franco-Gutiérrez *et al.*, 2017; Maheshkumar *et al.*, 2017; Monge *et al.*, 2018). The difference in the performance of agarose and polyacrylamide gel electrophoresis, depending on the primer pair used, is justified by what follows. Primer pairs 1 and 2 anneal on regions near the same intron of the *CHD* gene (Jensen *et al.*, 2003). This causes a difference of 16 bp between the alleles represented by the amplification by the pair of primers 1 and 20 bp when these are amplified by the pair of primers 2.

The Pair of primers 3 is distinguished from previous ones by flanking a region larger and closer to the 5' end of the *CHD-1* gene; Which in addition to generating larger product (s) (in the amplification), leads to the largest difference in size between the fragments generated (approximately 130 bp) (Baker *et al.*, 1999). According to Dos Remedios *et al.* (2010), separation by gel electrophoresis (2% agarose) works well when the PCR products differ in size by approximately 35 bp or more.

Based on the methodology developed in the present study, the sex of the 10 individuals studied was determined. The result of sexing resulted in 5 males and 5 females. These results were concordant among the pairs of primers employed (when they actually resulted in genotyping).

Finally, it was demonstrated the applicability of the protocol used in the present study (preferably with the use of Pair of primers 3), in studies involving the sexing of *A. aestiva*, including animals that are far from the sample processing site (in the field). Environmental conservation and management projects generally involve field sampling. The flexibility of the protocol described in the present work, for the collection of samples in the field, is sustained by the collection of blood having occurred from nail cutting and the material has been transported on filter paper at room temperature.

## 5. Conclusion

The present research carried out sexing of *A. aestiva* using an alternative method of DNA extraction, PCR using *CHD* gene primers, and agarose gel electrophoresis. The protocol selected as the most suitable protocol proved to be accurate and low cost. The biological material used for extraction, nail cut blood, was collected in the field and transported at room temperature; which demonstrates viability for the sexing of animals that are far from the place of processing of the samples. The alternative

protocol adopted for DNA extraction did not involve toxic reagents and generated considerable amounts of genetic material. The extracted material was shown to be pure enough to allow genotyping. Storage of the material at -20°C ensured the stability (not degradation) of this material in a period of at least 12 months after extraction. Among the three pairs of primers evaluated for amplification, set of primers 3 (2250F/2718R) was the most effective, allowing the sexing through 2% agarose gel electrophoresis. Such electrophoresis brings simplicity and speed to the process. Finally, it is recommended for the future, when not yet described in the literature, that an experimental study be carried out to select the best pair of primers for the sexing of each particular bird species.

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