Improvement of the Molecular Sexing of Parrots in the State of Bahia

Melhoria da Sexagem Molecular de Papagaios no Estado da Bahia

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Brazil presents the third largest bird diversity in the world, with about 1,800 species (Sabino & Prado, 2003). From this amount, 344 belong to the family Psittacidae; which includes parrots, macaws, parakeets, maracana, among others. Although there is a great variety of birds, there is a decrease in the number of specimens from different groups. This is especially due to human interventions, such as illegal trade or destruction of the natural habitat (Marini & Garcia, 2005). Owing to the considerable diversity of birds and their extinction rate, Brazil can be considered one of the most strategic countries in relation to conservation investments in this group of vertebrates (Sick, 1997).

According to the Wild Animals Screening Center (Centro de Triagem de Animais Selvagens - CETAS), Bahia has an average of 20,000 animals captured each year by operations carried out by the Brazilian Institute of Environment and Natural Renewable Resources (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renovįveis — IBAMA) in

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partnership with the Federal Highway Police. Leading the statistics of the arrests, are the birds. These represent 60 % of the illegal commercialization of wild animals. In recent years, the number of conservation and environmental protection programs has increased as a measure to prevent the disappearance of endangered species (Miyaki *et al.*, 1998). According to the IBAMA Normative Instruction 23 (from 2014), the main CETAS function is to receive and allocate wild animals coming from actions against animal trafficking, in addition to contribute with scientific research, education and extension.

The CETAS of Vitória da Conquista (inaugurated in 2000) is considered a reference center in the southwestern region of Bahia (Calazans & Japyasu, 2013). The center receives different specimens of birds coming from arrests of the traffic, being the majority of the Passeriformes and Psittaciformes orders. Psittaciformes require larger enclosures, more time for rehabilitation and adaptation, and generally don't present sexual (apparent) dimorphism. These characteristics justify the need for a faster and more rigorous evaluation of the birds, aiming the reintroduction of the specimens in the natural habitat. Among the Psittaciformes allocated in the CETAS of Vitória da Conquista, the *A. aestiva* parrots are the ones received in greater quantity. The success of programs to maintain or reintroduce specimens to the environment is influenced by the elaboration and implementation of reproductive management. As birds of the *A. aestiva* species don't present sexual dimorphism apparent between males and females, the correct determination of the sex of individuals is extremely important (Tell & Lasley, 1991).

There are several methods used for sexing birds. These include: (i) observation of reproductive behavior, (ii) morphometry, (iii) cloacal region palpation, (iv) surgical techniques (laparoscopy), (v) endoscopy, (vi) nuclear magnetic resonance tomography (TRMN), and (vii) molecular sexing. The last one is usually conducted in laboratories outside the institution that maintains the birds. Each method has relative advantages and limitations. It is preferable to use methods that bring the lowest risk to the bird life and bring reliable results (Dias, 2003). The determination of the sex of Psittaciformes by CETAS-Vitória da Conquista usually occurs by observing (i) reproductive behavior and (ii) morphometry. Males tend to have larger bodies and heads, being dominants. Females, as they place eggs, have the largest pelvic bones. However, these aspects are variable and don't always follow a defined pattern (Johnson, 1986).

Currently, one of the safest and most effective ways to identify males and females in several bird species is by molecular sexing. Genetic determination of sex in birds depends on the CHD system (*chromo-*

helicase-DNA-binding), allocated on sex chromosomes Z and W. This system shows two allelic forms, differently distributed on sex chromosomes. Males have two identical copies of the CHD-Z allele, allocated in the Z chromosomes. Female birds with different Z and W chromosomes have one copy of the CHD-Z allele and one copy of the CHD-W allele (GRIFFITHS *ET AL.,* 1998). Thus, the determination of the sex genotypes of the CHD system allows molecular sexing in birds. Molecular sexing can be performed from different biological samples such as blood, cloaca scraping and feather bulb (DUBIEC & NEUBAUER, 2006).

The objective of this study was to compare the use of cloacal swab and blood for molecular sexing and to determine the sex of true parrots (*A. aestiva*) seized from the trafficking of wild animals and kept in the CETAS of Vitória da Conquista (Ba, Brazil). In the sequence, the results of the molecular sexing were used to the reproductive management, being this one integrated in the process of reintroduction of the specimens of birds to the natural habitat.

MATERIAL & METHODS

SAMPLE AND COLLECTION

Blood samples and cloacal swabs of 33 true parrots (*A. aestiva*) kept at the CETAS, located in Vitória da Conquista - Ba (Brazil), were used. The birds had between 3 months and 2 years old. These birds, came from the state of Bahia (neotropical distribution), were obtained from arrests carried out by IBAMA and the Brazilian Federal Highway Police or by voluntary delivery. Among the 33 parrots received by CETAS, 16 have been seized in the region of Irecź, located in the central-north region of the state. The research project was approved by the Ethics Committee on the Use of Animals (CEUA) of the Multidisciplinary Institute of Health from the Federal University of Bahia (IMS-UFBA), protocol number 029/2015.

Blood samples were obtained by puncture of the brachial vein. Each individual was contained in the dorso-ventral position, using leather-shaved gloves, and the place of sample withdrawal was sanitized with cotton soaked in 70% alcohol. Approximately 1 ml of blood was collected (representing less than 1% of the birds body weight). The sample was conditioned in vancuteiner tubes with anticoagulant (powdered EDTA).

Cloacal swab samples were obtained by friction of swab moistened with saline solution inside the cloaca. Each Swab was conditioned in 1 ml of transport medium (Sucrose/Monobasic SodiumPhosphate/Dibasic Sodium Phosphate) (TAYLOR *ET AL.,* 1969). All samples were identified

and transported in refrigerated boxes to the Laboratory of Cellular and Molecular Biology of IMS-UFBA. The samples were kept at 4 ° C until processing.

DNA EXTRACTION AND STORAGE

DNA extraction from the birds was performed using blood and cloacal swab. Blood extraction was performed using guanidine isocyanate and covalent DNA binding on silica particles, following the manufacturer's protocol (Kit Brasilica - LGC Biotecnologia, SP, Brazil). Extraction from cloacal swab was performed using a boiling method and washes with 1X PBS buffer (Silva *et al.,* 2012). The volumes (i) of biological samples used at the beginning of the extraction protocol and (ii) of recovered DNA samples are described below. (i) 100 ul of blood sample from each individual and 1 ml of the transport medium (Sucrose/MonobasicSodium Phosphate/ Dibasic Sodium Phosphate) containing the swab sample dilution were used. (ii) DNA extracted from blood sample was eluted in 100 ul of elution buffer and the DNA extract from swab sample was eluted in 40 ul of phosphate-saline buffer (1x PBS). Genomic DNA from an individual of known sex was extracted from the blood sample. Sex was identified from DNA amplification by PCR and subsequent electrophoresis on 6% polyacrylamide gel. This DNA was used as a positive control for the PCRs and as a size parameter for the interpretation of the Z and W alleles on the gels. The DNA samples were stored at -20 ° C.

DNA CONCENTRATION, PURITY AND CONSERVATION ANALYSIS

DNA samples, obtained from venous blood and cloacal swab, were analyzed for concentration (ng/ģL) and purity by readings on NanoDrop 2000 (Thermo Scientific, MA, USA). As regards purity, (i) for the ratio 260/280 (DNA/Proteins): values between 1.7-2.0 indicate purity of the sample analyzed and (ii) for 260/230 ratio (DNA/Organic Contaminants): values between 2.0-2.2 indicate purity of the sample analyzed (Thermo Scientific, 2011). Regarding DNA conservation at -20ŗC, analysis was performed monthly by observing the presence and intensity of the bands (equivalent to the Z and W alleles) on silver nitrate stained 6% polyacrylamide gel.

AMPLIFICATION AND ELECTROPHORESIS OF GENE SYSTEM CHD-Z/W

Molecular sexing occurred through polymerase chain reaction (PCR), followed by electrophoresis. The reaction conditions were optimized from modifications of the Griffiths *et al.* (1998), using the pair of P2 allele-specific primers (5'-TCTGCATCGCTAAATCCTTT-3') and P8

(5'CTCCCAAGGATGAGRAAYTG-3'). Each PCR reaction was performed in a final volume of 20 ul using Taq DNAPolimerase (Invitrogen®, SP, Brazil), with the reagents in the following concentrations: 1x Buffer, 3mM MgCl2, 0.2mM dNTP, 1.0mM of each primer (P2 and P8), 0.17 unit /uL Taq DNAPolimerase and 4 ul DNA extracted. These primers can be applied in all species of birds, with the exception of ratites.

The thermocycling conditions were: (i) Initial denaturation at 95 ° C for 5 min (ii) Amplification by 36 cycles (95° C for 1 min, 41° C for 30 sec, 72° C for 30 sec), and (iii) Final extension at 72° C by 7 min, using the thermal cycler MyGenie96 (Bionner, Korea). For samples that initially presented non-specific bands, the annealing temperature of the primes was increased, from 1 to 3° C. The thermocycling conditions were modified from the conditions described by MIYAKI *ET AL.* (1998).

The amplified samples (4 ģL of each PCR reaction) were electrophoretically run on 4 % agarose gel stained with ethidium bromide (visualized on the transilluminator) and 6 % polyacrylamide gel stained with silver nitrate. The running occurred at approximately 90 V, for 120 minutes. A 9-band molecular weight standard, from 100 base pairs (bp) to 1 Kilobase (Kb) (BioBasic, SP, Brazil) was used in the gel, volume of 1.5 ģL/well.

MOLECULAR DETERMINATION OF THE SEX

The determination of the sex of each individual was obtained through the visualization of the bands stained in gel. (i) Males were identified based in the presence of a single band, referring to the Z allele in homozygosis, (ii) and the females by the presence of two bands, referring to the Z and W alleles. Depending on the bird species analyzed the allele size varies, for the Z allele ranges from 246 to 396 bp, whereas for the W allele variation of 254 to 412 bp is expected (Griffiths *et al.,* 1998). The expected and specific sizes of the parrots bands (*A. aestiva*), representative of the alleles, are: allele Z - 396 bp, and allele W - 412 bp (Vieira, 2009).

Sample of female quail (*Coturnix coturnix*) was used as a positive control of the sexing protocols, going through all the steps that passed the parrot samples, (i) Sample and collection, (ii) DNA extraction and storage, (iii) DNA concentration, purity and conservation analysis, (iv) Amplification and electrophoresis of Gene System CHD-Z / W, and (v) Molecular determination of the sex.

DETERMINATION OF THE PROPORTION OF SEX IN THE SAMPLE

The percentage determination of males and females in the sample was performed by simple frequency calculation, dividing the number

individuals of each sex by the total number individuals that had the sex determined.

EMISSION AND DELIVERY OF REPORTS OF SEXING

The reports, referring to sexing, were issued in standard printed form; and signed by a veterinary medical professional accredited to the Regulatory Council. The delivery of the reports ocurred in the institution where the parrots were being kept — CETAS of Vitória da Conquista. These reports were used for the reproductive management, being these used in strategies of reintroduction of birds in the nature. Prior to reintroduction, these birds underwent parasitological examinations and pretreatment adjustment. The individuals were reintroduced in the region of Condeśba (Southwest of Bahia), on 05/21/2015.

RESULTS

The main differences and results obtained from the use of the two genomic DNA extraction protocols, using blood and swab cloacal samples from *A. aestiva* as biological samples, are shown in Table 1. The biological samples differed in their yield (volume and concentration) and purity of the extracted DNA (data shown in the table). The execution time of the DNA extraction protocols, using the different biological samples, was different. The faster protocol was the one used for swab DNA extraction, which took on average 4 h, while the other 6 h. These times were measured for the simultaneous extraction of 20 samples. The difference in the total execution time of the protocols was influenced essentially by the number of steps used in each protocol, and not by waiting times in each step. Differences were also observed for the DNA's extracted from the biological samples, in relation to the shelf life at -20° C, as measured by 6 % polyacrylamide gel. While for the DNA extracted from blood no degradation was observed for five months, for DNA extracted from swab no degradation was detected for two months.

Analyzing the success of amplification of the CHD-Z/W gene system, the results obtained were different using the DNA's extracted from the different biological samples. Using DNA extracted from the blood sample, it was possible to obtain the amplification of the Z and W alleles satisfactory. This occurred after modifications in the PCR conditions of the Griffiths and Miyaki protocols (1998). Regarding the Griffiths protocol, the final concentration of $MgCl_2$ (from 1.5 mM to 3.0 mM) was modified. In the Miyaki protocol (1998), the annealing temperature of the primers (from 47° C to 41° C) was decreased.

For DNA samples extracted from cloacal swab, we obtained at first moment the amplification of the desired bands. However, such

amplification occurred in a few samples and with the simultaneous presence of nonspecific bands that made the precise definition of sex difficult. The interpretation of genotypes was almost impossible in the presence of nonspecific bands. To eliminate nonspecific bands, the

Table 1. Comparison of genomic DNA extraction protocols using blood samples and cloacal swabs of parrots (Amazona aestiva).

Variables	Blood sample	Swab sample
Initial volume of biological samples used per individual	100 uL	1 mL*
Final volume of DNA eluted by biological sample	100 uL	40 uL
Average (and median) concentration of DNA obtained (ng/µL)[g]	136,1[a] (median 80)	121,6[b] (median 28,9)
Average (and median) purity (DNA/Protein) of the DNA obtained [g]	1,77[c] (median 1,73)	0,68[e] (median 0,53).
Average (and median) purity (DNA/Organic contaminant) of the DNA obtained [g]	0,71[d] (median 0,675)	0,33[f] (median 0,28).
Average time for protocol execution (months)	6	4
Conservation time of DNA (extracted) storage (months) [h,i]	5	2
Success in the sex determining, by PCR amplification[j,k], of the DNA extracted	Yes	No

The protocols used were: (1) Kit Brasilica (LGC Biotecnologia, São Paulo, Brazil), for The protocols used were: (1) Kit Brasilica (LGC Biotecnologia, São Paulo, Brazil), for extraction of DNA from blood; and (2) Boiling and washing with PBS buffer (Silva et al.2012), for DNA extraction from cloacal swab. * Material of one swab per individual,initially dissolved in 5mL transport solution. [a] DNA concentration ranged from 23.8 to1.553 ng/il. [b] DNA concentration ranged from 3.2 to 952.2 ng/il. [c] The purity for theDNA/Protein ratio ranged from 1.51 to 2.51. [d] The purity for the DNA/Organic contaminant ratio ranged from 1.84 to 0.07. [e]The purity for the DNA/Protein ratio ranged from 1.62 to 0.33. [f] The purity for the DNA/ Organic contaminant ratio ranged from 1.68 to 0.12. [g] Measured on NanoDrop 2000 (Thermo Scientific, MA, EUA). [h] Extracted DNA stored at -20 ° C. [i] Criteria to determine DNA conservation were presence and intensity of the bands (equivalent to the Z and W alleles) on silver nitrate stained polyacrylamide gel. [j] PCR-polymerase chain reaction. [k] Optimized PCR protocol from Griffiths et al. 1998 and Miyaki et al. 1998.

annealing temperature of the primers was increased from 1 to 3° C. With the change in temperature, the bands of interest became even less clear in the gel (data not shown). It was impossible to carry out the molecular sexing of the birds from samples of cloacal Swab. On the other hand, using DNA extracted from blood, we obtained amplification of all the samples and it was possible to determine the sex of all the animals.

Electrophoresis performed on agarose and polyacrylamide gel showed significant differences in precision in sex determination. The agarose gel was not efficient for molecular sexing since the bands equivalent to the Z and W alleles were not separated and were visualized on gel as a single band. The polyacrylamide gel electrophoresis allowed the separation and visualization of the bands, which presented approximated sizes of 396 bp and 412 bp.

In figure 1, the different samples can be observed in the different wells, (i) the presence of a smaller male diagnostic band (equivalent to the Z allele), (ii) or the presence of two bands, determinants of the female genotype (equivalent to the Z and W alleles). In the figure it is also being shown, the result of sexing the positive control of the protocols (quail). As a result of the molecular sexing of the sample of 33 CETAS parrots, 25 males and 8 females were identified, corresponding to the following relative frequencies of 0.76 males and 0.24 females.

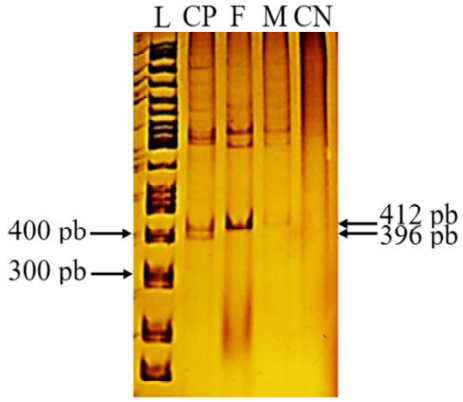

Fig. 1. Electrophoretic run of amplification of the CHD-Z/W gene system for sexing of parrots of the *Amazona aestiva* specie. 6 % polyacrylamide gel stained with silver nitrate. Electrophoresis at 90V, for 1 h and 40 minutes. Samples — L: Molecular weight standard, from 100 bp to 1 Kb (BioBasic, SP-Brazil); CP: Positive Control of female quail; F: female parrot; M: male parrot; CN: Negative Control (contains all PCR reagents minus DNA). The sizes of the ladder bands are highlighted on the left side of the gel (400 and 300 bp). The sizes of the expected alleles bands are highlighted on the right side of the gel (412 and 396 bp). Interpretation of bands — 396 bp band: Z allele; 412 bp band: W allele. Interpretation of genotypes - ZZ: Male; WZ: Female.

The protocols used were: (1) Kit Brasilica (LGC Biotecnologia, São Paulo, Brazil), for extraction of DNA from blood; and (2) Boiling and washing with PBS buffer (SILVA ET AL. 2012), for DNA extraction from cloacal swab. * Material of one swab per individual, initially dissolved in 5mL transport solution. [a] DNA concentration ranged from 23.8 to 1.553 ng/ġl. [b] DNA concentration ranged from 3.2 to 952.2 ng/gl. [c] The purity for the DNA/Protein ratio ranged from 1.51 to 2.51. [d] The purity for the DNA/Organic contaminant ratio ranged from 1.84 to 0.07. [e] The purity for the DNA/Protein ratio ranged from 1.62 to 0.33. [f] The purity for the DNA/Organic contaminant ratio ranged from 1.68 to 0.12. [g] Measured on NanoDrop 2000 (Thermo Scientific, MA, EUA). [h] Extracted DNA stored at -20 ° C. [i] Criteria to determine DNA conservation were presence and intensity of the bands (equivalent to the Z and W alleles) on silver nitrate stained polyacrylamide gel. [j] PCR-polymerase chain reaction. [k] Optimized PCR protocol from 1998 and MIYAKI ET AL. 1998.

DISCUSSION

The success of bird conservation programs is related to the correct and safe identification of the sex. It is estimated that approximately 30 % of the birds in the world don't present external sexual dimorphism (monomorphic birds). And often when there is dimorphism, this is usually subtle and can occur only in the period of sexual maturity (VIEIRA, 2009). The Psittacidae family (which includes the species *A. aestiva*) is one of the families that has a greater number of species that don't exhibit sexual dimorphism. In this case, the use of sex determination methods is recommended.

The identification of sex through molecular methods is considered by many laudists as safer methodology and method of choice: (a) presents a lower margin for variations in interpretation — influenced by the ability of the professional responsible for sexing, (b) presents results not influenced by phenotypic variations, resulting from mutations and environmental influences, (c) has an accuracy greater than 99 % (d) is considered simple (after standardization), (e) presents low cost (after acquisition of basic equipment) and (f) can be performed in birds of any age (GONÇALVES & WASKO, 2013).

Numerous methodologies/protocols were developed to determine the sex of birds using molecular techniques. Among them, the most frequently used methodology was developed by GRIFFITHS *ET AL.* (1998). This methodology is based on a molecular marker, which presents polymorphism when compared to the intron region between the two homologous CHD genes, allocated on the Z and W chromosomes. Amplification of the

CHD-Z and CHD-W genes produce two bands with sufficiently different sizes to be separated by gel electrophoresis (Lee *et al.,* 2008). Studies show that, although the CHD-Z and CHD-W genes have evolved independently, they have many similarities (Cerit & Avanus, 2007). As a consequence, the aminoacid sequences of the proteins encoded by the genes in question are quite similar. However, the proteins are different enough to be involved with the determination of the different sexes in birds.

The CHD gene is highly conserved even among very distant species, therefore is reliable as a marker for molecular sexing of birds. Griffiths *et al.* (1998) confirmed this assertion when comparing DNA sequences from the CHD gene of chicken and finch with rat CHD sequences. These sequences were determined by Delmas *et al.* (1993). Griffiths observed that despite the large taxonomic distance between the two bird species relative to the mouse, the important 3 'regions of the primers used (P2 and P8) were compatible with the target sequences of the genomic DNAs. We haven't found in the literature studies using primers P2 and P8 for X/Y allele amplification (of mammals). The primers used in the present study, P2 and P8, are useful for amplification of the CHD gene in all bird species, except in ratites (Marija *et al.,* 2013).

Comparing the CHD genes (CHD-Z and CHD-W), the introns have length variations. The difference in the length of the introns between the CHD-W and CHD-Z gene makes possible the molecular identification of sex in non-ratite birds (Cerit & Avanus, 2007). The pair of primers used in this study (P2 and P8) binds at two exons conserved sites, flanking an intron region (Jensen *et al.,* 2003). The difference in intron size, present in the two chromosomes, allows the visualization of two bands in the electrophoresis gel, in the case of females (Vucivec *et al.,* 2012). Bands resulting from the amplification of the Z and W alleles (of parrot) using primers P2 and P8 have proximal sizes, with the Z allele being close to 396 bp and the W allele about 412 bp.

Griffiths *et al.* (1998) used molecular sexing to determine the sex of 28 species of birds, including the order Psittaciformes (*Cyanopsitta spixii*, *Platycercus elegans*, *Calyptorhncus lathami*). This work diffused the use of primers P2 and P8 in PCR for the molecular sexing of birds. Miyaki *et al.* (1998) used the primers employed by Griffiths with efficiency for the sex identification of 31 species of parrots including *A. aestiva*.

The molecular techniques used for molecular sexing in the present study were (i) DNA extraction, (ii) followed by PCR for determination of the CHD system (using the P2/P8 primers) and (iii) electrophoresis,

the gels being stained with silver nitrate. In the present work, considering DNA extracted from blood samples, the amplification of the diagnostic fragments of the Z and W alleles were obtained with modifications of the protocols (i) of GRIFFITHS ET AL. (1998) and (ii) MIYAKI *ET AL.* (1998). The Griffiths protocol was used for the optimization of the PCR reactions, and the Miyaki protocol for thermocycling optimization. Regarding the conditions of the PCR reactions, when the original Griffiths protocol was applied, the determination of genotypes was difficult. This was caused by the lack of clarity of some bands, and no amplification of bands was obtained for some individuals in the sample. Two modifications were tested for protocol optimization, (i) Double the amount of $MgCl_2$ alone (1.5mM used by Griffiths for 3mM used here) and (ii) Double the amount of Primers and $MgCl_2$ (100 ng primers used by Griffiths for 1, 0 mM used here). From the visualization of the amplification of the Z and W alleles in gel, the double $MgCl_2$ condition was chosen. Regarding the conditions of thermocycling, the modifications made on the protocol of Miyaki *et al.* (1998) were: (i) 30 sec increase in initial denaturation during cycles and (ii) decrease at 6°C in annealing temperature of the primers. The first modification has the potential to maximize the annealing of the primers by denaturing more target DNA, and the second modification has the potential to facilitate hybridization of the primers in the target DNA.

Potential biological samples as a source for DNA extraction from birds are: (i) Blood, (ii) Feather bulb and (iii) Scraping material (collected from the oropharynx or cloaca, using swab). Considering the welfare of the animal, it is recommended to use the least invasive method to collect the sample. Thus, minimizing discomfort and stress. The prioritization of the use of blood as a sample for DNA extraction is justified by the results obtained: higher quantity, concentration and purity of extracted DNA (VIEIRA, 2009). Despite the numerous technical advantages (as to the product obtained) in the use of blood as a source of DNA for molecular sexing, other sources are desirable.

DNA extraction from cloacal Swab of parrots has been described in the literature. However, all the articles that mentioned it, used the cloacal swab to extract DNA from bird pathogens, not genomic DNA from them. SAIDENBERG *ET AL.* (2011) extracted cloacal swab viral DNA from 13 parrots (*Amazona vinacea* species). The results obtained, with detection of the virus investigated, were used as pre-release sanitary tests. Allgayer (2003) used DNA extracted from cloacal Swab to detect *Salmonella* sp. In 280 Psittacids (including *Amazona aestiva*) kept in captivity. The

presence of *Salmonella* sp. was efficiently detected in 37 of the 280 samples analyzed.

In molecular techniques, one of the factors influencing the performance of the results is the purity and integrity of the extracted DNA. In the present study, the DNA was extracted from two sources: blood and cloacal swab. The methods used for extraction were: silica resins to extract DNA from blood and boiling method with PBS buffer for swab.

Considering the biological samples and the extraction protocols tested, the DNA extracted from blood obtained the best performance. Considering that it was the only DNA well extracted, the results showed that it was the suitable DNA for the molecular sexing of birds through the identification of the CHD-Z/W gene system. Even after several attempts at DNA amplification from swab, including various modifications in the PCR conditions, consistent amplification of the bands equivalent to the Z and W alleles was not obtained. Inconsistency is interpreted as lack of amplification of several samples. And when the amplification was obtained, it wasn't possible to safely identify the sexes of the individuals, considering the existence of nonspecific bands in addition to the presence of the expected bands (these were weakly amplified).

The disadvantageous performance in DNA amplification extracted from cloacal swab (compared to blood sample) may have the same explanations for unsuccessful amplification from DNA extracted from feather bulb. According to Vucivec, *et al.* (2012), which extracted feather bulb DNA from 58 bird species, such results can be expected by the existence of several PCR inhibitors, such as: dead cells, RNA or various microorganisms (in the extracted samples). Such PCR inhibitors may also occur in cloacal swab, justified by the degree of exposure of the cloacal region to contaminants. These contaminants may be from the environment of the enclosure and the contact parrot-parrot. Allegretti (2009) in his work demonstrated that the intestinal tract of 52 species of *A. aestiva* was colonized by different types of bacteria of the genus *Lactobacillus*,*Bifidobacterium*, *Enterococcus*, *Pediococcus* and *Lactococcus*.

Although DNA/Protein purity values were adequate for DNA extracted from blood samples, DNA purity/organic contaminants indicated lack of purity for DNA extracted from both biological samples. DNA extracted from swab proved to be more impure than DNA extracted from blood samples. Contaminants may consist of residue from reagents used in the extraction or organic remains not removed during the process. For blood extracted DNA, the contaminants may consist of the resin (or silica) component of the extraction kit. And for DNA extracted from

swab, it may consist of PBS (Saline Phosphate). It is worth noting that the protocol used for the extraction of DNA from swab samples doesn't contain steps to remove lipid membrane and to precipitate the genetic material. These may have been potential inhibitors of DNA amplification by PCR.

In the present work, it was verified that the best time of preservation of DNA extracted was for the DNA of the blood, being 5 months. It's believed that DNA degradation may have been influenced by (i) factors such as sensitivity of the DNA molecule to nucleases, which facilitate its breakage (COELHO *ET AL.,* 2004), (ii) in addition to several cycles of freezing and defrosting, necessary to manipulate the material. Coelho (2001), using the extraction protocol of Sambrook et al. (1998) obtained better storage parameters of DNA extracted from blood, and it remained stable for a period of 3 to 9 months (stored at -20ţC). However, the Sambrook protocol uses phenol-chloroform, substances considered to be hazardous to health.

DNA degradation was also observed by VIEIRA (2009) and Jensen et al. (2003), who used different extraction protocols. Vieira (2009) extracted DNA from 14 species from two sources: (i) Feather Bulb and (ii) Blood. According to this author, DNA extracted from the feather bulb by the alkaline method showed complete degradation after 1 month and DNA extracted from blood by the Sambrook method showed signs of degradation after 8 months, both stored at 4ţC. JENSEN *ET AL.* (2003) extracted DNA from 47 bird species from two sources: (i) Blood and (ii) Egg membrane (using the Chelex 100 method, SigmaAldrich). For DNA extracted from egg membrane, storage at room temperature, there was limited degradation after 1 month. And for DNA extracted from blood, stored at -20° C, degradation occurred after 13 months.

In the present work, it wasn't possible to distinguish between the Z and W alleles by agarose gel electrophoresis in a safe manner. The similarity of size of the intronic regions between the alleles, amplified by the primers P2 and P8, results in the overlap of bands in the agarose gel. This is maximized by the accumulation of ethidium bromide between the bands in the gel (CERIT & AVANUS, 2007). In the agarose gel the overlap of bands influences the correct identification of the sex of the birds. Thus, a female (ZW) may be erroneously identified as male (ZZ). On the other hand, the polyacrylamide gel, besides having a higher resolution power (separation) of the bands, has a higher sensitivity (allowing the visualization of less intense bands) when developed with silver nitrate. These advantages of silver-stained polyacrylamide gel also contribute to better detect and deal with problems such as species-specific primer competition. Such competition occurs because eventually the primers

may bind preferentially to one of the CHD genes. The discrepancy in amplification between alleles may result in the visualization of either (i) only one band (ii) or one of the band with much less intensity. This is difficult to visualize on agarose gel, interfering with correct sexing (GRIFFITHS *ET AL.,* 1998). The visualization of two bands on the polyacrylamide gel was possible when amplified sample of female quail (*Coturnix coturnix*), used as reaction positive control.

The sexing of the 33 true parrots (*A. aestiva*), obtained through the present study, showed that there were 25 males (0.76) and 8 females (0.24). Similar proportions were found by CERIT & AVANUS (2007); Nebel *et al.* (2004) and FARIA *ET AL.* (2007). In the first mentioned article, the sex of 41 birds of the species *Nymphicus hollandicus* (order Psittaciformes) was determined, finding 23 males and 18 females. Nebel *et al.* (2004) in their study on molecular sexing of 24 birds of the species *Calidris mauri* (order Charadriiformes), identified 18 males and 6 females. FARIA *ET AL.* (2007) determined the sex of 21 birds of the species *Hylocryptus rectirostris* (order Passeriformes), and found 13 males and 8 females.

The molecular sexing methodology used in the present study has potential applicability in reproductive management and reintroduction programs of birds in the natural habitat. Patino *et al.* (2013) applied the molecular sexing in a management program, where he carried out the translocation of birds of the species *Pterodroma magentae* (order Procellariiformes) to a predator free area. MARIJA *ET AL.* (2013) in a study on sex determination of 20 bird species also highlights the importance of molecular sexing for biodiversity protection and enrichment programs.

CONCLUSION

Considering the DNA extraction techniques tested in the present study, blood samples (when compared to the use of cloacal swab) showed better performance regarding the quantity and quality of DNA extracted. In addition to being the only biological sample tested where the DNA extracted was adequate and reliable for molecular sexing of birds using the CHD-Z/W gene system. Regarding the modifications made in the PCR reaction, double $MgCl_2$ provided better amplification of the bands, visualized in gel. The molecular method employed here is the first known successful protocol for use on the molecular sexing of CETAS birds in Bahia. Previously, the sex determination of these animals was based only on reproductive behavior and morphometry, whose reliability is often questionable. The results obtained in this work have potential impact on studies of animal ecology, behavior and population structure. Besides, it

present potential to assist in the planning of couples training, contributing to maintenance and reintroduction programs of the animals in their natural habitat. Therefore, it's expected that the present work could have a significant impact on the preservation of bird biodiversity.

RESUMO

Estima-se que pelo menos 30 % das aves não têm dimorfismo sexual. Esta identificão pode ser feita por métodos moleculares utilizando diferentes materiais biológicos. O presente estudo objetivou comparar o uso de swab cloacal e sangue para a sexagem molecular e determinação do sexo de papagaios verdadeiros (*Amazona aestiva*) apreendidos no tráfico de animais selvagens em Vitória da Conquista (Ba), Brasil. Trinta e três papagaios verdadeiros, mantidos no Centro de Triagem de Animais Silvestres - Vitória da Conquista, foram anilhados. Amostras coletadas foram, submetidas a extração de DNA e os alelos do sistema CHD-Z / W foram amplificados por PCR. O material obtido foi submetido a eletroforese em gel de poliacrilamida e corado com prata. Os machos foram identificados pela presença de uma única banda (396 pb), referindo-se ao genótipo Z0 e fêmeas identificadas por duas bandas (396 pb e 412 pb) para o genótipo ZW. Os resultados mostraram uma composição de amostra de 25 machos (0,76) e 8 fêmeas (0,24). A determinação do sexo contribuiu para o pareamento dos papagaios e sua liberação no habitat natural. Os dados mostram que a técnica de sexagem molecular adotada é eficaz para a determinação sexual de papagaios selvagens apreendidos do tráfico.

PALAVRAS-CHAVE: Sexagem Molecular; CHD-Z/W; *Amazona aestiva*.

SUMMARY

It is estimated that at least 30 % of the birds don't have sexual dimorphism. Such identification can be made by molecular methods using different biological materials. The present study aimed to compare the use of cloacal swab and blood for molecular sexing and to determine the sex of true parrots (*Amazona aestiva*) seized from the traffic of wild animals in Vitória da Conquista (BA), Brazil. Thirty-three true parrots, kept in the Wild Animal Triage Center - Vitória da Conquista, were ringed. Biological samples were collected, DNA extraction, and the CHD-Z/ W system alleles was amplified by PCR. The material obtained was subjected to polyacrylamide gel electrophoresis and silver staining. The males were identified by the presence of a single band (396 bp), referring to genotype Z0 and females identified by two bands (396 bp and 412 bp) for the ZW genotype. The results showed a sample composition of 25 males (0.76)

and 8 females (0.24). The determination of sex contributed to the meeting of the parrots in pairs and their release in the natural habitat. The data show that the technique of molecular sexing adopted is effective for the sexual determination of wild parrots seized from trafficking.

KEYWORDS: Molecular sexing; CHD-Z/W; *Amazona aestiva*

RÉSUMÉ

On estime qu'au moins 30 % des oiseaux ont un dimorphisme sexuel. Cette identification peut-źtre effectuée par des procédés moléculaires ą l'aide de divers matériaux organiques. Cette étude visait ą comparer l'utilisation des écouvillon cloacal et de sang pour effectuer un sexage moléculaire et déterminer le sexe de vrais perroquets (*Amazona aestiva*) saisis dans le trafic de la faune ą Vitória da Conquista (BA), Brésil. Trente-trois vrais perroquets, conservés dans le Centre de Triage des animaux sauvages - Vitória da Conquista — ont été bagués. Les échantillons biologiques ont été collectés et aprčs extraction de l'ADN, les alleles du systčme CHD-Z/W ont été amplifiés par PCR. Les produits obtenus ont été soumis ą une électrophorčse sur gel de Polyacrylamide et coloré ą l'argent. Les māles ont été identifiés par la présence d'une seule bande (396 bp), se référant au génotype Z0. Les femelles on été identifié par deux bandes (396 pb et 412 pb) se référant au génotype ZW. Les résultats ont montré une que l'échantillon était finalement composé de 25 māles (0,76) et 8 femelles (0,24). La détermination du sexe a permis de former des couples de perroquets et leur libération dans l'habitat naturel. Les données montrent qu' une approche moléculaire a permis un sexage efficace des perroquets sauvages saisis lors de la traite.

MOTS-CLÉS: sexage moléculaire; CHD-Z/W; *Amazona aestiva*

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