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Simulating the Bird Eggs Illegal Trade and Improve the DNA Barcode Amplification to Combat Bird Trafficking Through Species Identification

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Abstract. The illegal bird trade is changing from capturing live animals to smuggling eggs, making trafficking even more dangerous. Eggs can exhibit intraspecific variations or show very similar traits between different species, making the morphological species identification inaccurate. Thus, genetic species identification has become an essential tool in solving wildlife crimes. However, the conditions to which eggs are subjected during trafficking, from capture in the wild to seizure, make egg samples increasingly difficult even for DNA analysis, limiting the availability of biological material for species identification. We simulated different scenarios where eggs could be found and determined the best DNA barcode size to amplify in each situation. Chicken muscle tissue (*Gallus gallus*) and japanese quail eggs (*Coturnix japonica*) were submitted to adverse conditions resulting from trafficking: dried eggs, decomposing eggs, broken eggshells, and egg fluid deposited on various substrates. We assessed three salting-out DNA extraction protocols to find a cheaper and less toxic method. Different molecular markers from cytochrome oxidase I (*COI*) gene, fragments of 747, 405 and 164 bp, were tested. We successfully amplified the DNA mini-barcode (164 bp) in all simulations. However, the amplification of larger fragments was more challenging in complex samples such as decomposed eggs, egg fluid, and broken eggshells. These results demonstrate that DNA barcoding and mini-barcoding are powerful tools for identifying bird egg samples in various states of preservation, including decomposed samples. To apply this methodology as evidence in combating environmental crimes, we present a standardized flowchart for identifying bird species using molecular techniques.

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Keywords: Case-type samples; DNA amplification; DNA mini-barcode; Forensic analysis; llegal bird trade.

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1. Introduction

According to estimates, five to ten million birds are illegally harvested from nature each year worldwide[1]. Over the years the *modus operandi* of illegal bird trade has changed, and the seizure of eggs has become more frequent[2,3]. Bird eggs are commonly illegally harvested from the wild in Central and South America and then sent to Europe, where are they are distributed to several countries[4–6]. Wildlife trafficking contributes to the loss of important ecological services and impacts human health and global economy. Furthermore, it directly interferes with the dynamics of animal and plant communities, both through population decline or species extinction, as well as the introduction of exotic species and the spread of diseases[7].

Species identification in smuggled eggs is an important step for determining legal enforcement and conservation actions[8–10]. However, eggs do not always hatch for morphological analysis of the young, due to difficulty to incubate eggs under captive conditions, where hatch failure can reach up 85%[11,12]. In addition, identifying eggs through morphology is a complex task due to interspecific similarities and intraspecific variations[13], making genetic species identification an essential tool for uncovering wildlife crimes[14]. The 5’ fragment of the cytochrome oxidase I gene (*COI*) is the best choice of molecular marker for bird species identification, as described in several studies with different bird taxa[15]. In cases of high DNA degradation and forensic samples, smaller fragments are required[16], such as the case of DNA mini-barcode, with a size between 100 and 300 bp[17].

Species identification in seized bird eggs has been previously reported[8–10,18,19]. Most studies have used only embryos and embryonic attachments for genetic species identification. However, this approach does not reflect the real situation of seized eggs, as demonstrated in the case report by Formentão et al.[10], in which there was no embryo in seven egg samples making necessary to use additional eggs components for DNA analysis.

The forensic application of animal DNA analysis still requires standardization of protocols due to the great species diversity and variety in nature of sample types[20], especially for seized bird eggs. An important gap for DNA analysis in wildlife forensic, is the lack of standardized protocols for analyzing egg samples[21–23], and insufficiency

of well trained-professionals managing seize bird eggs, who are unfamiliar with procedures for species identification in eggs, and the use of molecular techniques like DNA barcoding.

Simulating real forensic cases is an essential step in validating forensic DNA analysis protocols[24]. Our aim is to propose a standardized protocol for molecular species identification in illegally traded eggs, simulating illegal egg trade scenarios, and standardizing efficient DNA extraction and amplification protocols for recovering *COI* DNA barcode and mini-barcode fragments from these case-type samples. This cost-effective protocol can be used in less privileged areas where most trafficked bird eggs originate. Also, our work contributes to conservation efforts, as the increase in identified species of smuggled bird eggs highlights those species that need to be protected.

2. Materials and methods

A prior DNA extraction and amplification test was performed with eggs from four domestic species: chicken (*Gallus gallus*), Japanese quail (*Coturnix japonica*), canary (*Serinus canaria*) and cockatiel (*Nymphius hollandicus*). The test revealed no differences in DNA barcode and mini-barcode amplification between the eggs of the four species (data not shown). Thus, the model used for case-type samples consisted of unfertilized eggs from Japanese quail (*Coturnix japonica*) and muscle tissue of chicken (*Gallus gallus*), acquired from supermarkets in Florianópolis (Santa Catarina, Brazil). Due to the nature of the eggs acquisition, this study did not require ethics committee approval.

To determine the DNA concentration range of the primer set to allow the amplification of expected fragment in different sample types, DNA was extracted from muscle tissue, yolk, a yolk and albumen mixture, and broken eggshell membranes. The following nucleic acid concentrations were tested in all samples: 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 0.5, 1, 2, 5, 10, 30, 60, 90, 200, 300, 400 and 500 ng/µL. Concentrations of 800, 900, and 1000 ng/µL were used only for muscle tissue samples.

2.1. Case-type samples

The absence of an embryo in whole egg seizures was simulated using *in natura*, dried and decomposing whole eggs. Yolk and eggshell membranes were used to test the best sample type for analysis. The presence of an embryo in the seized eggs was

simulated by using muscle tissue under the same conditions. We simulated egg traces using eggshell membranes from broken eggshells and egg fluid (a yolk and albumen mixture) deposited on distinct substrates: polyester (100%), cotton (100%), jeans (polyester, elastane, and cotton), pantyhose (polyamide and elastane), Medium-Density Fiberboard (MDF) blocks, painted masonry wall, and paper towels, as trafficker often transport the trafficked eggs in their clothing. All egg trace samples were kept overnight in the laboratory environment. A total of 15 pieces of each: muscle, eggs, and egg fluid samples were used in each simulation, with five samples per DNA extraction protocol tested.

2.2. DNA extraction

For DNA extraction, 30-40µL of yolk, 50mg of muscle tissue, 0.5cm^2 of eggshell membrane, cloth and paper towel with egg fluid, and wet swabs for MDF and wall with egg fluid were used. Three DNA extraction protocols with different salts were chosen for being economically accessible and effective in precipitating proteins, which are major components of eggs:

1. Sodium Chloride Protocol: adapted from Aljanabi and Martinez[25] - 250 µg/mL final concentration of proteinase K; 4-5h incubation for yolk and overnight for eggshell membrane (in lysis buffer and proteinase K); overnight incubation (in isopropanol).

2. Potassium Acetate Protocol: adapted from Pilotto et al.[26] - 1000µL of lysis solution; 220µg/mL final concentration of proteinase K; 4-5h incubation for yolk and overnight for eggshell membrane (in lysis buffer and proteinase K); overnight incubation (in isopropanol).

3. Ammonium Acetate Protocol: identical to the protocol 2, replacing the potassium acetate with 4M ammonium acetate, according to Rivero et al.[27].

After DNA extraction, the concentration of nucleic acids and the presence of proteins were measured by spectrophotometry. This methodology provides only an estimation and was used to determine the appropriate sample dilution, as the amount of DNA and proteins could interfere with PCR success.

2.3. DNA amplification

Cytochrome oxidase I (*COI*) gene fragments were amplified using universal primers sets cited by Kerr et al.[28] in all case-type samples. A first Polymerase Chain Reaction

(PCR) was performed using the primer set BirdF1 and COIbirdR2 (expected amplicon size of 747 bp). Secondly, a nested PCR was performed using previous PCR reaction (samples that did not amplify the 747 bp fragment) with the primer set BirdF1 and AvMiR1 (expected amplicon size of 405 bp). In addition, we performed another PCR reaction for all samples using the primer set AvMiF1 and AvMiR1 for DNA mini-barcode amplification (expected amplicon size of 164 bp). The sensitivity test was performed only for the BirdF1 and COIbirdR2, and AvMiF1 and AvMiR1 primer sets because the BirdF1 and AvMiR1 pair was used to amplify the fragment from the PCR product, which contains more targets than the DNA extraction products.

The PCR reactions were performed according to the protocol described by Lijtmaer et al.[29] using DNA templates at concentrations between 1-200 ng/µL. We used both a short and long PCR program to verify the optimization of a faster PCR run. The short program consisted of one cycle at 94 °C for 40 s, followed by 35 cycles of 40 s at 94°C, 30s at 52°C and 40s at 72°C, and finally one cycle at 72°C for 5m. The long program added six cycles of 40s at 94°C, 30s at 45°C, and 40s at 72°C after the first cycle of the short program, continuing with the remaining steps as in the short program.

We tested three substances as PCR adjuvants: Dimethyl sulfoxide (DMSO) and formamide at final reactions concentrations of 1%, 2%, 3%, 4%, and 5%, and Tween-20 at final reactions concentrations of 0.5%, 0.75%, and 1%. The amplified fragments were visualized on a 1% agarose gel. The long PCR program and PCR adjuvants were tested only on samples that did not amplify in the first PCR.

2.4. DNA sequencing

Three samples were randomly selected for DNA mini-barcode sequencing. The samples included two from *in natura* yolk and one from egg fluid on cotton. These were purified with EXO and SAP enzymes and sent for sequencing at the Multiuser Laboratory for Studies in Biology (LAMEB) of the Biological Sciences Center (CCB) at the Federal University of Santa Catarina (UFSC). DNA sequencing and precipitation reactions were performed using standardized LAMEB protocols for the ABI3500 equipment. The electropherograms were qualitatively analyzed manually using Chromas Lite version 2.6.4 (Technelysium LTDA®) and the sequencing

results, in FASTA format, were uploaded to the BOLD Systems for species identification.

3. Results

Muscle tissue samples were successfully amplified with the primer sets BirdF1 and COIbirdR2, and AvMiF1 and AvMiR1 at nucleic acid concentrations ranging from 0.5 to 500 ng/µL, but failed at three other concentrations tested (800, 900 and 1000 ng/µL). For yolk, yolk and albumen mixture, and eggshell membrane samples, amplification with both primers pairs was successful at nucleic acid concentrations between 1 and 200 ng/µL.

DNA barcode and mini-barcode fragments were successfully recovered from all case-type samples. However, the success rate for amplifying the DNA barcode in some simulations remained low, particularly in the decomposing cases and eggshell membrane samples, while the DNA mini-barcode amplification was more successful. The primer pair used for the 405 bp barcode amplification should only be used in nested PCR, as it generates non-specific fragments when used in standard PCR. Both DNA barcode and DNA mini-barcode amplification were independente of the DNA extraction protocols.

There was no difference in the recovery of DNA barcode and mini-barcode fragments between the long and short PCR programs. Therefore, the time-optimized (short) PCR program is recommended. The PCR adjuvants did not enhance *COI* amplification, except for DMSO 3%, which improved DNA mini-barcode amplification in some samples (details provided in the following sections). Sequencing resulted in the correct identification of the species *Coturnix japonica* in all samples tested using DNA mini-barcode (98,18%, 98,94% and 100%).

3.1. Eggs conditions simulation

The in natura and dried muscle tissue simulations were successful in amplifying the DNA barcode using the BirdF1 and COIbirdR2 primer set (100% of the samples), as well as the DNA mini-barcode using the AvMiF1 and AvMiR1 primer pair (Figure 1). In the decomposing simulation, the recovery of the DNA mini-barcode was inefficient, succeeding in only two out of the 15 samples tested. DMSO 3% did not improve DNA mini-barcode amplification in this case (Figure 1B).

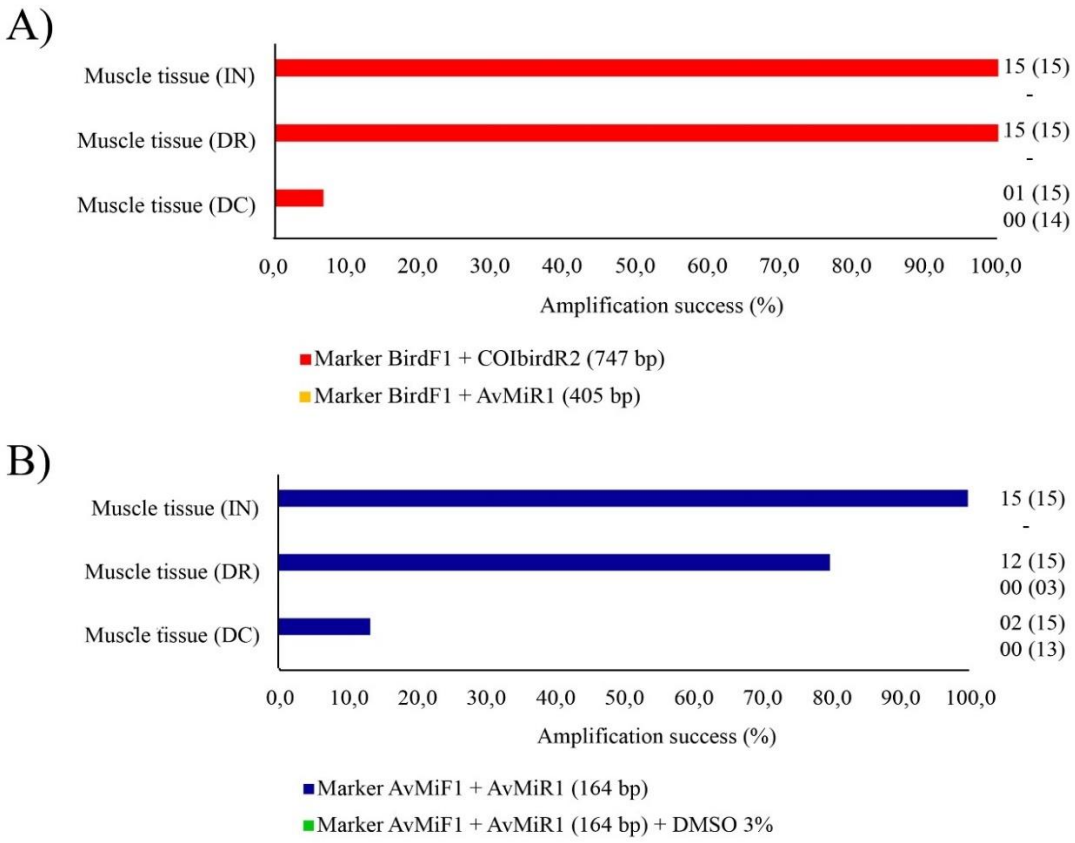

Figure 1. Percentage of muscle tissue egg case-type samples that were successful in amplifying the three different *COI* fragment sizes (747 bp, 405 bp, and 164 bp). IN = *in natura*, DR = dried, DC = decomposing. A: Amplification success for the BirdF1 + COIbirdR2 marker (747 bp) by standard PCR and BirdF1 + AvMiR1 marker (405 bp) by nested PCR. B: Amplification success for the AvMiF1 + AvMiR1 marker (164 bp) by standard PCR, with and without 3% DMSO. Numbers next to the bars represent the total number of samples that were successfully amplified, with the total number of samples tested shown in parentheses. Out of the 15 samples, five samples correspond to each DNA extraction protocol (sodium chloride, potassium acetate, and ammonium acetate). Nested PCR and the use of 3% DMSO for the 164 bp were performed only on samples that did not amplify expected fragments (747 bp and 164 bp without DMSO) in the first PCR.

The DNA mini-barcode achieved greater success in more samples than the DNA barcode in all simulations of eggshell membrane from whole eggs (Figure 2). In samples of the decomposing eggshell membrane, the use of 3% DMSO was required for DNA mini-barcode amplification (Figure 2B). The DNA barcode was successfully recovered in *in natura* and dried yolk samples (nested PCR was needed to amplify the 405 bp fragment, achieving 100% of samples amplification), as well as DNA mini-barcode (90-100% of samples amplification). Nevertheless, for the decomposing yolk simulation, the DNA mini-barcode was more efficient, and 3% DMSO increased the amplification success. The same occurred in samples of broken eggshells (Figure 2).

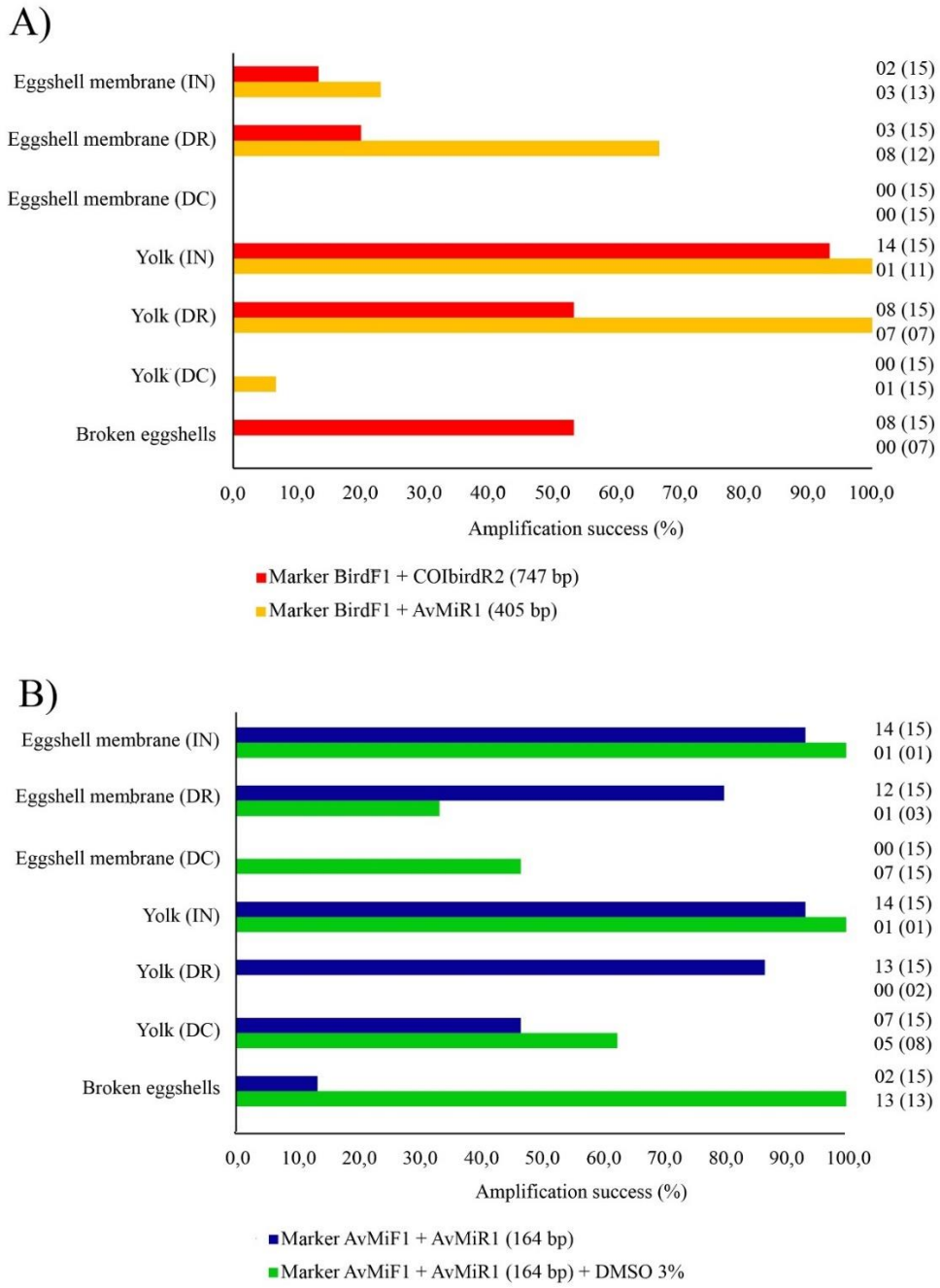

Figure 2. Percentage egg case-type samples successfully amplified for the three COI fragment sizes (747 bp, 405 bp, and 164 bp). IN = *in natura*, DR = dried, DC = decomposing. A: Amplification success for the BirdF1 + COIbirdR2 marker (747 bp) by standard PCR and BirdF1 + AvMiR1 marker (405 bp) by nested PCR. B: Amplification success for the AvMiF1 + AvMiR1 marker (164 bp) by standard PCR, with and without 3% DMSO. Numbers next to the bars represent the total number of samples that were successfully amplified, with the total number of samples tested shown in parentheses. Out of the 15 samples, five samples correspond to each DNA extraction protocol (sodium chloride, potassium acetate, and ammonium acetate). Nested PCR and the use of 3% DMSO for the 164 bp were performed only on samples that did not amplify expected fragments (747 bp and 164 bp without DMSO) in the first PCR.

3.2. Egg fluid on substrates simulations

The number of amplified egg fluid samples on cotton, jeans, and pantyhose was similar for both the DNA barcode and mini-barcode. Although the success rate for DNA barcode recovery in some samples (egg fluid on MDF, wall, and polyester) ranged from 53% to 60%, the DNA mini-barcode amplification achieved a 100% success rate. The use of 3% DMSO was required to amplify samples that initially failed (egg fluid on MDF and wall). For paper towel samples, both the DNA barcode and mini-barcode were successfully amplified in fewer than 40% of the samples (Figure 3).

4. Discussion

This study focused on the recovery and amplification of DNA barcode and mini-barcode in different forensic case-type samples. We tested different DNA extraction protocols and used primers previously described for the amplification of informative *COI* gene regions in birds. We did not perform DNA sequencing on all samples, and limited our analysis to demonstrating, through the sequencing of three randomly selected samples, that the amplified mini-barcode fragment corresponded to the expected result, rather than being a product of primer dimer formation.

The composition of eggs presents a challenge for the DNA extraction because they contain large amounts of protein and PCR inhibitors[30–32]. Additionally, the composition of eggs may vary during the decomposition processes[33]. No records of DNA extraction from egg parts were found using protocols other than commercial kits or organic methods[34,35], which are considered the most effective DNA extraction options for any type of sample. In this study, salting-out protocols were chosen to maintain the low cost of organic methods while reducing the toxicity of the process. This approach eliminates the need to resort to expensive DNA extraction kits, which is a limitation for many regions affected by egg trafficking.

Sodium acetate has the highest protein precipitation power, followed by potassium acetate, while ammonium acetate is an unstable salt with no ability to precipitate proteins[36]. In this study, the three salting-out DNA extraction protocols did not show differences in the proteins fraction (260/280) measured by spectrophotometry (data not shown) or in the number of samples that successfully amplified the *COI* fragments. We recommend using sodium chloride due to its easy

availability and low cost. However, depending on laboratory availability, potassium or ammonium acetate may also be used.

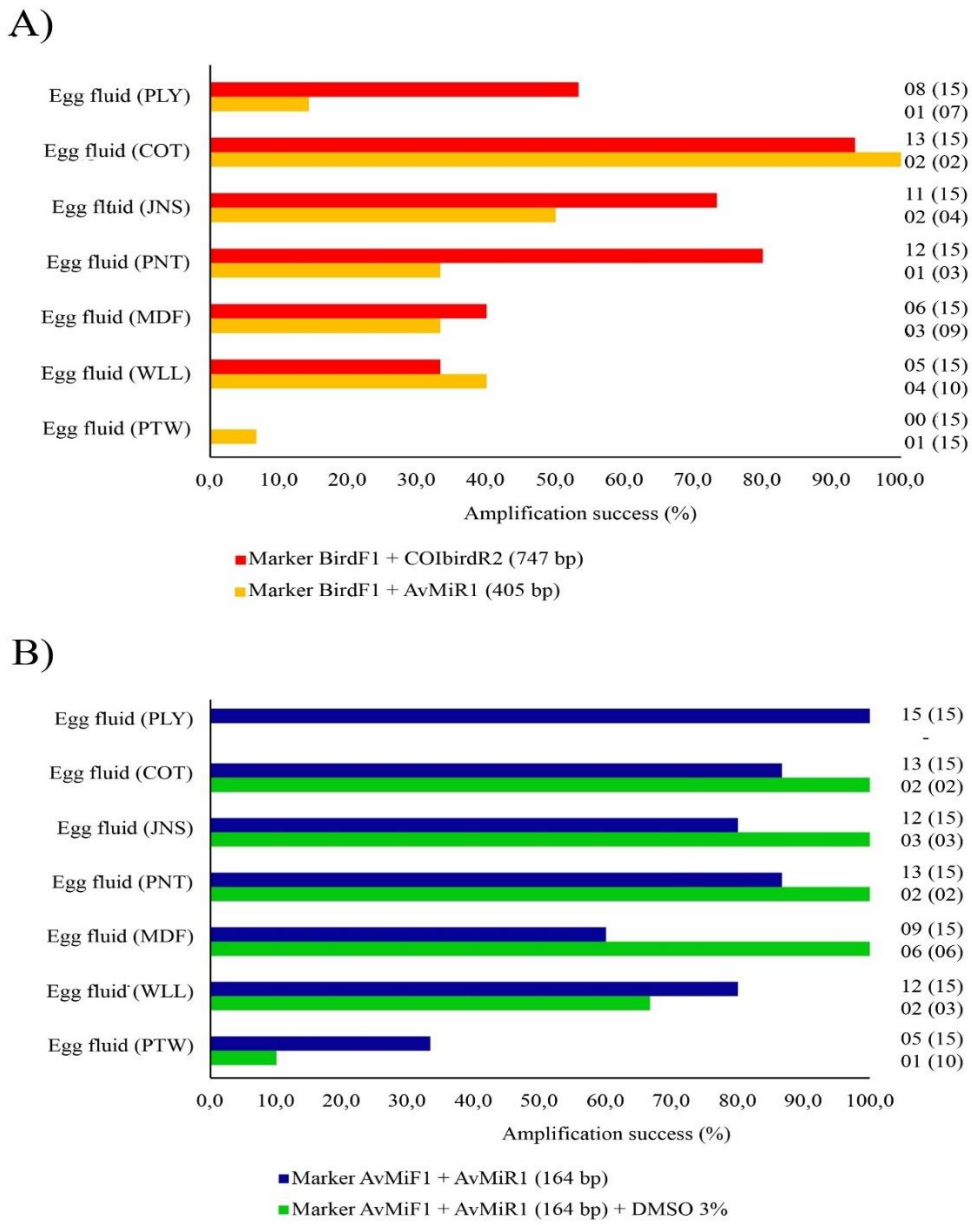

Figure 3. Percentage of egg fluid case-type samples successfully amplified for the three *COI* fragment sizes (747 bp, 405 bp, and 164 bp). PLY = polyester, COT = cotton, JNS = jeans, PNT = pantyhose, MDF = Medium Density Fiberboard, WLL = wall, PTW = paper towel. A: Amplification success for the BirdF1 + COIbirdR2 marker (747 bp) by standard PCR and BirdF1 + AvMiR1 marker (405 bp) by nested PCR. B: Amplification success for the AvMiF1 + AvMiR1 marker (164 bp) by standard PCR, with and without 3% DMSO. Numbers next to the bars represent the total number of samples that were successfully amplified, with the total number of samples tested shown in parentheses. Out of the 15 samples, five samples correspond to each DNA extraction protocol (sodium chloride, potassium acetate, and ammonium acetate). Nested PCR and the use of 3% DMSO for the 164 bp were performed only on samples that did not amplify expected fragments (747 bp and 164 bp without DMSO) in the first PCR.

We choose the Bird and COIbirdR2 primer set because they are reported as the most successful primers for DNA barcode amplification across several bird taxa[28,37,38]. The primers AvMiF1 and AvMiR1 were selected due to amplify the smallest *COI* fragment among bird mini-barcode primers, and have been extensively tested by Kerr et al.[28] on a wide range of bird species. Our strategy of verifying the recovery of different *COI* fragment sizes in case-type samples through successful amplification was highly effective and is recommended in forensic simulation studies[39].

The case-type samples were essential for determining the appropriate *COI* fragment size (barcode or mini-barcode) to use in various smuggled egg scenarios (Figures 1, 2 and 3). The amplification of only the DNA mini-barcode in some samples suggests DNA degradation due to natural process, as expected in decomposing samples. DNA mini- barcode amplification should be preferred in samples where DNA barcode fragments are difficult to amplify, as the success rate is higher with DNA mini-barcode PCR. Thus, it avoids repetition and allows for quicker analysis of the samples. When there is no difference in both fragment size amplification, DNA barcode should be prioritized, even though DNA mini-barcode efficiently identifies species[40,41]. Species identification regions may be compromised by artifacts from the capillary electrophoresis sequencing technique when using small fragments[42]. The increase in amplification success with DMSO suggests the presence of PCR inhibitors in some samples. DMSO functions by degrading and precipitating proteins during PCR, while also stabilizing DNA strands during denaturation, making the DNA template more accessible to Taq DNA polymerase[43,44].

A flowchart was designed to assist bird species identification in egg samples from illegal trade (Supplementary material SM1). Several possible scenarios were included, such as the presence or absence of an embryo, as well as different substrate types where the sample could be collected. Markers of different sizes are also provided, depending on the type of sample being analyzed, highlighting the direct influence of DNA quality. This flowchart was successfully applied in the case reported by Formentão *et al.*[10].

Currently, different technologies have been developed for species DNA detection. The most recent ones include isothermal amplification techniques and the CRISPR-Cas system[45–47]. Although rapid and cost-effective, these techniques are

based on the detection of species-specific sequences. This presents a particular challenge for species identification in illegally traded bird eggs, as the number of possible species is vast, and species-specific assays may not cover all these possibilities[48].

On the other hand, metabarcoding analysis is a powerful tool in these cases[49]. Our work focused on the identification of a single species and did not include the validation of sample analysis with species mixtures. However, species mixtures are a plausible scenario in the case of egg traces, such as broken eggshells or fluid in substrates. The use of next-generation sequencing (NGS) technologies allows for the identification of multiple species within the same sample[20,49]. In this application, all the pre-sequencing steps proposed here can be maintained; the only difference lies in the sequencing technology used, which would be NGS instead of Sanger sequencing. The sequencing performance of the DNA barcode and mini-barcode fragments proposed in our study should be validated on NGS platforms before being applied in a forensic context.

5. Conclusion

The limited number of reports on egg genetic species identification in the scientific literature may stem from the inefficiency in detecting smuggled eggs, due to the lack of accurate detection procedures implemented after seizures. This study proposes a guideline to assist professionals in post-seizure actions, enabling the identification of species quickly and safely. Standardizing procedures in wildlife forensic laboratories ensures both the reliability and efficiency of obtaining results, while also allowing for their comparison.

Future studies could focus on the development or enhancement of molecular techniques. A technical enhancement perspective for our species identification protocol in illegally traded bird eggs includes the validation of NGS technologies for species identification in mixtures. Additionally, efforts to generate DNA barcodes for bird species should continue, aiming to make the database more representative of avian diversity. This is particularly important because DNA barcoding is the fastest and most cost-effective technique, especially for application in developing countries that are most affected by the illegal trade of bird eggs.

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