**SUPPLEMENTARY ELECTRONIC MATERIAL (APPENDIX 1)**

ARDEOLA 69(2)

# OBTAINING DNA SAMPLES FROM SENSITIVE AND ENDANGERED BIRD SPECIES: A COMPARISON OF SALIVA AND BLOOD SAMPLES

## OBTENCIÓN DE MUESTRAS DE ADN DE ESPECIES DE AVES SENSIBLES Y EN PELIGRO: COMPARACIÓN DE MUESTRAS DE SALIVA Y SANGRE

Christopher CAMBRONE[1, 2, 3 *], Sébastien MOTREUIL[2], Francis O. REYES[3, 4], Miguel A. LANDESTOY[5], Frank CÉZILLY[1, 2] & Etienne BEZAULT[1, 2]

[1] UMR BOREA (MNHN, SU, UCN, CNRS-8067, IRD-207), Université des Antilles, Guadeloupe, France.
[2] UMR CNRS 6282 Biogéosciences, Université de Bourgogne-Franche Comté, Dijon, France.
[3] Caribaea Initiative, Département de Biologie, Université des Antilles - Campus de Fouillole, 97157 Pointe-à-Pitre, Guadeloupe, France.
[4] Dirección de Biodiversidad, Ministerio de Medio Ambiente y Recursos Naturales, Santo Domingo, República Dominicana.
[5] Escuela de Biología, Universidad Autónoma de Santo Domingo, Av. Alma Mater, Santo Domingo, República Dominicana.

* Corresponding author: christopher.cambrone@yahoo.com

**Detailed protocol for DNA extraction and amplification, and statistical analyses**

*DNA extraction*

DNA extraction was performed using the DNeasy Blood & Tissue (Qiagen Inc., Valencia, CA, USA). For blood samples, we collected a piece of the blood clot weighing about 20.82 ± 9.15mg (mean ± S.D.) with a sterile spatula, since blood precipitates on contact with absolute ethanol. In order to remove as much as possible of storage ethanol within clots, we performed two rinsing cycles with 500µL of Phosphate Buffered Saline (PBS). Each time we added PBS, we vigorously vortexed the tube and discarded the supernatant after centrifugation for five minutes at high speed (13,500 rpm). After the second rinsing round, we followed the standard protocol of the commercial kit with an overnight initial lysis. Buccal swab samples were processed using 600µL of PBS and 50µL of proteinase K as the first lysis step, which lasted overnight. Such a volume was necessary to fully immerse the swab tip. Following overnight lysis, we used centrifugation to extract all remaining liquid from swabs. Instead of using commercially available filters, such as used by Vilstrup *et al.* (2018), we used a new sterile 2mL Eppendorf tube into which we inserted a sterile 0.5mL Eppendorf tube, previously cut at its lower end, in which the swab tip was placed (Figure A1). We extracted remaining lysis solution by centrifugation (13,500 rpm for two minutes) and then added it to the initial lysis solution. Prior to the DNA-binding stage, the volumes of buffers and ethanol were proportionally adjusted to the volume of the lysis solution. In order to pass the entire lysis mixture through the same spin column, the DNA-binding process was repeated several times, each time pipetting 500µL and discarding the flow-through after each centrifugation. DNA was eluted in 100µL of elution buffer.

All DNA extracts were quantified by using Qubit 4 (Thermo Fisher Scientific, Waltham, MA, United States) and Qubit dsDNA broad-range reagents (detection range 0.2 to 4,000ng/µL; Thermo Fisher Scientific). The quality of DNA extracts from both blood and saliva was assessed by electrophoresis, passing 3µL of extracted DNA solutions through a 1% agarose gel, stained using Midori-green.

*DNA amplification*

We amplified and sequenced two mitochondrial genes of different lengths in order to compare DNA extracted from buccal and blood samples. The shorter, the D-loop (DLP, ~430 bp), was amplified using PigDovF (5'-TACCATATTCATRACCCYCATA-3'; developed for the study) and PAL-2 reverse (5'-GGCCTGAAGCTAGTCGTGAT-3'; Butkauskas *et al.*, 2019), whereas the longer, the NADH dehydrogenase 2 (ND2, ~1040 bp), was amplified with H6313 (5'-CTCTTATTTAAGGCTTTGAAGGC-3') and L5219 (5'-CCCATACCCCGAAAATGATG-3') as primers (Johnson & Sorenson, 1998). We developed the PigDovF primer because other forward primers developed by Seki *et al.* (2007) or Butkauskas *et al.* (2008), for, respectively, the Japanese

Wood-pigeon *Columba janthina* and the Common Wood-pigeon *C. palumbus*, did not perform well with White-crowned Pigeon.

The same PCR protocol was used for DNA samples from both blood and buccal swabs for each mtDNA gene. Statistical comparisons relied on these one-shot PCRs. Where necessary, we performed a second round of PCR by increasing both DNA and Bovine Serum Albumin (BSA) concentrations for samples that failed to amplify during the one-shot PCR so as to confirm their use. However, they were not taken into account for statistical comparisons. PCR mixes were performed in 25µL, including 10ng of DNA, 200pM of each primer, and 200µM of each desoxyribonucléoside triphosphate (dNTP), 0.625 U of Taq DNA polymerase, 1X of Qiagen PCR buffer (Qiagen Inc.) and 0.80µg/µL of BSA. Temperature cycles began with initial denaturation at 95°C for five minutes, followed by 35 cycles of 95°C for 45 seconds (denaturation), 50°C for 45 seconds (annealing), 72°C for 45 seconds (elongation), and ended by a final elongation at 72°C for ten minutes. The PCR protocol was identical for the two mtDNA genes, except that the annealing was conducted at 58°C and elongation lasted 85 seconds for the ND2 gene. PCR products were visualised by electrophoresis on a 2% agarose gel to validate the amplification success.

Sequencing was performed by Eurofins Genomics (Ebersberg, Germany) according to the BigDye Terminator v3.1 cycle sequencing kit protocol from ABI (Applied Biosystems 3730x, Life Technologies, Grand Island, NY, USA). Two complementary sequences were produced for each sample and each mtDNA gene. Electropherograms were analysed using Geneious Prime (Kearse *et al.*, 2012), and the consensus (*i.e.,* final) sequences from the alignment of the reverse and forward complementary sequences were extracted. Final sequences of blood and buccal samples produced from the same individual were aligned to assess their similarity.

To compare sex identification, we relied on universal primers 2550F (5'–GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3'), amplifying the intron of Chromo-Helicase-DNA binding protein (CHD1; CHD1-W ≈ 450 bp and CHD1-Z ≈ 750 bp), as developed by Fridolfsson & Ellegren (1999). PCR mixes were performed in 25 µL, including 20ng of DNA, 400pM of each primer, and 300µM of each dNTP, 1.25 U of Taq DNA polymerase, 1X of Collared Qiagen PCR buffer (Qiagen Inc.) and 1.50µg/µL of BSA. Temperature cycles with an initial denaturation at 95°C for five minutes, followed by 35 cycles at 95°C for 45 seconds (denaturation), 46°C for 45 seconds (annealing), 72°C for 45 seconds (elongation), and ended by a final elongation at 72°C for ten minutes. PCR results were visualised on a 2% agarose gel. According to sex identification performed on other columbid species (*e.g.,* Monceau *et al.*, 2013; Ayadi *et al.,* 2016), two bands are expected for females and a single one for males (females being ZW and males ZZ).

*Statistical analyses*

We relied on a two-tailed paired Wilcoxon signed-rank test to compare DNA concentrations obtained from blood and buccal samples. Using the two mtDNA genes, we compared amplification success, forward and reverse sequence quality, and sequencing success between the two sample types.

Amplification success was accounted as the percentage presence of correct band size for either blood or buccal swabs samples based on agarose gel electrophoresis.

The assessment of sequence quality relied on the Phred quality scores, which are stored in chromatogram files such as *.ab1* and *.scf* files (Ewing & Green, 1998; ranging from 4 to about 60). Geneious automatically provides the percentage of bases of high (HQ; Phred score > 40), medium (MQ; 20-40), and low quality (LQ; < 20) for each forward and reverse sequence. We performed the reverse calculation to obtain the number of bases in each class quality per sequence, and we summed up to obtain the total number of bases in each class quality over all forward or reverse sequences. Since percentages are rounded to two decimals in Geneious, the number of bases in each class quality was also rounded in order to obtain a whole number of bases. To calculate the total percentage of HQ, MQ and LQ bases, we divided the number of bases of each quality class by the total number of bases, taking into account all quality classes. Forward and reverse sequences were analysed independently as the sequencing performance may differ between primers. Sequencing success corresponded to the percentage of the final sequence produced after the alignment of the forward and reverse sequences, and after manual correction for inconsistent base-calls using a reference sequence.

We relied on Fisher Exact tests to assess to what extent the quality of sequenced bases, amplification success and sequencing success were dependent on the sample type used.

We used Mc Nemar tests to test for the consistency of amplification and sequencing success between blood and buccal samples collected from the same birds.

Because of failures associated with molecular sexing from buccal samples, we relied on Fisher exact tests to assess to what extent DNA quality influenced amplifications and whether sexing was biased according to males or females. To that end, we classified DNA quality into four categories (Figure 1 and Supplementary Material, Appendix 2, Table B1 and B2).

We also relied on a two-tailed Mann-Whitney-Wilcoxon test to compare DNA concentration between samples that failed to identify sex and those that succeeded.

All statistical analyses were performed using R software 4.1.0 (R Core Team, 2021), with a significance level set at 0.05.

**Figure A1.** Additional steps to extract remaining lysis solution from swab tips. Step (A) consisted in cutting the 0.5mL tubes at their lower ends, ensuring that the hole diameter was smaller than the tip of buccal swabs used. In (B), the 0.5mL tube was placed in a 2mL tube, and then the buccal swab, with the wood handle trimmed previously to fit the 0.5mL tube, is introduced within the smaller tube. The last step (C) consisted in centrifuging the tube arrangement at 13.5 rpm for two minutes. Afterward, the remaining lysis solution, in the bottom of the 2mL tube, was added to the stock lysis solution.

*[Pasos adicionales para extraer la solución de lisis restante de las puntas de los hisopos. El paso (A) consistió en cortar los tubos de 0,5 mL por sus extremos inferiores, asegurándose de que el diámetro del orificio fuera menor que la punta de los hisopos bucales utilizados. En (B), el tubo de 0,5mL se colocó en un tubo de 2mL, y luego el hisopo bucal, con el mango de madera recortado previamente para ajustarse al tubo de 0,5 mL, se introduce dentro del tubo más pequeño. El último paso (C) consistió en centrifugar la disposición del tubo a 13,5 rpm durante dos minutos. A continuación, se añadió la solución de lisis restante, en el fondo del tubo de 2mL, a la solución madre de lisis.]*