Title: A novel method to optimise the utility of underused moulted plumulaceous feather samples for genetic analysis in bird conservation.

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ABSTRACT

Non-invasive sampling methods are increasingly being used in conservation research as they reduce or eliminate the stress and disturbance resulting from invasive sampling of blood or tissue. Here we present a protocol optimised for obtaining usable genetic material from moulted plumulaceous feather samples. The combination of simple alterations to a 'user-developed' method, comprised of increased incubation time and modification of temperature and volume of DNA elution buffer, are outlined to increase DNA yield and significantly increase DNA concentration ($W = 81$, $p < 0.01$, Cohens's $d = 0.89$). We also demonstrate that the use of a primerless Polymerase Chain Reaction (PCR) technique increases DNA quality and amplification success when used prior to PCR reactions targeting avian mitochondrial DNA (mtDNA). A small amplicon strategy proved effective for mtDNA amplification using PCR, targeting three overlapping 314-359bp regions of the cytochrome oxidase I barcoding region which, when combined, aligned with target-species reference sequences. We provide evidence that samples collected non-invasively in the field and kept in non-optimal conditions for DNA extraction can be used effectively to sequence a 650bp region of mtDNA for genetic analysis.

Keywords: *Non-invasive sampling, feather samples, Polymerase Chain Reaction (PCR), primerless PCR, mtDNA, small amplicon strategy*

53

54 *INTRODUCTION*

55

56 Working with cryptic, rare or elusive species can make obtaining invasive samples such as 57 tissue or blood logistically difficult (Mills et al. 2000; Horváth et al. 2005). Moreover, for endangered 58 and sensitive species, it can be difficult to obtain permits for more intrusive sampling methods, which 59 in some cases are considered unethical (Segelbacher 2002). In these cases, biological samples such as 60 feathers, hair, buccal cells, faecal matter and shed skin can be collected in the field with minimal 61 disturbance to the study species (Mills et al. 2000; Bohmann et al. 2014). However, some types of 62 non-invasive sample collection such as buccal swabbing and hair plucking still require trapping and 63 handling of the animal (Broquet et al. 2006; Dai et al. 2015), and these methods have the potential to 64 cause stress and affect the behaviour of an individual, even if such effects are short term (Broquet et 65 al. 2006; Rudnick et al. 2009; Dai et al. 2015). Highly non-invasive sample collection of material such 66 as moulted feathers, shed skin, faecal samples or environmental DNA (eDNA), which can be 67 collected opportunistically in the field, can eliminate the need to interact with the study species 68 (Bayard De Volo et al. 2008; Bohmann et al. 2014). This is advantageous, particularly for research on 69 sensitive species and ecosystems, as it minimises the level of disturbance to the wildlife and 70 prioritises the welfare of individuals being investigated (Dai et al. 2015).

71 Despite the advantages of using highly non-invasive sampling methods, it is often difficult to 72 identify the biological material collected to species-level without genetic analysis (Mills et al. 2000; 73 Rudnick et al. 2009). This is a particular problem for samples such as feathers, hair and faecal matter 74 that can often come from a variety of species sharing the same habitat with similar somatic features 75 (Waits and Paetkau 2005; Coghlan et al. 2012; Ahlers et al. 2017). Sample type also has an impact 76 upon the likelihood of successful DNA extraction, for example, large primary, secondary and tail 77 feathers are preferable for obtaining usable genetic material compared to smaller plumulaceous 78 feathers (Dove 2000; Vili et al. 2013). Larger feathers are embedded deeper in the body of the bird 79 and so are more likely to retain DNA containing biological material such as epithelial cells (Seki

80 2006; Gebhardt and Waits 2008). Primary feathers in particular can contain the umbilicus blood clot
81 in the shaft of the quill which is a by-product of feather development and, if present, provides a
82 plentiful source of DNA (Segelbacher 2002; Vili et al. 2013). However, opportunistic sample
83 collection methods often remove the ability to select for sample type (Broquet et al. 2006).
84 Furthermore, there is often no indication of how long the sample has been in the field and thus
85 exposed to a variety of environmental conditions before collection (Hogan et al. 2008; Vili et al.
86 2013). Hot and humid environments provide non-optimal conditions for biological samples intended
87 for genetic investigation (McNally et al. 1989; Hanson and Ballantyne 2005) as this can lead to a
88 higher prevalence of decomposing microorganisms such as fungi, mould spores and keratin-degrading
89 microorganisms, which can damage the DNA (Sangali and Brandelli 2000; Vili et al. 2013). The
90 increased likelihood of degraded DNA in such samples reduces and often prevents the selection of
91 non-invasively collected samples for use in genetic analyses (Vili et al. 2013). Therefore, an improved
92 method is needed to increase the biological value of low quality samples which have been kept in
93 non-optimal conditions; particularly for endangered or elusive species for which invasive sampling
94 methods are not possible (Broquet et al. 2006; Hogan et al. 2008; Presti et al. 2013).

95 In this study we focused on the utility of non-invasively collected feather samples of the
96 critically endangered Grenada dove (*Leptotila wellsi*). We provide a three-step process to allow
97 successful extraction and amplification of mitochondrial DNA (mtDNA) from plumulaceous feather
98 samples. Firstly, we describe improvements to a user-developed protocol for DNA extraction that
99 increases DNA yield, followed by primerless PCR to improve quality, along with a small amplicon
100 strategy that enabled effective mtDNA amplification using PCR, targeting three overlapping regions
101 of the cytochrome oxidase I barcoding region.

102

103 *METHODS AND RESULTS*

104

105 *STUDY SPECIES AND SAMPLE COLLECTION*

106 The Grenada dove is a critically endangered columbid, endemic to the island of Grenada
107 (Rosenberg and Korsmo 2001; BirdLife International 2019) with a population size of 160±30
108 individuals (Rivera-Milán et al. 2015). Habitat loss and degradation means that this species exists in
109 small fragmented populations (Rosenberg and Korsmo 2001; Rusk 2008; Rivera-Milán et al. 2015). It
110 is threatened by predation from a number of mammalian species as well as habitat destruction from
111 natural damaging events such as hurricanes (Bolton et al. 2016). Low encounter rates and cryptic
112 behaviour make surveying this species particularly difficult, requiring intensive monitoring that may
113 cause disturbance (Rivera-Milán et al. 2015; Bolton et al. 2016; Rusk 2017). Therefore, non-invasive
114 sampling methods are required to obtain samples for genetic analysis with minimal disruption to this
115 endangered species.

116 Feather samples used for this study were obtained non-invasively, as moulted feathers, and
117 collected opportunistically from known occupied dove territory: Mount Hartman estate and
118 Perseverance, Grenada (Rusk 2008, 2017). Habitat consists of both dry and mixed broadleaf
119 evergreen-deciduous second-growth forests (Rusk, 2017). This tropical dry forest habitat has a
120 minimum temperature of 22°C and temperatures that can reach up to 32°C, with a maximum rain fall
121 of 259mm in the rainy season and a minimum of 67mm in the dry season (Meteostat 2018; Nelson et
122 al. 2018). Due to the opportunistic nature of the sample collection, the feathers used in this study
123 spent an unknown amount of time in the litter bed of this hot and humid environment before
124 collection. Samples were stored in sample bags at 4°C until transportation by airmail to the UK in
125 June 2018. On arrival in the UK, the samples were cleaned with 70% ethanol and stored at -20°C. The
126 sample set (n= 160) used in this study was comprised of 152 plumulaceous (Figure 1), three
127 secondary and three primary feathers, as well as two egg shells recovered from the forest floor.

128

129 *DNA EXTRACTION*

130 The commercially available QIAGEN DNeasy® Blood and Tissue kit was used to conduct DNA
131 extraction. Extractions were carried out as per the 'User-Developed Protocol: Purification of total
132 DNA from nails, hair, or feathers using the DNeasy® Blood & Tissue Kit' (QIAGEN Inc., Crawley)

133 with the following alterations to incubation time and temperature and volume of DNA elution buffer.
134 Feather samples were cut into 1cm pieces directly into a sterile 1.5ml microcentrifuge tube containing
135 the lysis buffers using sterile scissors tp increase surface area (Presti et al. 2013). The incubation step
136 was increased to 48 hours in order to achieve complete sample lysis on samples that are particularly
137 difficult to digest (Bush et al. 2005; Bayard De Volo et al. 2008). To increase DNA yield we heated
138 buffer AE (elution buffer) at 70°C for 10 minutes before use. Buffer AE contains the organic
139 compound Tris (tris(hydroxymethyl)aminomethane, $(HOCH_2)_3CNH_2)$) and EDTA
140 (Ethylenediaminetetraacetic acid ($C_{10}H_{16}N_2O_8$)) which functions to rehydrate the nucleic acids and
141 release DNA from the silica membrane. This process is improved when the DNA and silica are
142 exposed to higher temperatures (Bruns et al. 2007; Zhou and Ling 2011; Haddad et al. 2017). We
143 used an elution volume of 100µl in a two-step process, giving a final volume of 200µl which was
144 subsequently stored at -20°C. Although using half the recommended volume of elution buffer
145 decreases the DNA yield, the aim was to increase the final concentration of DNA as it is well
146 documented that a higher concentration of starting DNA in PCR reactions improves the likelihood of
147 successful DNA amplification (Kishore et al. 2006; Rohland and Hofreiter 2007; Graziano et al.
148 2013).

149 In order to assess whether the alterations to the protocol had optimised DNA concentration
150 following extraction, we compared the concentration of DNA from samples extracted using the
151 standard manufacturer's protocol and the protocol we have outlined above. DNA concentrations for
152 samples extracted using the standard protocol were obtained from a feather set that arrived in the
153 laboratory in 2016 (n=50). We used a number generator to randomly select 20 plumulaceous feather
154 samples from the feather set obtained in 2016 and extracted using the standard protocol and from the
155 feather set obtained in 2018 and extracted using the optimised extraction method. The DNA
156 concentration of each sample was quantified using the Invitrogen™ Qubit™ 3.0 Fluorometer, which
157 has a DNA detection range of 0.50 - 600ng/ml, using dsDNA High sensitivity settings following the
158 manufacturer's protocol (Table 1). A Shapiro Wilk test (Shapiro and Francia 1972) indicated that the
159 data were not normally distributed ($p<0.05$) therefore a Wilcox test (Wilcox 2008) to compare the

160 difference in DNA extracted when using the standard protocol and the optimised protocol outlined in
161 this study. All analyses were performed in R (R Core Team 2013).The results of the test showed that
162 samples that underwent the optimised protocol had a significantly higher extracted DNA
163 concentration than the standard protocol (W = 81, p-value < 0.01, Cohens's *d*= 0.89). Samples that
164 were lower than the detection range of the Invitrogen™ Qubit™ 3.0 Fluorometer were assigned the
165 value 0.49ng/ml. Table 1 shows that 30% of the samples extracted using the standard protocol were of
166 too low concentration to evaluate. In comparison, only 5% of the samples that were extracted using
167 the optimised protocol had a concentration below the range of the Invitrogen™ Qubit™ 3.0
168 Fluorometer. Therefore, both DNA extraction success rate and DNA concentration were increased by
169 the optimised protocol.
170 To test that target DNA, rather than that of subsidiary material, had been extracted and to
171 ascertain the potential presence of PCR inhibitors, which are common when using non-invasively
172 collected samples (Waits and Paetkau 2005; von Thaden et al. 2017), avian cytochrome b (cyt b), was
173 amplified. We designed primers using Primer3 (Koressaar and Remm 2007; Untergasser et al. 2012)
174 based on a cytochrome b sequence from the white-tipped dove (*Leptotila verreauxi*), whose whole
175 mitochondrial genome is available on GenBank, accession number: NC_015190.1 (Pacheco et al.
176 2011). The Primer3 output for the designed primers is provided in in Online Resource 1. This species
177 was chosen as it has been suggested, based on the ecology of the species, that it is closely related to
178 the Grenada dove (Blockstein and Hardy 1989), although molecular phylogenetic analysis has not yet
179 been carried out for the Grenada dove. The primers were designed to include a 200-250bp region of
180 the cyt b gene, with a length of around 20 bases long, a GC content of around 50% and melting
181 temperatures that are no more than 5 degrees apart (Dieffenbach et al. 1993; Naqib et al. 2019). The
182 chosen primers targeted a 203bp region of cytochrome b gene: CYTB_2 Forward: 5'-
183 CTGCCTACTAACCCAGATCCT-3' and CYTB_2 Reverse: 5'-AGGAGCCGTAGTAGAGTCCT-
184 3'. To prevent contamination of samples, PCR was conducted in a PCR hood where tube racks,
185 pipettes and tubes were exposed to UV light for 20 minutes prior to setting up the reaction (Bayard De
186 Volo et al. 2008). PCR was conducted using illustra™ PuReTaq Ready-To-Go™ PCR Beads (GE

187 HEALTHCARE; Chicargo) with a final volume of 25µl containing ~ 2.5 units of recombinant
188 PuReTaq DNA polymerase, 200µM of dNTP's in 10mM Tris-HCl, 50 mM KCl and 1.5 mM $MgCl_2$
189 stabilizers, BSA and reaction buffer, 5µl of DNA template and a negative control using sterile H_2O.
190 PCR was conducted on a TECHNE TC-3000 thermocycler (Bibby Scientific Ltd; Stone) using the
191 following conditions optimised for this primer set: initial denaturation at 95°C for 5 minutes, 45
192 cycles of 95°C for 30 seconds, 48°C for 30 seconds, 72°C for 60 seconds and a final extension at
193 72°C for 5 minutes. PCR products were separated by electrophoresis (Westermeier 2005) on a 2%
194 (Mardis and McCombie 2017) agarose gel (Thermo Fisher Scientific, Waltham) and visualised using
195 a BioRad Gel Doc™ EZ Imager and Image lab 4.0 software (Bio-Rad Laboratories, 2017). DNA
196 extraction using the optimised method resulted in a visible band on the gel at the 203bp target region
197 of avian cyt b for 154 out of 158 (97.5%) of the feathers being used in this study (Table 2) thus
198 confirming target DNA rather than subsidiary material had been amplified.
199
200 *PRIMERLESS PCR*
201 Primerless PCR, also known as 'DNA shuffling' or 'sexual PCR', exposes the DNA template
202 to Taq DNA polymerase, dNTPs and a heating and cooling cycle which serves to denature the sample
203 into smaller fragments which then anneal to each other (Stemmer 1994; Melnikov and Youngman
204 1999; Brakmann and Schwienhorst 2004; Suenaga et al. 2005; An et al. 2011). This is known as self-
205 priming and functions to repair DNA damage such as nicks, fragmentation, abasic sites and blocked
206 3'-ends in degraded DNA samples that may inhibit amplification (Diegoli, Farr, Cromartie, Coble, &
207 Bille, 2012).
208 Primerless PCR reactions were conducted using illustra™ PuReTaq Ready-To-Go™ PCR
209 Beads with a final volume of 25µl including 5µl of DNA template. Samples were subjected to a PCR
210 cycle with the cycling parameters: initial denaturation at 95°C for 5 minutes, 10 cycles of 95°C for 30
211 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 35 cycles of 95°C for 30 seconds, 50°C for 30
212 seconds, 72°C for 60 seconds and a final extension at 72°C for 5 minutes. A negative control was also

213 generated at this stage by exposing 25µl of PCR reaction mix and no DNA template to the same PCR
214 cycle to ensure no contamination or false amplification occurred during the primerless PCR process.
215 In order to investigate the efficacy of the primerless PCR process, low quality and quantity
216 samples, as identified by lack of gel band presence, brightness and/or clarity following optimised
217 DNA extraction and amplification (Jacobs et al. 2013; Thiel et al. 2014) of the 203bp region of
218 cytochrome b gene outlined above, were chosen. Samples consisted of: 10 plumulaceous feathers, one
219 secondary feather, and one egg shell. Each sample was used as a substrate for the amplification of the
220 barcoding region of the cytochrome oxidase I (COI) gene using primer set AWCF1 and AWCintR2
221 (C1; 328bp) (Patel et al. 2010), as described in the Small Amplicon Strategy section below, with and
222 without a prior primerless PCR stage. A standardised dilution factor was used to ensure the same
223 amount of DNA template was used in each primered PCR reaction. Following amplification, the
224 samples were visualised on the UV transilluminator as a comparison for effective amplification with
225 and without exposure to primerless PCR. 'This can be seen for feather samples in Figure 2 and the
226 egg shell sample in Figure 3, which show that more bands were present and were more defined after
227 the addition of the primerless PCR step. Nine of the twelve samples failed to amplify without the
228 addition of primerless PCR but successfully amplified and presented clear bands when exposed to
229 primerless PCR. For example, sample 69 (Figure 2) shows a brighter and more defined band is
230 present after under-going the primerless PCR process. The increased prevalence of bands and the
231 improved clarity, brightness and definition of the present bands after primerless PCR indicates the
232 improved quality of DNA after exposure to this process.
233

234 SMALL AMPLICON STRATEGY

235 The third step we adopted was a small amplicon strategy to successfully amplify and
236 sequence three small overlapping amplicons, which were combined to construct a longer and more
237 informative section of the gene. Small amplicons have an increased likelihood of amplification
238 (Broquet et al. 2006; Fischer et al. 2016; Debode et al. 2017) thus we targeted 200-250bp amplicons
239 (Rohland & Hofreiter, 2007; Stiller et al., 2009). We performed primered PCR on our samples,

240 following inclusion of the primerless PCR step, along with a primerless negative control and a
241 standard negative control, using the following overlapping primer sets, which are known to amplify
242 *Columbiforme* COI barcoding region: AWCF1 and AWCintR2 (C1; 328bp), AWCintF2 and
243 AWCintR4 (C2; 314bp), AWCintF4 and AWCR6 (C3; 350bp) (Patel et al. 2010). The PCR reaction
244 was subjected to the same cycling parameters as used in the primerless PCR stage: initial denaturation
245 at 95°C for 5 minutes, 10 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds,
246 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds and a final extension at
247 72°C for 5 minutes. PCR products were separated and visualised using agarose-gel electrophoresis.
248 All three primer sets successfully amplified DNA from our non-invasively collected samples (Figure
249 4). Samples were purified using the QIAquick PCR Purification as per the manufacturer's protocol
250 (QIAGEN Inc., Crawley). Samples were then prepared for sequencing using the Mix2Seq Kit
251 (Eurofins Genomics, Luxembourg) according to the manufacturer's instructions and submitted to
252 Eurofins Genomics, where they were sequenced using Sanger sequencing methods.
253 Sequence data for the three overlapping regions were reconstructed to create an
254 approximately 650bp sequence (Figure 5A). Firstly, consensus sequences were obtained for each
255 amplicon by aligning the forward and reverse sequence data in NCBI Basic Local Alignment Search
256 Tool (BLAST®) (Johnson et al., 2008) and were used in all further reconstructions and alignments.
257 Obtaining consensus sequence is particularly important when using primerless PCR as random
258 fragmentation and self-priming can introduce artificial recombinants or base errors but this can be
259 counteracted by using consensus sequence (Weber et al., 2000). To assemble the longer combined
260 sequence, each of the small amplicons were aligned using a global alignment algorithm with Emboss
261 Explorer merger tool (Figure 5B). A heuristic approach is used to select the base with the best local
262 sequence quality score in the case of any mismatches (Bell and Kramvis 2013). Highly degraded
263 DNA can produce poor read length or low quality base calls in the sequence data, particularly at the
264 ends of the trace producing "messy" end sequences (Bell and Kramvis 2013). Therefore, most
265 probably due to the highly degraded nature of the samples used in this study, some base
266 inconsistencies were recognised. In these cases the Emboss Merger local quality score, along with the

267 electropherogram obtained from Eurofins for each sequence (Figure 5C), were assessed and the base
268 with the highest quality score as per both the emboss and Eurofins sequence quality assessment was
269 selected (Bell and Kramvis 2013). Due to the non-invasive sample collection method used for these
270 samples, along with the inability to identify the species from the morphological features of the feather
271 alone, each sequence was run in NCBI's Basic Local Alignment Search Tool (BLAST®) to predict
272 species identification (Johnson et al. 2008). This search indicated that the sample, presented in Figure
273 5, was from the Caribbean columbid, white-crowned pigeon (*Patagioenas leucocephala*), with a
274 BLAST total score of 989 and a query coverage of 91% to Genbank sequence JJQ175689.1 (Schindel
275 et al. 2011). Combined sequence data obtained from the three small amplicons were aligned with the
276 corresponding COI barcoding region sequence data from a known *L. wellsi* specimen (unpublished
277 data – Genbank reference sequence not yet available for this species) and *P. leucocephala* (accession
278 number JQ175689.1) (Schindel et al. 2011) to confirm the expected nitrogenous base positions
279 (Johnson et al., 2008), which is particularly important given the possibility of potential base errors
280 introduced by primerless PCR (Weber et al., 2000). Target-species was confirmed for the 650bp
281 length of the COI barcoding region from non-invasively collected plumulaceous feather samples.
282
283 *DISCUSSION*
284 The methods outlined in this study: an optimised user-developed DNA extraction protocol,
285 use of a primerless PCR technique, and a small amplicon PCR strategy, facilitated the attainment of
286 target-species mtDNA sequence data of 650bp in length from non-invasively collected plumulaceous
287 feather samples. Problems arising due to poor quality of DNA extracted from non-invasively collected
288 samples can often dissuade researchers from proceeding with genetic analysis, causing the potential of
289 such samples to go unrecognised (Horváth et al. 2005; Speller et al. 2011). This can also lead to a
290 preference in invasive as opposed to non-invasive sample collection methods due to the higher
291 confidence in invasive sample quality (Johnson and Clayton 2000a; Harvey et al. 2006). The methods
292 we have outlined allow such samples, which previously may have been discounted for genetic
293 analysis, to be successfully used to obtain informative sequence data.

294 It is widely documented that feather type influences the success and yield of DNA extraction
295 from feather samples (Bayard De Volo et al. 2008; Hogan et al. 2008). Primary feathers are widely
296 used as a recourse for genetic monitoring of wild bird populations, as they often contain a blood clot
297 located in the superior umbilicus of the feather shaft (Horváth et al. 2005). Along with assessing the
298 genetic variation within populations (Nichols et al. 2001), feather samples have been used to
299 investigate phylogeny and biogeography (Johnson and Clayton 2000b), and to understand the impact
300 of anthropological processes on genetic structure and gene flow (Fourcade et al. 2016) of bird species.
301 However, a study on molecular sexing and microsatellite genotyping of hyacinth macaw
302 (*Anodorhynchus hyacinthinus*) found that despite using larger moulted feathers (with a size greater
303 than 20cm) than in this study, feathers that spent more than seven days in the field and were of poor
304 physical quality, had a low success rate in yielding sufficient DNA for genetic analysis (Presti et al.
305 2013). Nevertheless, we have demonstrated that plumulaceous feathers that are found in the field do
306 not necessarily need to be excluded from sample sets as they can still be utilised to yield informative
307 data.

308 The use of primerless PCR increased the amplification success in subsequent PCR reactions
309 for samples that had proven difficult to amplify. Bands from samples exposed to primerless PCR were
310 visually clearer and brighter, which suggests a higher quality of DNA (Hughes-Stamm et al. 2011;
311 Jacobs et al. 2013; Lucena-Aguilar et al. 2016). Primerless PCR has been used in studies of ancient
312 DNA, including that of Weber *et al.* (2000), who increased the successful amplification of ancient
313 DNA from bone samples when investigating the population bottleneck of the northern elephant seal
314 (Weber et al. 2000). The same technique was adopted using museum samples from the African
315 horseshoe bat (*Rhinolophus darlingi*) to investigate the phenotypic convergence of its evolutionary
316 history (Jacobs et al. 2013). Both studies reported that primerless PCR improved the recovery of DNA
317 from ancient samples but did not comment on differences in DNA quality post primerless PCR
318 (Weber 2004; Jacobs et al. 2013). To the best of our knowledge, our study is the first to demonstrate
319 the utility of primerless PCR to increase DNA quality for degraded non-invasive feather samples and

320 demonstrates that this technique can be applied to a variety of non-invasive samples collected in the
321 field.

322 Although the primerless PCR technique recovers the quality of DNA obtained from the
323 degraded samples this process does not completely repair damaged DNA, particularly when the DNA
324 damage is highly fragmented, which is a common problem with samples kept in non-optimal
325 conditions (Diegoli et al. 2012). The varying quality of sequence data at ends of the trace producing
326 "messy" end sequences is a particular problem for overlapping regions resulting in base
327 inconsistencies (Stiller, Knapp, Stenzel, Hofreiter, & Meyer, 2009). However, this is not specific to
328 the techniques we describe here and is prevalent in many genetics datasets, though it is not widely
329 documented (Sobel et al. 2002; Hackett and Broadfoot 2003; Bonin et al. 2004). Sequencing error can
330 lead to misidentification of individuals, misinterpretations and erroneous conclusions to be drawn
331 from genetic analysis of relatedness and population structure (Hogan et al. 2008). Sequencing error
332 can occur at any stage when obtaining genetic information but there are a number of actions that can
333 be taken to minimise these errors (Bonin et al. 2004). The following have been adopted in this study
334 and are proposed for future studies to limit the risk of sequencing error following primerless PCR: 1)
335 the amplification of small amplicons because genotyping error correlates with amplicon size (Vili et
336 al. 2013); 2) the inclusion of negative controls (Waits and Paetkau 2005; Alda et al. 2007; Boonseub
337 et al. 2012); 3) sequence quality assurance by using only forward and reverse consensus sequences
338 with further analysis and inspection of electropherogram scoring levels; and 4) cross-reading and
339 aligning sequence data with reference samples (Weber et al. 2000; Bonin et al. 2004). For downsteam
340 analyses, it is also recommended to include a sequencing error rate when using genetic data for
341 population and phylogenetic analyses (Bonin et al. 2004).

342 We have outlined methods that achieve successful extraction and amplification of an
343 informative length of mtDNA from non-invasively collected plumulaceous feather samples. The
344 analysis of mtDNA has many practical applications in conservation including species identification as
345 well as presence/absence detection. The high mutation rate of mtDNA in comparison to nDNA allows
346 the identification of Evolutionarily Significant Units (ESUs), giving an insight into the phylogenetics

347 of a species (Cronin 1993; Wan et al. 2004; Gupta and Bhardwaj 2013). Data pertaining to
348 phylogenetics have multiple conservation implications including evolutionary divergence and
349 speciation, along with phylogeography and rates of change relating to morphology and behaviour of a
350 species (Huang et al. 2009). Determining the point at which speciation occurs or defining a species as
351 genetically distinct – known as the phylogenetic species concept – is particularly useful for resolving
352 taxonomic uncertainties, outlining wildlife legislation, and in identifying conservation priorities
353 (Hazevoet 1996; Wan et al. 2004; Pellens and Grandcolas 2016; Chen et al. 2018). Analysis of
354 mtDNA can be used to make long-term species conservation action plans from an evolutionary
355 perspective (Nielsen et al. 2017) and to inform short-term demographic management of populations
356 through identification of population change and connectivity therefore providing information on the
357 effects of habitat fragmentation (Cronin 1993; Moritz 1994; Nabholz et al. 2009). Therefore, our
358 improved methods of extraction and amplification of mtDNA from non-invasively collected, low
359 quality feather samples, have the potential to extend the applicability of molecular analyses in studies
360 aimed at the conservation of endangered bird species, for which it is typically difficult to obtain high
361 quality samples.
362
363 *CONCLUSION*
364 In conclusion, the optimised user-developed DNA extraction protocol, along with the use of
365 the primerless PCR technique, and a small amplicon PCR strategy, are sufficient to enable DNA
366 extraction and mtDNA amplification from moulted plumulaceous feathers collected non-invasively
367 and opportunistically in the field. This not only provides evidence in support of using non-invasive
368 sampling methods for genetic analyses, in particular when applied to endangered species, but also
369 highlights the utility of biological material kept in non-optimal conditions, may previously have been
370 discounted (Rawlence et al. 2009; Vili et al. 2013). Data collected in this manner is informative for
371 species identification, presence/absence detection, population structure and phylogenetic analyses of
372 rare and elusive species (Bonin et al. 2004; Marucco et al. 2011; Adam et al. 2014), all of which are
373 key questions in conservation research.

374

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Table 1: Concentration of DNA (ng/ml) from samples extracted using the standard QIAGEN user developed DNA extraction protocol and samples extracted using the optimised extraction protocol.

Standard Protocol		Optimised Protocol	
2016 Samples	DNA Conc. (ng/ml)	2018 Samples	DNA Conc. (ng/ml)
P2	<0.50*	M31	7.45
P4	26.9	M40	10.8
P5	<0.50	M44	1.13
P7	13.0	M46	5.18
P8	<0.50*	M63	3.60
P12	<0.50*	M68	4.60
M1	6.09	M83	1.76
M2	1.28	M95	217.67
M5	0.56	M100	58.17
M8	0.81	M108	1.33
M18	0.82	M114	50.7
M19	7.92	M115	4.64
M22	1.74	M118	<0.50*
M23	1.35	P37	32.1
M28	15.9	P38	307.33
F3	1.32	P39	4.50
F4	1.23	P45	45.43
F5	0.51	P49	34.13
F6	<0.50*	P69	2.23

F8	<0.50*	F12	1.87
Mean	4.1185	Mean	39.7555
Standard Error	±1.56	Standard Error	±17.82
Median	1.03	Median	4.91

619 *DNA concentration was too low to read and therefore out of range of the Invitrogen™ Qubit™ 3.0

620 Fluorometer (range: 0.50 - 600 ng/ml) using dsDNA High sensitivity settings.

621 **Table 2**: Number of successful and failed amplifications of a 203bp target region of avian cytochrome
622 b gene, recorded by feather type, following DNA extraction using our optimised technique (prior to
623 including the primerless PCR step). Amplification was considered successful if a visible band was
624 present on a 2% agarose gel for the target region.

Feather type	Number of feathers	Number of successful amplifications	Number of failed amplifications
Secondary	3	2 (66.7%)	1 (33.3%)
Primary	3	3 (100%)	0 (0%)
Plumulaceous	152	149 (98%)	3(2%)
Total	158	154 (97.5%)	4 (2.5%)

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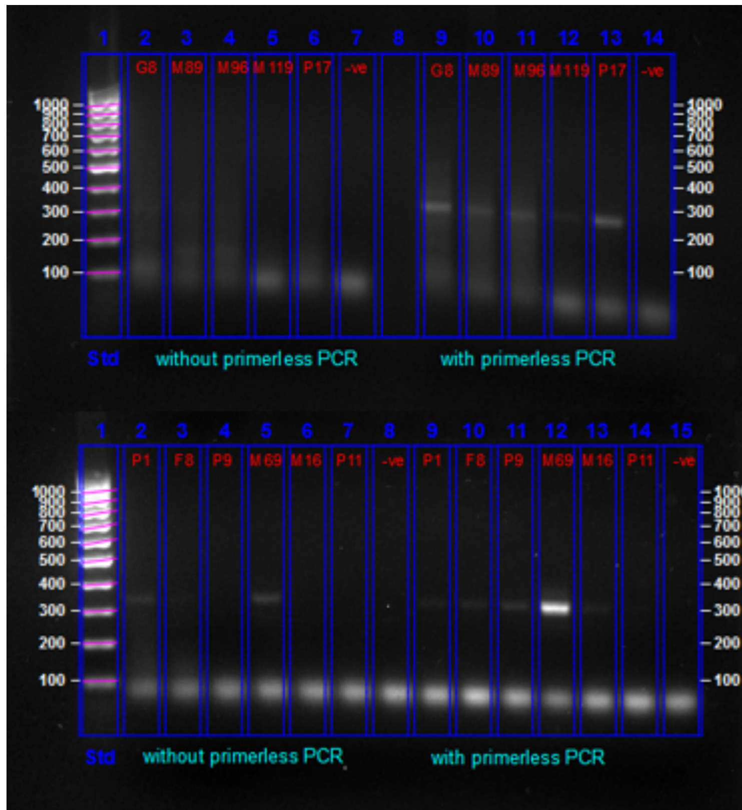
632 Figure 1: Examples of the plumulaceou*s* feathers used in this investigation.

633

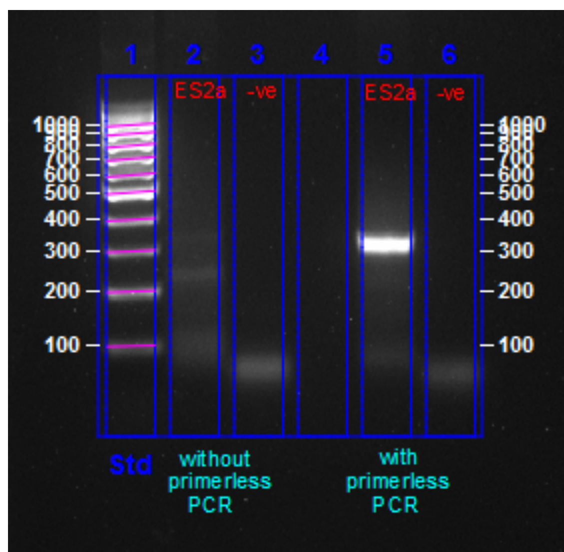


634

635 Figure 2: Gel image showing the amplification of non-invasively collected plumulaceous feather
636 samples (with the exception one secondary feather (G8)) using primer set AWCF1 and AWCintR2
637 (328bp) (Patel et al. 2010) both with and without exposure to primerless PCR.

638

639



640

641 Figure 3: Gel image showing the amplification of a non-invasively collected egg shell sample using

642 primer set AWCF1 and AWCintR2 (328bp) (Patel et al. 2010) both with and without exposure to

643 primerless PCR.

644

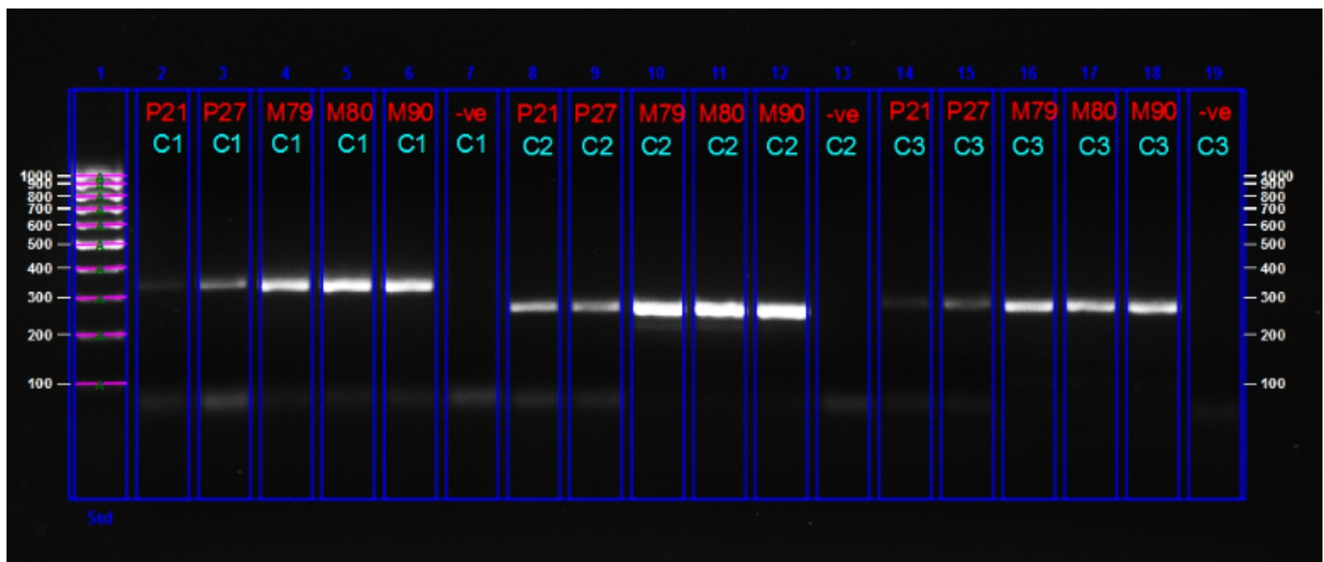
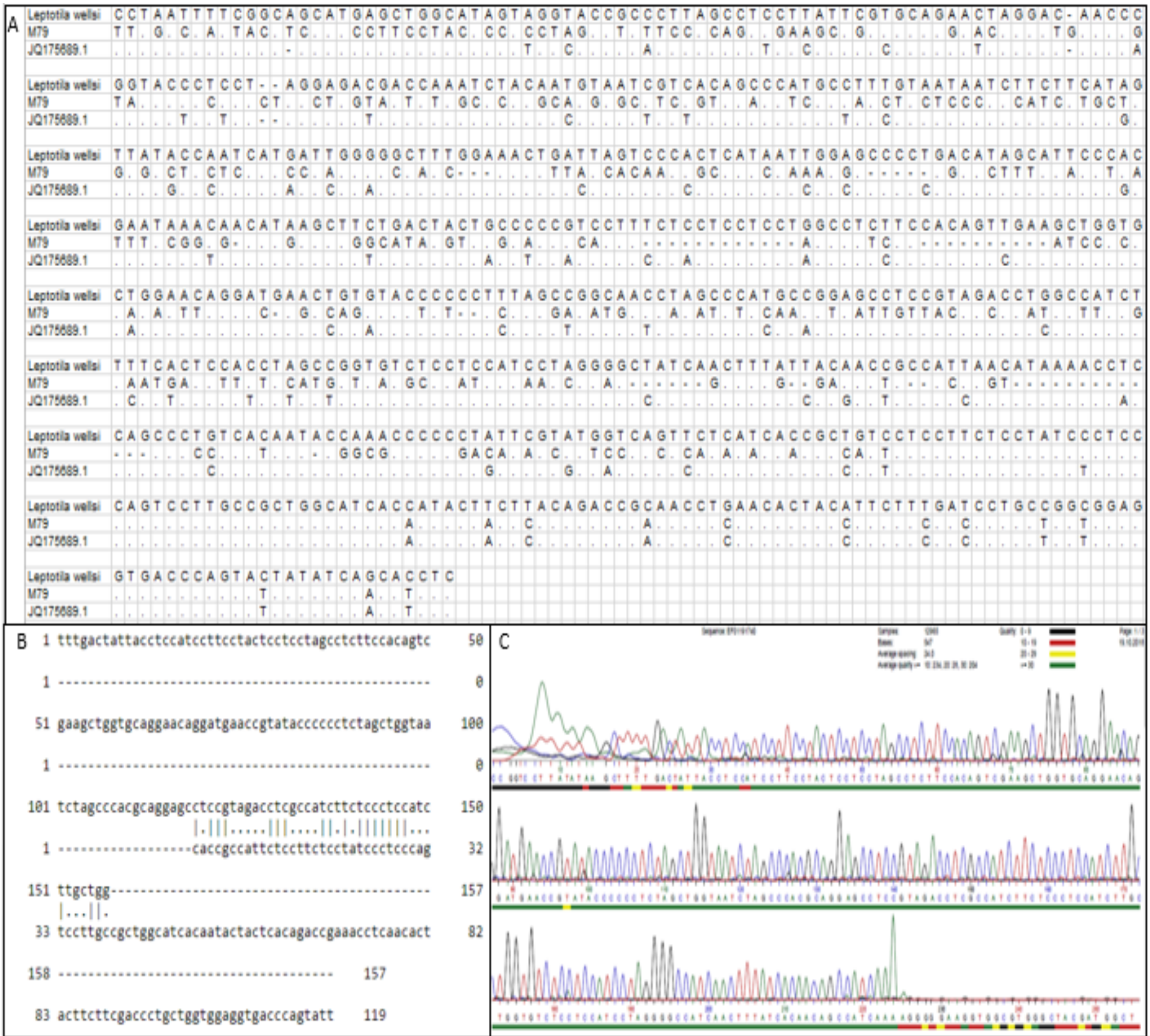


Figure 4: Gel image showing successful amplification of the three small amplicons using primer sets AWCF1 and AWCintR2 (C1; 328bp), AWCintF2 and AWCintR4 (C2; 314bp), AWCintF4 and AWCR6 (C3; 350bp) (Patel et al. 2010) from moulted plumulaceous feathers collected non-invasively and opportunistically in the field.

654



655 **Fig. 5**: Sequence data from sample M79. **A**. The 650bp sequence obtained for sample M79 aligned
656 with the corresponding COI barcoding region sequence data for *Leptotila wellsi* and *Patagioenas*
657 *leucocephala,* labelled with its accession number JQ175689.1 (Schindel et al. 2011). **B**. Emboss
658 Explorer (Bell and Kramvis 2013) OUTPUT example for two overlapping sequences from sample
659 M79 using forward and reverse consensus sequences. C. Electropherogram example with base quality
660 score as assigned by Eurofins for the forward sequence of COI_2.

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