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Assessment of *CHD*-specific primers for gender determination in Red-billed Oxpeckers *Buphagus erythrorhynchus*

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Red-billed Oxpeckers *Buphagus erythrorhynchus* are morphologically similar and do not display distinctive phenotypic difference between males and females. The development of DNA-based gender determination techniques constituted a breakthrough in reliable sex determination in birds. Two DNA-based methods of gender determination were evaluated to determine the preferred method for the Red-billed Oxpeckers. DNA-based gender determination of the Red-billed Oxpeckers was conducted so that specific sexes could be relocated to new release sites within South Africa. The two primer sets used were 2550F/2718R and P2/P8. When comparing the results of the two primer sets, it was determined that 17% (n = 25) of individuals that were identified as having one sex by the 2550F/2718R primer set changed their DNA gender determination when the P2/P8 primer set was used. Based on molecular evidence and the pathology results for three recorded mortalities at the National Zoological Gardens of South Africa, it was determined that the P2/P8 primer set would be preferable to the 2550F/2718R primer set for DNA gender determination of Red-billed Oxpeckers.

Introduction

Gender determination in birds is useful for studying population dynamics and structure, habitat use, behaviour and mating systems (Donohue and Dufty 2006). However, Griffiths et al. (1998) indicated that an estimated 50% of all bird species are sexually monomorphic, having no external morphological differences between males and females. Different PCR-based methods have been developed to determine the sex of several different bird species. In birds, unlike in mammals, females are the heterogametic sex, i.e. have two different sex chromosomes (ZW), while males are the homogametic sex (ZZ). Two different primer sets have been developed by Fridolfsson and Ellegren (1999) and Griffiths et al. (1998). These primer sets amplify an intron from the Chromo Helicase DNA binding (*CHD1*) gene and amplify across an intron in both *CHD-Z* and *CHD-W*. Determination of sex is then obtained via an observed difference in amplified fragment lengths. Heterogametic (ZW) females are expected to have two different-sized introns while homogametic (ZZ) males should only have one intron size. Gel electrophoresis therefore reveals one band in males and two bands in females in most of the bird species.

Red-billed Oxpeckers (*Buphagus erythrorhynchus*) are endemic to sub-Saharan African savannas and feed on ixodid ticks, dead skin, mucus, saliva, blood, sweat and tears (Bezuidenhout and Stutterheim 1980). The Red-billed Oxpecker is a passerine bird in the starling and myna family Sturnidae. The birds are sexually monomorphic and thus gender determination is not possible via outward appearances. Gender determination in the Red-billed Oxpeckers was conducted in order to assist the Endangered Wildlife Trust of South Africa (EWT) in a relocation project referred to as 'Operation Oxpecker'. DNA-based sex identification results were used in order to ensure an equal sex distribution of relocated birds. Red-billed Oxpeckers were either released in three different areas of South Africa or were kept at the Mokopane Biodiversity Conservation Centre, Limpopo province, as part of an ongoing research project.

In this study, gender determination using the 2550F/2718R and the P2/P8 primer sets was conducted and compared in the red-billed Oxpeckers. Results from the DNA analysis were compared to post-mortem results for three birds that died during the study. Data obtained from this study indicated that the P2/P8 primers are better suited to determine gender in Red-billed Oxpeckers than the 2550F/2718R primer set.

Methods

The molecular investigation was conducted using a group of Red-billed Oxpeckers from the Platjan farming area, adjacent to the Limpopo River, and a second population from Skukuza, Kruger National Park, Mpumalanga, South Africa. Red-billed Oxpeckers were captured within mist nets. Within the Platjan farming area these nets were erected around water troughs and holding pens used by the livestock in the area. At Skukuza these nets where errected around and on the walkways of the rhino holding bomas. Red-billed Oxpeckers caught in these nets were promptly removed, measured and ringed with a uniquely numbered AFRING bird ring. Blood samples were collected by sterilising the area with a webcol and then pricking the ulna vein, where it crosses over the distal end of the humerus, of the wing with a 27G × 1″ dental needle. Approximately 50 µl

blood was then collected in a capillary tube. The drop of blood was placed on Flinders Technology Associates (FTA) filter paper, air dried and stored in a 1.5 ml sterile tube. The tube was placed in a cooler box to keep the sample cool and out of ultraviolet (UV) light (sunlight).

Genomic DNA was isolated from 147 Red-billed Oxpeckers from blood collected on the filter paper using the Qiagen DNeasy Blood and Tissue Kit®. The concentration of DNA was determined using a nanodrop, the DNA yield obtained was 10–20 ng μl^{-1} and the A_{260}/A_{280} ratio was between 1.5 and 1.9. Amplification of the *CHD1* gene was conducted using the P2/P8 (Griffiths et al. 1998) and the 2550F/2718R (Fridolfsson and Ellegren 1999) primer sets. PCR amplification was carried out in a total volume of 25 µl. PCR was conducted with Promega GoTaq® DNA polymerase, which consists of a 1× buffer containing 10 mM Tris®-HCl (pH 9.0), 50 mM potassium chloride (KCl) and 0.1% Triton® X-100. The final reaction conditions were as follows: 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM 2'-deoxynucleotide triphosphate (dNTP), 5 pmol of each of the forward and reverse primer, 0.25 U *Taq* DNA polymerase and 10 ng genomic DNA template. The conditions for PCR amplification were as follows: 2 min at 94 °C denaturation, 30 cycles for 30 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C, followed by extension at 72 °C for 10 min. PCR products were added to tracking dye and were separated by electrophoresis in a 2% (w/v) agarose gel for 45 min at 100 V in 1× Tris-borate-EDTA buffer.

Results

Amplification of the *CHD1* gene using the 2550F/2718R primer set (Fridolfsson and Ellegren 1999) was observed in 65% (*n* = 95) of the samples tested and amplification using the P2/P8 primer set (Griffiths et al. 1998) was observed in 90% (*n* = 133) of the samples (Figure 1). When comparing the results of the two primer sets, discrepancies were observed and an overrepresentation of males was observed for the 2550F/2718R primer set (Table 1). It was determined that 17% (*n* = 25) of individuals that were identified as having one gender by the 2550F/2718R primer set changed their DNA gender identification when the P2/P8 primer set was used. Post-mortem results were obtained from the pathologist for three birds that died during the quarantine period. Gender determination of the birds as determined via the pathologist corresponded with DNA results obtained from the P2/P8 primer set (Table 2). One bird that was identified as female by post-mortem results and via the P2/P8 primer set, but was identified as male using the 2550F/2718R primer set, was sequenced. The amplified sequence of the Z-chromosome was analysed using both the forward and reverse primers (2550F/2718R) and was aligned to sequences in GenBank (BLAST) as indicated in Appendix 1. Sequence differences due to mutations in the Z-chromosome were not observed in either the forward or reverse primer binding sites, indicating that mutations in the priming site were not the cause of the error. However, as the W-chromosome did not amplify, mutations in the priming site of this chromosome cannot be discounted.

Table 1: Results obtained for the molecular gender differentiation of Red-billed Oxpecker by analysis of the fragment *CHD1*, amplified with the primer sets P2/P8 and 2550F/2718R

Primer set	Male		Female	
	No.	%	No.	%
2550F/2718R	66	70%	29	30%
P2/P8	57	43%	76	57%

Table 2: Comparison between the two methods of molecular gender differentiation and results obtained from the post-mortem inspection of the three Red-billed Oxpeckers

No.	2550F/2718R	Post-mortem inspection	P2/P8
1	Male	Male	Male
2	Female	Male	Male
3	Male	Female	Female

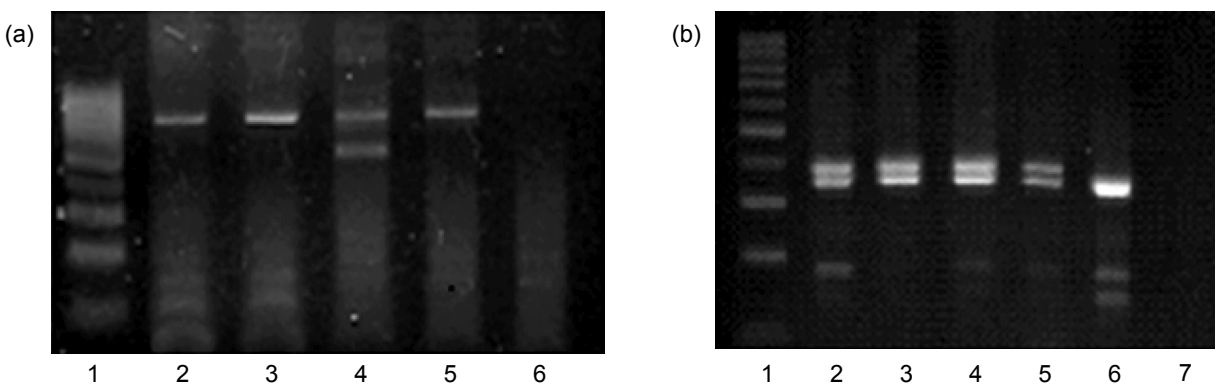

Figure 1: Molecular gender differentiation of Red-billed Oxpeckers by analysis of the fragment *CHD1*. Electrophoresis of the PCR products was conducted on a 2% agarose gel. (a) PCR products of the 2550F/2718R primer set; lane 1: 100 bp molecular marker (Fermentas); lanes 2, 3, 5: male; lane 4: female; lane 6: negative control. (b) PCR products of the P2/P8 primer set; lane 1: 100 bp molecular marker (Fermentas); lanes 2–5: female; lane 6: male; lane 7: negative control

Discussion

DNA sex identification in Red-billed Oxpeckers using the primer sets 2550F/2718R (Fridolfsson and Ellegren 1999) and P2/P8 (Griffiths et al. 1998) produced different results. It was observed that only amplification results obtained from the P2/P8 primer set matched the pathological report. Thus, using results obtained from the 2550F/2718R primer set would have hampered the success of the annual relocation operations.

Based on these results it can be determined that it would be recommended to use the P2/P8 primer set when performing DNA gender determination of Red-billed Oxpeckers. Differences between the two primer sets and bird gender results have been reported previously. Ong and Vellayan (2008) conducted gender determination in various birds from the Anatidae family using feathers. The authors indicated that the P2/P8 primer set was easily able to resolve males and females and produced clear fragments on the agarose gel, whereas the 2550F/2718R primer set only produced a single fragment for both male and female birds. Dawson et al. (2001) indicated that in the Eurasian Blackbird *Turdus merula* the 2550F/2718R primer set amplified a single product for both sexes. Fridolfsson and Ellegren (1999) indicated that in three passerine bird species (White Wagtail *Motacilla alba* and two species of tit *Parus* spp.) the 2550F/2718R primer set was unable to resolve differences between males and females. The authors indicated that this was not due to the absence of intron size difference between the W and Z chromosomes (Fridolfsson and Ellegren 1999). However, the amplification of a single fragment for both males and females is due to sequence divergence in the priming site of the W-chromosome, which prevents female-specific amplification. This finding is substantiated by the authors' observation that the W-chromosome is often more weakly amplified in females of other passerine species (D Dalton pers. obs.).

Thus it is recommended that all bird species of known sex be tested with both primer sets in order to develop a species-specific DNA sex-determination test.

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Appendix 1: Alignment of *CHD1Z* sequences for the Red-billed Oxpecker *Buphagus erythrorhynchus* and other bird species in the GenBank database (BLAST) using the 2550F/2718R primer set. A sequence of 431 bp for the Red-billed Oxpecker was amplified and is indicated below in reverse (from 431 to 1 bp). The number on either side of the sequence indicates the size of the sequence. For ease of reference, the adjoining sequences are divided into eight sections. Each dot (.) in the sequence indicates a nucleotide (A, G, C or T) that is identical to the one at the corresponding position in the sequence of the Red-billed Oxpecker. A dash (—) indicates gap differences in the sequence, where a nucleotide is absent. An A, G, C or T in the rows of species other than the Red-billed Oxpecker indicates a nucleotide substitution difference between the bird species

```
Buphagus erythrorhynchus          431 CAAAGAGCCAGTCCC-TT-T-C-AAAAAAAGAA-GACATTTTC-TTCC----A-TGTTT- 386
Corvus corone corone              431 ...........-C.-.-T.G........T.-.......-....-----.C.-...- 386
Corvus macrorhynchos japonensis   431 ...........-C.-.-T.G........T.-.......-....-----.C.-...- 386
Corvus frugilegus                 431 ...........-C.-.-T.G........T.-.......-....-----.C.-...- 386
Agelaius phoeniceus               417 ...........-C.-C-.TGG........-.........-A...----T-.T...- 372
Emberiza stewarti                 415 ...........-C.-C-.TGG........-.........-A...----T-.T...- 370
Emberiza hortulana                413 ...........-C.-C-.TGG........-.........-A...------.-...- 370
Emberiza citrinella               414 ...........-C.-C-.TGG........-.........-A...----T-.T...- 369
Oporornis philadelphia            410 ...........-C.-C-.TGG........-....-....AA...----T-.T...- 365
Oporornis tolmiei                 410 ...........-C.-C-.TGG........-....-....AA...----T-.T...- 365
Emberiza calandra                 418 ...........-C.-C-.TGG........-.........-A...------.-...- 375
Emberiza cirlus                   412 ...........-C.-C-.TGG........-.........-A...------.T...- 368
Ficedula albicollis               356 ...........T.-..-.C.-.G.......T-.........-....----.-..C..- 309
Ficedula hypoleuca                356 ...........T.-..-.C.-.G.......T-.........-....----.-..C..- 309
Ficedula parva                    356 ...........-.T-..-.-.T.G.......T-.........-....----.-..C..- 309
Sialia sialis                     319 ...........T..-..-.-.T.G........-.........-....----.-.....- 280
Sialia mexicana                   319 ...........T..-..-.-.T.G........-.........-....----.-.....- 280
Perisoreus infaustus              319 ...........-C.-.-T.G........C.-.......-....-----.C.-...- 274
Erithacus rubecula                316 ...........-..-.-.T.G........-.........-....-----.-..C..- 268
Corvus corax                      319 ...........-C.-.-T.G........T.-.......-....-----.C.-...- 274
Tiaris olivacea                   323 ...........-C.-C-.TGG........-.........-C...TTTTT-.T...- 274
Prunella modularis                322 ...........A..A.---.G.......T-.........-A...----.-.....- 277
Loxigilla portoricensis           321 ...........-C.-C-.TGG........-...T.....-C...T---T-.T...- 275
Emberiza citrinella               319 ...........-C.-C-.TGG........-.........-A...----T-.T...- 274
Troglodytes aedon                 323 ....A...G..T-..-.-.T.G.T.....-.........-....----.-CA...- 278
Cardinalis cardinalis             324 ...........-C.-C-.TGG........-.........-A...TT--.-.T...- 277
Loxigilla portoricensis           321 ...........-C.-C-.TGG........-...T.....-C...T---T-.T...- 275
Coereba flaveola                  304                      ........-.........-A...T---T-.T...G 276
Emberiza leucocephalos            321 ...........-C.-C-.TGG........-.........-A...----T-.T...- 276

Buphagus erythrorhynchus          385  TTTTAATCAAAGGGGTAAAGGTC-ACATAA-C-CGCAAACAAAGCTGAAAGGGACTGCT- 330
Corvus corone corone              385 .......TG.........C....-......-T-.A.G.....................- 330
Corvus macrorhynchos japonensis   385 .......TG.........C....-......-T-.A.G.....................- 330
Corvus frugilegus                 385 .....T.TG.........C....-......-T-.A.G.....................- 330
Agelaius phoeniceus               371 ...........A.........-.T......-T-.A...........C...A........- 316
Emberiza stewarti                 369 ...........A.....G...-.T......-T-.A...........C...A........- 314
Emberiza hortulana                369 ...........A.........-.T......-T-.A...........C............- 314
Emberiza citrinella               368 ...........A.........-.T......-T-.A...........C...A........- 313
Oporornis philadelphia            364 ......................-.T......-T-.A...........C............- 309
Oporornis tolmiei                 364 ......................-.T......-T-.A...........C............- 309
Emberiza calandra                 374 ....T......A.........-.T......-T-.A...........C............- 319
Emberiza cirlus                   367 ...........A.........-.T......T.A.AA..........C...A........- 310
Ficedula albicollis               308 .........C..T..........-...G..-T-.A.......................- 253
Ficedula hypoleuca                308 .........C..T..........-..CG..-T-.A.......................- 253
Ficedula parva                    308 .........C..T..........-...G..-T-.A.......................- 253
Sialia sialis                     279 A.......................-......-T-.A.........T............- 224
Sialia mexicana                   279 A.......................-......-T-.A.........T............- 224
Perisoreus infaustus              273 .......TG..............-......-T-.A.G.....................- 218
Erithacus rubecula                267 A........C..T.........---.--..-----..C.............T......- 219
Corvus corax                      273 .......TG.........C....-......-T-.A.G.....................- 218
Tiaris olivacea                   273 ......................-.T......-T-.A...........C............- 218
Prunella modularis                276 ......................C.-...C..-T-.A.......................- 221
Loxigilla portoricensis           274 ............A.......-.T......-T-.A...........C............- 219
Emberiza citrinella               273 ...........A.........-.T......-T-.A...........C...A........- 218
Troglodytes aedon                 277 ......................-......---TAT.......................- 223
Cardinalis cardinalis             276 ......................-.T......-T-.A...........C.G.........- 221
Loxigilla portoricensis           274 ............A.......-.T......-T-.A...........C............- 219
Coereba flaveola                  275 ......................-.T..G...-T-.A...........C............- 220
Emberiza leucocephalos            275 ......................-.T......-T-.A...........A............G 219
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Appendix 1: (cont.)

Buphagus erythrorhynchus	329	GAGGGTCATCTTGTCTAAATCCCCTACTCAGGCAGGGACAGCTGAAGTATGCTACCCAAG	270
Corvus corone corone	329	.....A.....A.....A..A...C.....G.....	270
Corvus macrorhynchos japonensis	329	.....A.....A.....A..A...C.....G.....	270
Corvus frugilegus	329	.....A..C.....A.....A..A...C.....G.....	270
Agelaius phoeniceus	315	...A.....A...C.....G.....	256
Emberiza stewarti	313	...A.....A...C.....G.....	254
Emberiza hortulana	313	...A.....A...C.....G.....	254
Emberiza citrinella	312	...A.....A...C.....G.....	253
Oporornis philadelphia	308	..CA.....A...C.....G.....	249
Oporornis tolmiei	308	..CA.....A...C.....G.....	249
Emberiza calandra	318	...A....C.....T.....A...C.....G.....	259
Emberiza cirlus	309	...A.....A...C.....G.....	250
Ficedula albicollis	252	.....A...C.....G.....	193
Ficedula hypoleuca	252	.....A...C.....G.....	193
Ficedula parva	252	.....C.....A...C.....G.....	193
Sialia sialis	223	.T.....G.....	164
Sialia mexicana	223	.T.....G.....	164
Perisoreus infaustus	217	.....A.....A.....A...C.....G.....	158
Erithacus rubecula	218	.....-.....--..G....A...C.....G.....	162
Corvus corax	217	.....A..C.....A.....A..A...C.....G.....	158
Tiaris olivacea	217	...A.....A...C.....G.....	158
Prunella modularis	220	.....G.....A.A...C.....G.....A	161
Loxigilla portoricensis	218	...A.....A...C.....G.....	159
Emberiza citrinella	217	...A.....A...C.....G.....	158
Troglodytes aedon	222	.....T.....A.....T.C..A...C.C...G.....A	163
Cardinalis cardinalis	220	...A.....T.....A...C.....G.....	161
Loxigilla portoricensis	218	...A.....A...C.....G.....	159
Coereba flaveola	219	...A.....A...C.C...G.....	160
Emberiza leucocephalos	218	..-A.....T.....A...C.....G.....	160

Buphagus erythrorhynchus	269	G--T-C---ATGT--CCAA-G-TAGCTTTT-G-GACCTCAACAAGAGACAGAAATTTCAT	223
Corvus corone corone	269	.--.-.---...---....G.-.....-.-.....C.....-.....C...	223
Corvus macrorhynchos japonensis	269	.--.-.---...---....G.-.....-.-.....C.....-.....C...	223
Corvus frugilegus	269	.--.-.---...---....G.-.....-.-.....C.....-.....C...	223
Agelaius phoeniceus	255	.--.-T---....--....-.-.....-.-T.....A.....	208
Emberiza stewarti	253	.--.-T---....--....-.-.....-.-T.....A.....	206
Emberiza hortulana	253	.--.-T---....--....-.-.....-.-T.....G.....A.....	206
Emberiza citrinella	252	.--.-T---....--....-.-..A.....-.-T.....A.....	205
Oporornis philadelphia	248	.--.-.---G..G--....-.-.....-.-T.....A.....	201
Oporornis tolmiei	248	.--.-.---G..G--....-.-.....-.-T.....A.....	201
Emberiza calandra	258	.CA.A.TTT....--....-.-.....-.-T.....C.....T.....	205
Emberiza cirlus	249	.--.-T---....--....-.-.....-.-T.....A.....	202
Ficedula albicollis	192	.--.-.---....--....-.-...G....-.-.....A.....	145
Ficedula hypoleuca	192	.--.-.---....--....-.-...G....-.-.....A.....	145
Ficedula parva	192	.--.-.---....--....-.-.....-.-.....	145
Sialia sialis	163	.--.-.---..A.AT....-.-.....-.-.....T...G.....	114
Sialia mexicana	163	.--.-.---..A.AT....-.-.....-.-.....T...G.....	114
Perisoreus infaustus	157	.--.-.---...---....-.G.....-.-.....C.....-.....C...	111
Erithacus rubecula	161	.--.-.---....--....-.-.....-T-.....G.....	114
Corvus corax	157	.--.-.---...---....-.G.....-.-.....C.....-.....C...	111
Tiaris olivacea	157	.--.-.---....--....-.-.....-.-T...C.....A.....	110
Prunella modularis	160	.--.-.---....--T...-.-.....-.-C.....A...A.....	113
Loxigilla portoricensis	158	.--.-.---.C..--....-.-.....-.-T.....A.....	111
Emberiza citrinella	157	.--.-T---....--....-.-..A.....-.-T.....A.....	110
Troglodytes aedon	162	.--.-.---....--....-.-.....T.-.....T.T.....	114
Cardinalis cardinalis	160	.--.-.---....--...C-.-.....G..-.-T.....G...A.....	113
Loxigilla portoricensis	158	.--.-.---.C..--....-.-.....-.-T.....A...C.....	111
Coereba flaveola	159	.--.-.---....--....-.-.....-.-T.....A.....G.	112
Emberiza leucocephalos	159	.--.-.---....--....-.-.....-.-T.....A.....	112

Appendix 1: (cont.)

Buphagus erythrorhynchus	222	AACCTCTGGACAATCTG-TTCCAGTGCTTTGACACCCTCGCAGTAAAGAAAAA-GAT-TT	167
Corvus corone corone	222	.............C...-.....................A...C....-....-...-..	167
Corvus macrorhynchos japonensis	222	.............C...-.........C.........A...C....-....-...-..	167
Corvus frugilegus	222	.............C...-.........C.........A.......-....-...-..	167
Agelaius phoeniceus	207	..........A..C...-.....................A.............-A.A-AA	151
Emberiza stewarti	205	..........A..C...-.....................A.............-A.A-AA	149
Emberiza hortulana	205	..........A..C...-.....................A.............-A.A-AA	149
Emberiza citrinella	204	..........A..C...-.....................A.............-A.A-AA	148
Oporornis philadelphia	200	..........A..C...-......A.............A.............-A.A-AA	144
Oporornis tolmiei	200	..........A..C...-......A.............A.............-A.A-AA	144
Emberiza calandra	204	..........A..C...-.....................A.............--.--AC	150
Emberiza cirlus	201	..........A..C...-.....................A.............-A.A-AA	145
Ficedula albicollis	144	................-.....................A.........-------.-..	94
Ficedula hypoleuca	144	................-.....................A.........-------.-..	94
Ficedula parva	144	................-......A.............A.........-------.-..	94
Sialia sialis	113	................G.....A....C......T...AT.............-...-..	56
Sialia mexicana	113	................G.....A....C......T...AT.............-...-..	56
Perisoreus infaustus	110	.............C...-.........AC..........A.............--...-..	55
Erithacus rubecula	113	................-.....................A.............-...-..	57
Corvus corax	110	.............C...-.........C.........A.............--...-..	55
Tiaris olivacea	109	..........A..C...-.....................A.............-A.A-A.	53
Prunella modularis	112	...A......A..C...-...................AT..A.............-A.A-A.	56
Loxigilla portoricensis	110	..........A..C...-.....................A.............A..A-..	53
Emberiza citrinella	109	..........A..C...-.....................A.............-A.A-A.	53
Troglodytes aedon	113	.............C...-..G..C...............A.............-A.A-A.	57
Cardinalis cardinalis	112	..........A..C...-...................T...A.............--..AAA	56
Loxigilla portoricensis	110	..........A..C...-.....................A.............A..A-..	53
Coereba flaveola	111	..........A..C...-.....................A.............-A.A-AA	55
Emberiza leucocephalos	111	..........A..C...-.....................A.A.............-A.A-AA	55

Buphagus erythrorhynchus	166	-T-TTAA-TATTCAAGATCAAAGCTTCTTAGCTGCTATTACCAGCAAGCTTCTTACCTGA	109
Corvus corone corone	166	-.C....-.....................................T...........	109
Corvus macrorhynchos japonensis	166	-.C....-.....................................T...........	109
Corvus frugilegus	166	-.C....-.....................................T...........	109
Agelaius phoeniceus	150	-.---..-.............................T.......T...........	96
Emberiza stewarti	148	-.-.-..-.............................T.......T...........	93
Emberiza hortulana	148	-.-.-..-.............................T.......T...........	93
Emberiza citrinella	147	-.---..-.............................T.......T...........	93
Oporornis philadelphia	143	-.-.-..-..C..........................TT.C....T...........	88
Oporornis tolmiei	143	-.-.-..-..C..........................TT.C....T...........	88
Emberiza calandra	149	-A-....-.............................T.......T...........	93
Emberiza cirlus	144	-.-.-..-..........----................T.......T...........	93
Ficedula albicollis	93	-.-....-........G.....................................T...........	37
Ficedula hypoleuca	93	-.-....-........G.....................................T...........	37
Ficedula parva	93	-.-....-..G.....G.....................................T...........	37
Sialia sialis	55	-.-.C..-.G.....................AT..............T.........	1
Sialia mexicana	55	-.-.C..-.G.............T...AT..............T.........	1
Perisoreus infaustus	54	-.C....-.....................................T.......	1
Erithacus rubecula	56	-.-.A..A.....................A.............T.....T.........	1
Corvus corax	54	-.C....-.....................................T.......	1
Tiaris olivacea	52	-.-.C.--.....................................T.......	1
Prunella modularis	55	-.-C...-.............T..T.....................T.........	1
Loxigilla portoricensis	52	-C---.--.............................C.........T.........	1
Emberiza citrinella	52	-----..-.............................T.......T.........	1
Troglodytes aedon	56	G.-....-.............G...........C.............T.........	1
Cardinalis cardinalis	55	-.-....-..........G.....................................T.........	1
Loxigilla portoricensis	52	-C---.--.............................C.........T.........	1
Coereba flaveola	54	-.-.C.--.....................................T.........	1
Emberiza leucocephalos	54	-.-.-..-.........................T.....TT......T.........	1

Appendix 1: (cont.)

Buphagus erythrorhynchus	108	AATGGGAATTGACGATATTTCAGATATTCTGCCAAGATGTCCAGCATCCTTACCATCTGA	49
Corvus corone corone	108	.....G.....T	49
Corvus macrorhynchos japonensis	108	.....G.....T	49
Corvus frugilegus	108	.....G.....T	49
Agelaius phoeniceus	95	.....A.....G.....G.....T	36
Emberiza stewarti	92	.....A.....G.....G.....T	33
Emberiza hortulana	92	.....A..C..G.....G.....T	33
Emberiza citrinella	92	.....A.....G.....G.....T	33
Oporornis philadelphia	87	.....A.....G.....T	28
Oporornis tolmiei	87	.....A.....G.....T	28
Emberiza calandra	92	.....A.....G.....G.....T	33
Emberiza cirlus	92	..C..A.....G.....G.....T	33
Ficedula albicollis	36	.....C.....	3
Ficedula hypoleuca	36	.....C.....	3
Ficedula parva	36	.....C.....	3

Buphagus erythrorhynchus	48	GAAAAAATCAGGACTCTGTTGCCGCGTTCTCGTAGACGAATCAGTAA	1
Corvus corone corone	48	..G.....A.....	2
Corvus macrorhynchos japonensis	48	..G.....A.....	2
Corvus frugilegus	48	..G.....A.....	2
Agelaius phoeniceus	35	.....A.....	1
Emberiza stewarti	32	.....A.....	1
Emberiza hortulana	32	.....A.....	1
Emberiza citrinella	32	.....A.....	1
Oporornis philadelphia	27	.....A...	1
Oporornis tolmiei	27	.....A...	1
Emberiza calandra	32	.....A.....	1
Emberiza cirlus	32	.....A.....	1