Short communication

Molecular identification of two threatened pigeon species (Columbidae) using faecal samples

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Bolle's Laurel Pigeon *Columba bollii* and the White-tailed Pigeon *C. junoniae* are two endangered endemic frugivorous pigeons from the relict laurel forest of the Canary Islands (Eastern Atlantic Ocean, Spain). These two columbids probably evolved in different habitats (laurel and thermophilous forest, respectively), but the almost total destruction of these latter forests could force the White-tailed Pigeon to occupy laurel forest areas (Martín *et al.* 2000). In 1985, the total population sizes were estimated at 1160–1315 and 1200–1480 birds, respectively (Emmerson 1985). Once widespread on the archipelago, their current distribution and abundance are affected by habitat destruction, illegal hunting and predation by rats (Hernández *et al.* 1999, Martín *et al.* 2000), which has led to them being listed as Near Threatened (*C. bollii*) and Endangered with a decreasing trend (*C. junoniae*; IUCN 2007). The threatened status of these Columbidae species and their elusive behaviour makes it difficult to observe and handle individuals. Therefore, non-invasive samples collected from the field are required to carry out ecological studies (Piggott & Taylor 2003).

Faecal samples provide a valuable source for analysing diet and distribution, but previous reliable identification of faeces is required in order to identify the target species. This task can be more complex when congeneric species are present in sympatry, and when their faeces cannot be distinguished based on morphological characteristics. In

this situation, faecal DNA analysis will permit correct assignment at species level. Molecular studies for identification of congeneric and sympatric species from faecal samples have been developed for mammals (Farrell *et al.* 2000, Palomares *et al.* 2002), but as far as we know, no studies have been carried out on birds. Faeces are morphologically indistinguishable between pigeon species because they have similar body sizes and probably share similar food resources (fruits and leaves). Molecular techniques provide a potential method of identification. The main aim of this study was to identify the faecal samples of two sympatric *Columba* species from the Canarian Archipelago.

To achieve this, we sequenced the whole control region of the mitochondrial genome in order to define the genetic variation and then identified intra- and inter-specific polymorphisms in these pigeon populations. On the basis of these sequence data, faecal samples were distinguished by restriction fragment length polymorphism (RFLP). This technique is cheaper and more rapid than sequencing PCR products (Paxinos *et al.* 1997, Hansen & Jacobsen 1999).

METHODS

In order to determine the exact sequences of the hypervariable control region (CR) and flanking tRNAs (tRNA-Glu and tRNA-Phe) in mitochondrial DNA (mtDNA), we initially extracted muscle tissue DNA from carcasses of both pigeon species by a standard proteinase K–phenol–chloroform protocol (Gilbert *et al.* 2004). DNA amplification was performed by using a Long Expand high-fidelity PCR system (Boehringer Mannheim, Germany) and highly conserved primers (ND6, 5'-CCCAAAAAAAGCACAAATAAGT-CAT-3'; CL4, 5'-CTTGGCATCTTCAGTGCCATGCT-3') designed (Thompson *et al.* 1994) from the alignment of bird sequences available in the GenBank by the Clustal W program. PCR conditions were 35 cycles of 45 s at 94 °C, 45 s at 60 °C and 2 min at 72 °C. The total length of the sequences was 1902 bp for *C. bollii* and 1896 bp for *C. junoniae*. These sequences were deposited in GenBank with accession numbers DQ229348 (*C. bollii*) and DQ229349 (*C. junoniae*).

In the previously amplified CR sequences, domains I and II contain the main mutations that define the haplotype diversity between species and individuals (Brown *et al.* 1986). We determined the genetic diversity at inter- and intraspecific levels of the pigeon populations, via DNA extracted from moulted tail feathers (the species are readily distinguished by their different colour patterns), for different individuals of each species (Gilbert *et al.* 2004). Internal primers were: CR3F, 5'-CCCCCTGCACITGTGTCTTA-3'; CR2R, 5'-CGATCACGGACTGAAATGGTG-3'. PCR conditions were an initial denaturation cycle of 94 °C for 2 min, followed by 35 cycles of 15 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C. All reactions ended with a final extension cycle at 72 °C for 5 min. A fragment of 637 bp for *C. bollii* and 638 bp for *C. junoniae* was amplified, which

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corresponded to sequences in domains I and II of the CR.

As the faeces often contain small amounts of degraded DNA, Eggert *et al.* (2005) recommended amplifying short DNA fragments (approximately 100–300 bp). Therefore, taking into account the previously identified inter- and intraspecific variation, a second set of internal primers within 637- to 638-bp fragments were designed to amplify shorter fragments of 196 bp for *C. bollii* and 197 for *C. junoniae*. RA3 (5'-AAACCAGCAACTCGACGCGAGA-3') and CR2R were at conditions of 35 cycles of 10 s at 94 °C, 15 s at 58 °C and 15 s at 72 °C.

DNA was extracted from faecal samples using guanidium thiocyanate (GuSCN), a strong chaotropic agent often used as protein denaturant in cellular lysis (Boom *et al.* 1990). However, we found that the main problem in DNA amplifications of faecal samples was not DNA yield, but the *Taq* polymerase inhibition observed from the lack of primer dimer formation in several experimental reactions; these dimers were present in the negative controls. Endemic Canary Island pigeons are probably highly frugivorous, although their feeding ecology is poorly known (Martin *et al.* 2000). In fact, the Madeira Laurel Pigeon *C. trocaz*, which is closely related to Bolle's Laurel Pigeon, consumes fruits and vegetation according to temporal variations in food resource availability (Oliveira *et al.* 2002, Marrero *et al.* 2004). Therefore, their faeces may contain plant inhibitors in quantities sufficient to inhibit PCR reactions (Khanuja *et al.* 1999). Silica particles were added to capture the low amount of faecal DNA and successive washes with different organic solvents such as

acetone or chloroform were performed to reduce the effect of the inhibitors. When this problem still persisted, DNA extracts were diluted and the amount of *Taq* DNA polymerase and/or bovine serum albumin was increased in the PCR reactions (Eggert *et al.* 2005).

Finally, as a considerable quantity of faecal samples should be collected to describe the trophic ecology of species correctly, we developed a diagnostic RFLP test to reduce sequencing costs and time. On the basis of alignment of the shorter sequences (196–197 bp) from different individuals of both species, restriction enzymes were tested by the GeneRunner program (Hastings Software, Inc.). The enzyme *Hae*III (5'-GG/CC-3') gave the simplest and most suitable restriction pattern, which clearly identified the extracted DNA from faeces of *C. bollii* and *C. junoniae*, with regard to haplotype diversity. *Hae*III has two restriction sites in the *C. bollii* sequence and one cut site in *C. junoniae* (Fig. 1). Fresh faecal samples were collected from the same areas as feathers (laurel forest of Los Tiles, La Palma Island). To reduce pseudoreplication, transects and water points were sampled once and both samples (feathers and faeces) were individualized in plastic bags for later study.

Wildlife rehabilitation centres on the islands supplied labelled faeces and feathers from three individuals of *C. bollii* and five of *C. junoniae*, which were temporarily kept in captivity. The identity of these known samples was corroborated by the molecular method described above, providing positive controls to the test; the absence of contamination was tested by use of a negative control. The digested products were analysed by electrophoresis

<i>Columba bollii</i>	AAACCAGCAACTCGACGCGAGAAGTATCCATCAGGACTAG	(40)
<i>Columba junoniae</i>		
<i>Columba bollii</i>	CTTCAGGCCATTCTTTCCCCCTACACCCTAGCAGACTT	(80)
<i>Columba junoniae</i>	A....	
	<i>Hae</i> III	
<i>Columba bollii</i>	GCTCTTTGCGCCTCTGGTTCCTATGTCAGGGCCATAAGC	(120)
<i>Columba junoniae</i>	CG.....AA.....C.	
	<i>Hae</i> III	
<i>Columba bollii</i>	TTGCCAAATCCCAT-GACCTCGCTCTTCACAGATACATCT	(160)
<i>Columba junoniae</i>	• GTTG • C • • T • CA • • • • T • C • • • • • • • • • •	
<i>Columba bollii</i>	GGTCGGGGTCATACCTCACCATTTCAGTCCGTGATCG	(197)
<i>Columba junoniae</i>	T.....	

Figure 1. Fragments of 196–197 bp of mtDNA control region (CR) sequences of Bolle's Laurel Pigeon *Columba bollii* and White-tailed Pigeon *C. junoniae* from the Canary Islands. *Hae*III enzyme-restriction sites (5'-GG/CC-3') are underlined and identified. A dot indicates the same base as on the upper line; a dash indicates an alignment gap.

Table 1. Intraspecific DNA sequence variation in 637- to 638-bp PCR products of the CR (domains I and II) and the corresponding haplotype frequencies of Bolle's Laurel Pigeon and White-tailed Pigeon from La Palma, Canary Islands. The primer pair used was CR3F/CR2R (sequences described in the text).

Haplotype	<i>C. bollii</i>					<i>C. junoniae</i>				
	Variable site location					Variable site location				
	2	3	4	4	–	1	2	4	4	–
	2	8	4	6	–	9	3	2	3	–
	6	3	7	4	–	1	5	6	5	–
I	A	A	C	T	24	A	G	C	A	33
II	G	.	.	.	44	G	.	T	.	8
III	.	G	.	.	42	.	A	.	G	1
Total					110					42

Figure 2. *HaeIII* restriction enzyme-digested 196- to 197-bp PCR products amplified with primer pair RA3/CR2R (sequences described in the text) from the mtDNA control region (CR). Three restriction products (47, 65 and 84 bp) were obtained for Bolle's Laurel Pigeon (B) and two (47 and 150 bp) for White-tailed Pigeon (J). DNA was electrophoresed in a 10% acrylamide gel. M, 50-bp DNA marker; PB and PJ, positive controls of known faecal sample DNA from individuals in captivity (Bolle's and White-tailed Pigeons, respectively); N, negative control.

through a 10% acrylamide gel followed by ethidium bromide staining.

RESULTS AND DISCUSSION

DNA samples from 152 feathers (110 from *C. bollii* and 42 from *C. junoniae*) were amplified using the 637- to 638-bp fragments. Nucleotide diversity (Rozas *et al.* 2003) between the two species was 0.16 ($se = \pm 0.07$). Intraspecific haplotype diversity for *C. bollii* and *C. junoniae* (Table 1) shows that these two pigeons have identical values (0.002 ± 0.001), and correspond to those found for other bird species (Johnson & Clayton 2000, Primmer *et al.* 2002).

For the DNA extracted from 28 faecal samples, RFLP analysis of 196- to 197-bp PCR products with *HaeIII* resulted in two obviously different intraspecific banding

patterns. Two restriction products of 47 and 150 bp were present in *C. junoniae* and three of 47, 65 and 84 bp in *C. bollii* (Fig. 2). Faecal sample identification resulted in 70% *C. bollii* and 30% *C. junoniae*. This was very similar to the frequencies obtained for feathers collected in the field (72.4% *C. bollii* and 27.6% *C. junoniae*; $G_1 = 0.010$, $P = 0.91$). Control samples from the eight captive birds were identified with a success rate of 100%, coinciding with that of the feathers in each case.

Faecal samples of both endemic pigeon species in the Canary Islands, sharing the same laurel forest areas, were successfully identified by their *HaeIII* restriction profiles of PCR-amplified products. As far as we know, this is the first case of two congeneric sympatric bird species being identified via molecular analysis of their faeces. These two species are of conservation concern (IUCN 2007), yet due to their arboreal habits and cautious nature, their ecology is poorly understood. The application of the present methodology, based on the use of non-invasive samples, could provide the basis for further studies of these species, for example through mapping species ranges, identification of key habitats, studies of trophic ecology and analyses of genetic variability. More generally, the protocol presented in this study can be used in a broader context for other congeneric vertebrate species.

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