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Short communication

The development of a molecular assay to distinguish droppings of black grouse *Tetrao tetrix* from those of capercaillie *Tetrao urogallus* and red grouse *Lagopus lagopus scoticus*

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Counts of faecal droppings are a useful approach for the non-invasive monitoring of species presence and abundance. However, for game birds, it is often difficult to determine unequivocally the species origin of the droppings. Here, we compare the utility of two molecular approaches which exploit variation in the cytochrome b region of mitochondrial DNA to distinguish the droppings of black grouse *Tetrao tetrix* from those of red grouse *Lagopus lagopus scoticus* and capercaillie *Tetrao urogallus*. A sensitive detection method is required as DNA extracts from droppings tend to yield poor quality DNA in low copy number. The first approach adopted a real-time PCR method in which primers were used to amplify a small fragment in the mitochondrial cytochrome b region, and fluorogenic probes complementary to species-specific SNPs were designed. The success of this test was compared with that of conventional, end point PCR followed by DNA sequencing of a 346 bp mitochondrial cytochrome b region containing the shorter sequence used in the real-time PCR test. In samples which produced results with both the real-time and sequencing approaches, the results were always in agreement. However, the sequencing approach, when used in conjunction with hot-start Taq PCR, proved superior as it worked in a greater number of samples than the real-time method. The length of clear sequence generated allowed secure identification based on several species-specific SNPs. The development of a molecular approach based on a commercially available DNA extraction kit followed by off-site sequencing now offers a secure method of identifying the species origin of field-collected grouse droppings and requires only basic knowledge of molecular techniques and inexpensive molecular equipment.

Key words: black grouse, cytochrome b, DNA, droppings, hot-start PCR, real-time PCR, sequencing, species identification, Tetrao tetrix

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The black grouse *Tetrao tetrix* is a large game bird closely related to the capercaillie *Tetrao urogallus*. Although widespread in Britain until the latter half of the 19th century, it is now one of the most rapidly declining bird species in the UK with a distribution confined to some upland areas of Scotland, Wales and the North of England (Gibbons et al. 1993). Black grouse has now been identified as a species of high conservation concern in the UK and conservation activities have centred on improving black grouse habitat. This has involved activities to encourage black grouse such as reducing herbivore

impacts on moorland edges, as well as thinning trees in commercial plantations to create patchy forest edges and more open ground. Attention has also been focussed on understanding whether techniques such as heather burning and cutting can benefit this species.

Monitoring the success of these conservation activities is difficult because black grouse are both rare and reclusive. To date, population monitoring relies on counting males during the display period when they are at their most visible (Hancock et al. 1999). However, this is time-consuming and the number of actively displaying males can vary with time of day and weather (Cayford & Walker 1991). An alternative approach is to count the faecal droppings of black grouse, but this has proven somewhat problematic as it is sometimes difficult to distinguish the droppings of black grouse from those of other tetraonid bird species (Ball et al. 2000). In studies of other species in which similar difficulties in identifying the species origin of samples have been encountered, improvements in the efficiency of DNA extraction have allowed modern molecular methods based on the PCR reaction to be used to improve accuracy of discrimination. These methods have frequently been based on mitochondrial DNA (mtDNA) due to its multiple number of copies per cell and robust circular structure (Mugue et al. 2008). For species discrimination in forensic, food and faecal samples, a range of PCR methods based on species-specific single nucleotide polymorphisms (SNPs) in mtDNA have been used. These include methods based on: species-specific primers (Mugue et al. 2008), universal primers followed by restriction digest (Borgo et al. 1996) or sequencing (Kitano et al. 2007) of the product to reveal species-specific variation, or universal primers combined with species-specific probes in real-time PCR (O'Reilly et al. 2008). The disadvantage of some of these methods is that they rely on gel-based electrophoresis and staining for detection of the PCR product, and therefore, they require a large quantity of PCR product for effective visualisation. This is also a limitation of the sequencing approach which also requires sufficient amplified DNA for generation of a clear sequencing trace. In contrast, the real-time PCR approach is reported to be several orders of magnitude more sensitive (Valasek & Repa 2005). Real-time PCR may, therefore, be an appropriate method for field-collected faecal samples, which tend to yield poor quality DNA in low copy number (Regnaut et al. 2006). The superiority of the real-time PCR approach over conventional gel-based detection methods for poor quality DNA has been demonstrated previously in studies which aimed to distinguish between red fox *Vulpes vulpes* and pine marten *Martes martes* scats. O'Reilly et al. (2008) obtained a 95% rate of successful amplification using real-time PCR, which is much higher than the 53% success rate achieved using a different set of similar samples by Davison et al. (2001) using conventional PCR based methods. However, there are examples where a traditional PCR approach based on universal primers followed by sequencing have been successfully used to identify the species of origin of DNA from faecal samples (Parsons et al. 1999, Verma et al. 2003, Green et al. 2007). Here, we describe the development of a real-time PCR assay to distinguish between faecal droppings from black grouse and two other tetraonid species that can occur sympatrically in the UK; red grouse *Lagopus lagopus scoticus* and capercaillie. We then compare the results from this assay with those obtained using conventional hot-start, end point PCR followed by sequencing. Both assays were designed around species-specific variation in the cytochrome b region of mitochondrial DNA.

Methods and results

In silico search for DNA sequences

Several candidate DNA sequences from a number of mitochondrial regions were obtained for black grouse, red grouse and capercaillie from the NCBI nucleotide sequence database (Available at: http://www.ncbi.nlm.nih.gov/). Homologues were aligned using CAP3 sequence assembly software (Available at: http://deepc2.psi.iastate.edu/aat/cap/cap.html). A region in the cytochrome b sequence was selected which contained species-specific SNPs that allowed common primers and species-specific real-time probes to be designed *in silico* which should enable species identification when applied in practice. The NCBI accession numbers for the aligned sequences are as follows: black grouse (AF230174, EF571183), red grouse (AF230170, EF571187) and capercaillie (AB120132, AB120133, AB120134, AB120135, AB120136, AB120137, AB120138, AB120139, AB120140, AB120141, AB120142, AB120143, AB120144, AB120145, AB120146, AB120147, AB120148, AB120149, AB120150, AB120151, AB120152, AB120153, AB120154, AB120155, AB120156, AB120157, AB120158,

AB120159, AB120160, AB120161, AB120162, AB120163).

DNA sequencing, primer and probe design

To assess whether intra-specific variation could confound the species-diagnostic assay, we sequenced the cytochrome b region in a further 11 black grouse (seven from the UK, two from Finland and two from Sweden), 10 red grouse (from the UK), three Swedish willow grouse *Lagopus lagopus* and one capercaillie (from Germany). For the sequencing exercise, DNA was extracted from muscle or feathers using a Qiagen Tissue Extraction kit (Qiagen Inc.) according to the manufacturer's instructions for muscle samples, and according to Segelbacher (2002b) for feathers. A 346 bp region of the cytochrome b gene was PCR amplified using the primers 5'-cttcagtmgccacacatgc-3' and 5'-aagaatcgggtaagggttgg 3'. The PCR was carried out in a total volume of 20 µl containing 1µl of DNA (20 ng). The reaction mix contained 10 mM KCl, 10 mM $(NH_4)SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1 % Triton X-100 at pH 8.8, 0.2 mM of each dNTP and 0.5 unit Taq polymerase (New England Biolabs, UK). Primers were at a concentration of 0.4 µM. PCR conditions were: 94°C for 3 minutes followed by 33 cycles of 94°C for 40 seconds, 55°C for 30 seconds and 72°C for 30 seconds. A final extension step of 72°C for 6 minutes was then carried out. An aliquot of PCR product was checked on an agarose gel and if a single clean band of appropriate size was obtained the sample was sequenced off-site on an ABI3730 using a BigDye v3.1 kit (The Gene Pool, School of Biological Sciences, University of Edinburgh, Edinburgh, UK).

The sequences obtained were high quality and have been deposited on the NCBI database with the following accession numbers: black grouse (EU590829, EU590830, EU590831, EU590832 EU590833, EU590834, EU590835 EU590836, EU590837, EU590838, EU590839), red grouse (EU590841, EU590842, EU590843, EU590844, EU590845, EU590846, EU590847, EU590848 EU590849) and capercaillie (EU590850). The three willow grouse sequences were homologous to the red grouse in the region used for primer and probe design in the real-time test (EU672842, EUEU672843, EU672844).

These new sequences and those previously used for the *in silico* design of the real-time PCR test were aligned using CAP3 software. These aligned sequences contained species-specific SNPs of which six, five and two were specific to black grouse, red grouse and capercaillie, respectively (Appendix 1). No intraspecific variation was apparent in the region used for the real-time PCR test among the 13 black grouse, 12 red grouse, or 33 capercaillie sequences that were aligned. Primers and probes for use in the real-time PCR test were designed using Primer Express Version 3 software (Applied Biosystems, UK). The primers (Primer pair 1 = F 5'-gcaaacggcgcttcattc-3' and R 5'-tttctttrtayarrtaggagccgtagt-3') amplified an 85 bp region containing the species-specific SNPs located within the original 346 bp sequenced fragment (Fig. 1). The reverse primer contained four degenerate bases to allow it to amplify DNA from the three study species. A second set of primers (Primer pair 2 = F 5'-gcaaacggcgcttcattc-3' and R 5'-agattactcctgtgtttcaggttt-3') which amplified a 110 bp region was also tested (see Fig. 1). This primer pair contained no degenerate bases and was designed to amplify DNA from black and red grouse, but not capercaillie. The species-specific TaqMan® MGB probes (Applied Biosystems, UK) were designed and fluorescently labelled as follows: black grouse 6FAM - CTT CAT CTG TAT TTT C, red grouse VIC - TTT ATC TGC ATT TTC CTC, and capercaillie NED - CTT CAT CTG CAT TTT C. A second probe for red grouse (Probe II), VIC - CTT TAT CTG CAT TTT C, was also tested in an attempt to improve stringency. The real-time PCR reactions were performed in Microamp Optical 96-well reaction plates using an ABI Prism 7300 sequence detection system and the following Applied Biosystems default PCR programme: 2 minutes at 50°C, 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. PCR reactions were set up as follows: 2x TaqMan Universal Mastermix (Applied Biosystems), 0.2 µM each of forward and reverse primer, 0.2 µM each of black grouse, red grouse and capercaillie probe and 5 µl diluted DNA in a final volume of 25 µl. When the degenerate reverse primer was used, the concentration of this primer added to the reaction was increased by a factor of five. Filtered pipette tips were used throughout to reduce the potential for sample cross-contamination through aerosol.

The real-time-assay was initially tested using primer pair 1 on DNA extracted from tissues of black grouse (N = 11), red grouse (N = 10) and capercaillie (N = 1) and mixtures containing equal amounts of DNA from two or three of the test species. In every case where only black grouse DNA was present in the test, the species was correctly identified based on the accumulation of fluorescence

Figure 1. The relative locations of the primers and probes used to identify species-specific SNPs in black grouse (AF230174), red grouse (AF230170) and capercaillie (AB120132). Red I and Red II represent two separately designed probes for red grouse. The species-specific SNPs are indicated in bold, the probe sequences are highlighted in grey and the forward and reverse primers are boxed.

from the black grouse probe. There was no accumulation of fluorescence from the red grouse or capercaillie probes in the presence of only black grouse DNA. Similarly, when red grouse DNA alone was present, there was only fluorescence from the red grouse probe. However, with capercaillie DNA alone, there was a rise in the fluorescence of both the capercaillie and the red grouse probes, but there was no rise in the fluorescence from the black grouse probe. The black grouse and capercaillie probes were therefore species-specific, but the probe designed for red grouse fluoresced in the presence of both red grouse and capercaillie DNA. Attempts to redesign the red grouse probe to make it more specific were unsuccessful in resolving the lack of specificity (see Fig. 1, probe Red II). When using primer pair 1, the rise in the fluorescence was slow so that the Ct threshold (i.e. the point when fluorescence surpasses a set baseline) was crossed relatively late (35 cycles).

As the test failed to differentiate between red grouse and capercaillie, we tested a second primer pair (primer pair 2), which contained no degenerate bases and which was specifically designed to differentiate black grouse from red grouse DNA. Although this primer pair did not increase the number of species identifications achieved, the development of fluorescence occurred much faster and the Ct threshold was crossed much earlier than with primer pair 1 (31 cycles). However, when this primer pair was tested with capercaillie DNA, once again there was non-specific amplification and fluorescence was observed from both the red grouse and capercaillie probe. This was despite the fact that neither the reverse primer sequence nor the red grouse probe was exactly complimentary to the capercaillie DNA primer and probe site. However, given that the underlying aim of the assay was to allow unambiguous identification of black grouse droppings, a lack of specificity between red grouse and capercaillie was relatively unimportant.

DNA extraction of droppings for validation of the molecular tests

Ten DNA extractions were performed from 10 fresh droppings of each of the three species. These were obtained from single species enclosures in the Highland Wildlife Park, Kincraig, Kingussie, Invernessshire, Scotland. Eight field-collected droppings of unknown species origin collected from Balmoral Estate, Scotland were also assayed. The Highland Wildlife Park droppings were known to be fresh and were processed within two days. Those from Balmoral Estate appeared to be relatively fresh and DNA was extracted from both sets of droppings within two days of collection. Two extraction methods were tested, because the high concentrations of uric acid and other PCR inhibitors contained in bird droppings can result in poor amplification (Segelbacher 2002a). In the first method, DNA was extracted by placing 0.2 g of dropping into a 2 ml eppendorf tube containing 1 ml S.T.A.R (Stool Transport and Recovery) buffer (Roche Ltd). The tube was vortexed for 15 seconds and left at room temperature for at least 30 minutes, after which 100 µl of chloroform was added. Each tube was then vortexed and centrifuged at 1000 g for 1 minute and a sub-sample of the supernatant was transferred into a clean 1.5 ml tube. DNA was then extracted using a Roche High Pure PCR Template Preparation kit following the manufacturer's instructions.

In the second method, the QIAamp DNA stool kit (Qiagen, UK) was used following the protocol of Regnaut et al. (2006). The final elution volume was 200 µl in both methods. The second method was used in all subsequent extractions because it decreased the Ct number by one cycle compared to DNA extracted from the same sample using the first method. The second method also produced a stronger amplication product, which facilitated better sequencing results. For both methods, tubes with no faecal sample were taken through the whole process from extraction to PCR in order to act as negative extraction controls.

Using the Qiagen extraction kit, a further set of 31 field-collected droppings were extracted. These were the product of collections performed once per year by RSPB staff along a transect in Abernethy Forest, Scotland. Consequently, some of these samples could have remained on the forest floor for several months prior to collection. Eleven and 20 samples were analysed from the 2007 and 2008 collections, respectively.

Comparison of real-time PCR vs conventional end point PCR followed by DNA sequencing

In order to compare the real-time PCR approach with that based on conventional end point PCR followed by sequencing, samples of DNA extracted from the Highland Wildlife Park and Balmoral Estate droppings were also subjected to PCR using primer pairs that either amplified the 346 bp or the 110 bp fragments followed by DNA sequencing. The end point PCR reactions for sequencing were set

up using either standard or hot-start Taq polymerase. The PCR reaction using standard Taq was carried out in a total volume of 25 μl. The reaction mix contained 5 μl undiluted DNA sample, 10 mM KCl, 10 mM $(NH_4)SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1 % Triton X-100 at pH 8.8, 0.2 mM of each dNTP and 0.5 unit Taq polymerase (New England Biolabs, UK). For the hot-start Taq PCR reaction, 5 μl undiluted DNA sample, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 0.1% Tween 20, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.5 unit Superhot Taq polymerase (Bioron International) was used. Primers were at a concentration of 0.4 μM in both reactions. For the standard PCR, the cycling conditions were 94°C for 3 minutes followed by 35 cycles of 94°C for 40 seconds, 57°C for 54 seconds and 72°C for 40 seconds. A final extension step of 72°C for 6 minutes was then carried out. The hot-start PCR protocol was the same, but with a 7-minute initial denaturation at 94°C. An aliquot of each PCR product was run on a 1.4 % agarose gel and visualised under UV light. Those samples which produced a single, clean band were sequenced off-site on an ABI3730 using a BigDye v3.1 (The Gene Pool, School of Biological Sciences, University of Edinburgh, Edinburgh, UK).

Amplification of faecal DNA samples prior to sequencing always failed when standard Taq polymerase was used and successful amplification was only observed with hot-start Taq. Clear sequence traces of sufficient quality to enable species discrimination were often achieved when the 346 bp fragment was used (Table 1). In contrast, traces were of poor quality when the short 110 bp fragment was amplified and sequenced. For every extract of fresh droppings taken from captive birds of known species the sequencing approach correctly identified the species of origin. The real-time PCR test also efficiently distinguished black grouse from the other two species. In the field-collected samples from Balmoral Estate of unknown species origin, there was agreement between the real-time PCR and the sequencing approaches which identified that none of the samples were black grouse (see Table 1).

In order to examine the application of these tests on potentially older samples, a further set of 31 field-collected droppings from Abernethy Forest, Scotland, were analysed with both methods. A successful identification was achieved in only slightly over half of these field-collected droppings (see Table 1). The lower percentage of successful identifications in this set compared with the fresher samples tested previously demonstrates that the time from pellet deposition to DNA extraction may be a factor in determining the success of the tests. Of the Abernethy samples, 29% tested using real-time PCR crossed the Ct threshold, whereas 55% provided sequence data of sufficient quality to allow secure species identification. The species identification always agreed across the two test approaches. In addition to identifying the origin of a greater proportion of samples, the sequencing approach had the added advantage of effectively discriminating between red grouse and capercaillie. Where species identification was obtained, the diameter range of the black grouse droppings (6-12 mm) overlapped the size range of those from red grouse (7-8 mm).

Discussion

The observation that the size range of the red and black grouse droppings overlapped demonstrates that size alone cannot be used as an indicator of species origin, and this highlights the need for a molecular approach to tetraonid species identification. Although some authors state that a molecular approach can be prohibitively expensive in terms of time and equipment, the ever decreasing cost of molecular techniques now make this approach a viable option. The cost consists of two major components: the DNA extraction step and the molecular means of species discrimination. Commercial DNA extraction kits have now become mainstream and, although they remain a significant part of the cost, their use is justified by the improvement in consistent quality and yield of DNA they provide. Our study demonstrated that, although the two commercial extraction kits that were tested provided a level of success, the modified protocol for the QIAamp kit in our study generated superior extracts for PCR amplification. For the subsequent species identification stage, the sequencing and real-time approaches do not differ greatly in terms of cost once the probes are developed , and the choice of discrimination method is therefore based on the one which provides the highest success rates and the most reliable results.

A molecular approach to species identification of faecal samples is dependent on a DNA extract of sufficient quality and quantity for further analysis with PCR. With only a few exceptions, a species identification was achieved when fresh droppings were used as the source of DNA, and this supports

Table 1. The size and species identification based on real-time PCR or sequencing of a fragment of the cytochrome b gene in mtDNA (NR = not recorded, bg = black grouse, rg = red grouse, cp = capercaillie).

		Species identification		
Sample number	Diameter of dropping (mm)	Real-time method using primer pair 1	Real-time method using primer pair 2	Sequencing of 346 bp fragment
Balmoral 1	NR	rg/cp	rg/cp	rg
Balmoral 2				
Balmoral 3	NR	rg/cp	rg/cp	rg
Balmoral 4				
Balmoral 5	NR	rg/cp	rg/cp	rg
Balmoral 6	NR	rg/cp	rg/cp	rg
Balmoral 7	NR	rg/cp	rg/cp	rg
Balmoral 8	NR	rg/cp	rg/cp	rg
Abernethy (2007) 1				
Abernethy (2007) 2	7	rg/cp	rg/cp	rg
Abernethy (2007) 3				
Abernethy (2007) 4	7			rg
Abernethy (2007) 5	7	bg	bg	bg
Abernethy (2007) 6				
Abernethy (2007) 7				
Abernethy (2007) 8				
Abernethy (2007) 9				
Abernethy (2007) 10				
Abernethy (2007) 11	9	bg	bg	bg
Abernethy (2008) 1	6			bg
Abernethy (2008) 2				
Abernethy (2008) 3	6			bg
Abernethy (2008) 4	6			bg
Abernethy (2008) 5				
Abernethy (2008) 6	7			bg
Abernethy (2008) 7	7	bg	bg	bg
Abernethy (2008) 8	7			bg
Abernethy (2008) 9	7			bg
Abernethy (2008) 10				
Abernethy (2008) 11				
Abernethy (2008) 12	8			bg
Abernethy (2008) 13	8	bg	bg	bg
Abernethy (2008) 14				
Abernethy (2008) 15				
Abernethy (2008) 16	8	rg/cp	rg/cp	rg
Abernethy (2008) 17	9	bg	bg	bg
Abernethy (2008) 18	10	bg	bg	bg
Abernethy (2008) 19				
Abernethy (2008) 20	12	bg	bg	bg

the findings of Marrero et al. (2008) who published the only other mtDNA-based test for identification of congeneric bird species based on faecal samples. However, when extractions were made from field-collected droppings of uncertain age, the success rate was considerably lower. This is possibly due to a combination of both physical weathering, e.g. UV exposure, rain or warm temperatures (Murphy et al. 2007, Hájková et al. 2006) and enzymatic degradation, e.g. microbial DNase (Regnaut et al. 2006). Therefore, frequent field visits to collect droppings in as fresh a state as possible followed by storage in an ultra-low temperature freezer is recommended in order to maximise the chances of successful PCR amplification (King et al. 2008).

The sequencing approach proved to be more successful than that of real-time PCR both in terms of the number of identifications made and, concomitantly, its ability unambiguously to discriminate all three species. The greater number of identifications achieved via sequencing compared to real-time PCR was an unexpected result, given the reported greater sensitivity of the latter approach (Yang et al. 2006), as well as the difference in size of the amplified

fragment. The sequenced fragment was much longer than that amplified for the real-time PCR test, and this might be expected to amplify less well due to degradation of the DNA in the field-collected samples. However, previous studies have reported that, for fragments of mtDNA <400 bp, there was no effect of length on ability to provide clean sequences (Frantzen et al. 1998). Therefore, length of fragment seems to be less important in determining PCR success in mtDNA compared to nuclear DNA (Frantzen et al. 1998). The reason for the greater proportion of species identifications achieved by the sequencing approach is unclear, but it may be due to several factors. The PCR amplification step was performed at a lower temperature for the sequencing compared to the real-time method, and this may have allowed greater primer binding which, in turn, resulted in more PCR product. The annealing temperature in the real-time PCR reaction was not reduced as this may have lowered the specificity of probe binding. Given that lack of probe specificity was already an issue for identifying red grouse and capercaillie DNA, lowering the annealing temperature was therefore not considered a viable option. It is also possible that the buffer supplied with the hotstart Taq provided greater protection against PCR inhibitors present in the extract than the buffer in the real-time PCR master mix.

The specificity of the probe in the real-time PCR test relies on a maximum of two species-specific SNPs. In contrast, species identification using good quality sequence is based on a greater number of species-specific SNPs thereby allowing very secure identification. Furthermore, the sequencing approach offers the added advantages that it may reveal the cases where the droppings are not from one of the suspected species, and similarly, the method could be applied to a broader range of game birds such as rock ptarmigan *Lagopus mutus* without further development work. The sequence information for the cytochrome b region that is now available for a large number of species should, in many cases, enable the species origin of an unknown scat to be determined (Parson et al. 2000).

The primer/probe combinations used in the real-time test only distinguished DNA of black grouse from that of red grouse and capercaillie and as these two species do not tend to coexist in the same habitat, this lack of specificity may not be a problem. The three willow grouse sequences were homologous to the red grouse sequences used for primer and probe design, indicating that this test could also be useful for distinguishing willow grouse from black grouse in a broader European context. If the application of the real-time test is solely to differentiate black grouse from red/willow grouse, then primer pair 2, designed to amplify only red/willow or black grouse DNA, proved to be the most appropriate choice of primers as these result in faster accumulation of fluorescence so that the Ct threshold is crossed much earlier. This is presumably due to superior amplification of the PCR product resulting from the absence of degenerate bases in primer pair 2.

The real-time probe could unambiguously distinguish black grouse in amplified samples, whereas the red grouse probe failed to differentiate capercaillie from red grouse. The specificity of the black grouse probe may be due to the centrally located base, which was different between black grouse and both red grouse and capercaillie. In contrast, despite the fact that the red grouse and capercaillie probes differed by up to two SNPs, they were unable to differentiate between these two species. The failure of either of the red grouse probes to be species-specific when presented with capercaillie DNA may be explained by the location of the SNPs within the probe. It is recommended that the SNP site is located in the middle third of the sequence or towards the 3' end, but not in the last two bases of the 3' end (Anonymous 2004). As the sequence in the central part of the red grouse and capercaillie probes was identical, the SNPs that distinguished these two species from each other may not have been optimally positioned. Although this particular real-time assay did not distinguish effectively between red grouse and capercaillie, there are other regions of mtDNA which contain species-specific SNPs around which it should be possible to construct probes with centrally positioned SNPs. However, the high cost of fluorogenic labelled probes makes the development of such a test expensive because probes designed *in silico* do not always work in practice.

In conclusion, the sequence information from the 346 bp fragment of the cytochrome b gene allowed the unambiguous identification of the three tetraonid species from amplified DNA extracted from field-collected droppings of unknown age. In comparison to the real-time test, this offers a better approach for identification for these species, and in addition, has the potential to be utilised for the identification of droppings from a number of related species. Both methods require exploratory sequencing to find species-specific SNPs, and this is a relatively inexpensive exercise. However, the construc-

tion of a real-time test requires development and testing of fluorogenic probes, and the expense of these may restrict the extension of this approach to other species. Another advantage of the sequencing approach is that, with the commercial, off-site sequencing services that are now widely available, the molecular laboratory work is relatively simple and can be done using very basic molecular equipment. In contrast, the real-time method requires highly skilled staff and an expensive real-time PCR machine.

The ability to identify black grouse DNA from faecal droppings represents a major step forward in being able to assay the presence of black grouse, and by doing so, allow the effect of habitat management on black grouse presence and retention to be recorded.

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Appendix 1. Cytochrome b sequences in mtDNA of black grouse, red grouse and capercaillie samples showing in grey highlight the location of the primer sites for the 346 bp fragment and as boxed letters the location of the species-specific SNPs.

>gi|6970190|gb|AF230174.1|AF230174 *Tetrao tetrix* cytochrome b (CYTB) gene, partial cds; mitochondrial gene for mitochondrial product

ATCATTACCGGCCTCCTACTGGCCATACATTACACCGCAGACACCTCACTCGCATTCTCTTCAGTAGCCC
ACACATGCCGAAACGTCCAATACGGCTGACTTATCCGCAATCTCCATGCAAACGGCGCTTCATTCTTCTT
CATCTGTATTTTCCTTCACATCGGACGTGGACTTTACTACGGCTCCTACTTATACAAAGAAACCTGAAAC
ACAGGAGTAATCTTACTTCTCACACTCATAGCAACTGCCTTCGTGGGATATGTTCTTCCATGAGGACAAA
TATCATTCTGAGGGGCCACTGTCATTACAAACCTATTCTCAGCAATCCCCTACATTGGACAAACCCTAGT
AGAGTGAGCCTGGGGGGGATTCTCAGTTGATAACCCAACCCTTACCCGATTCTT

>gi|6970182|gb|AF230170.1|AF230170 *Lagopus lagopus* cytochrome b (CYTB) gene, partial cds; mitochondrial gene for mitochondrial product

ATCCTCGCTGGCCTCCTACTGGCCATACACTACACTGCAGACACCTCTCTTGCATTCTCTTCAGTCGCCC
ACACATGCCGAAACGTCCAATACGGCTGACTTATCCGCAATCTCCACGCAAACGGCGCTTCATTCTTCTT
TATCTGCATTTTCCTCCACATCGGACGTGGACTCTACTACGGCTCCTACCTGTATAAAGAAACCTGAAAC
ACAGGAGTAATCTTGCTTCTCACACTCATAGCAACTGCCTTCGTAGGATATGTTCTCCCATGAGGACAAA
TATCATTCTGAGGGGCCACCGTCATTACAAACCTATTCTCAGCAATCCCTTACATCGGACARACCTTAGT
GGAATGAGCGTGGGGGGGATTCTCAGTAGATAACCCAACCCTTACCCGATTCTT

>gi|42557324|dbj|AB120132.1| *Tetrao urogallus* mitochondrial cytb gene for cytochrome b, complete cds

ATGGCACCCAACATCCGAAAATCACACCCCCTTTTAAAAATAATTAACAATTCCCTAATTGACCTCCCCG
CCCCATCCAACATCTCTGCTTGATGAAACTTTGGCTCCCTACTAGCAGTATGTCTTACCACCCAAATCCT
CACTGGCCTCCTATTGGCCATGCACTACACCGCAGACACCTCACTCGCATTCTCTTCAGTAGCCCACACA
TGCCGAAACGTCCAATACGGCTGACTTATCCGTAATCTCCATGCAAACGGCGCTTCATTCTTCTTCATCT
GCATTTTCCTTCACATCGGACGTGGCCTTTACTACGGCTCCTATCTATATAAAGAAACCTGAAACACAGG
AGTAATCCTGCTTCTCACACTCATAGCAACTGCCTTCGTAGGATATGTCCTCCCATGAGGACAAATATCA
TTCTGAGGAGCCACTGTCATCACAAACCTATTCTCAGCAATCCCCTACATTGGACAAACCTTAGTAGAAT
GAGCCTGAGGCGGATTCTCAGTTGACAACCCAACCCTTACCCGATTCTT