



Reliable and robust molecular sexing of the hen harrier (*Circus cyaneus*) using PCR-RFLP analysis of the CHD 1 gene

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3 **Reliable and robust molecular sexing of the hen harrier (*Circus cyaneus*) using**
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5 **PCR-RFLP analysis of the CHD 1 gene**
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ABSTRACT

The hen harrier (*Circus cyaneus*) is a bird of prey that is persecuted in the UK and there is a need for a DNA-based individual identification and sexing system for use in forensic investigations. This study reports a new set of PCR primers for the chromo-helicase-DNA binding protein 1 gene which allows sexing using PCR-RFLP. Instead of exonic primers which amplify across a large intron, this set consists of a primer within the intron, enabling reduction in amplicon sizes from 356 bp to 212 bp and 565 bp to 219 bp in W and Z chromosomes. DNA degradation and dilution experiments demonstrate that this set is significantly more robust than one that amplifies across the intron and sequencing of the intronic primer binding region across several individuals shows that it is highly conserved. Whilst our objective is to incorporate this primer set into an STR-based individualisation kit, it may in the meantime prove useful in forensic or conservation studies.

Keywords: forensic science, wildlife forensics, bird of prey, wildlife persecution, molecular sexing, chromo-helicase-DNA binding protein 1

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3 The hen harrier (*Circus cyaneus*, Falconiformes, Accipitridae, Circinae) has had a history of
4 persecution in parts of the UK because its diet includes red grouse (*Lagopus lagopus*) that is
5 managed on grouse moors for hunting (1,2). Despite being protected under UK legislation,
6 illegal killing of hen harriers continues and numbers remain low in many regions (3). In order
7 to establish a reliable forensic tool for use in cases of illegal persecution, there is a need for
8 a DNA-based individual identification and sexing system. Development and validation of a
9 STR based multiplex is currently underway in this laboratory but we present here a new set
10 of PCR primers which can reliably sex hen harrier samples using PCR-RFLP.
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21 Molecular sexing of non ratite birds has most commonly been achieved using PCR
22 amplification of the sex linked chromo-helicase-DNA binding protein 1 (CHD 1) gene, where
23 primers within conserved exons amplify across introns that differ in size on the avian sex
24 chromosomes (Z and W). Upon size separation, females being heterogametic (ZW), display
25 two bands of different sizes while males being homogametic (ZZ), display only one band.
26
27 The P2/P8 (4) and the 1237L/1272H (5) primer sets amplifying across a CHD 1 intron have
28 been used successfully to sex species across several avian orders. However, Ito et al. (6)
29 reported that amplification across this intron failed to produce size differences in six species
30 of raptors within Accipitridae, including the marsh harrier (*Circus spilonotus*), a species
31 closely related to the hen harrier. We consequently used primers 2550F and 2718R
32 described by Fridolfsson & Ellegren (7) which have also been successfully used to sex
33 diverse species from 11 avian orders and amplify products of 400-450 (W) and 600-650 bp
34 (Z) across a different CHD 1 intron. After sequencing homologous hen harrier sequences,
35 we redesigned a set of primers (one within a highly conserved region of the intron) that
36 amplifies smaller W (212 bp) and Z (219 bp) fragments. Due to smaller sizes of products,
37 this new primer set is significantly more robust for sexing hen harrier samples containing
38 degraded and/or very low amounts of DNA such as those likely to be encountered in forensic
39 casework. We demonstrate this by carrying out DNA degradation and dilution experiments
40 followed by PCR. We also evidence that the intronic primer binding region is conserved in
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3 hen harriers across a wide geographical range within the UK by carrying out sequencing of
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5 several hen harrier individuals across this region.
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8 9 **Methods**

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11 DNA was extracted using 25 mg of muscle tissue from a male and a female bird that had
12
13 died of natural causes in England using the QIAamp DNA Mini kit (QIAGEN, Hilden,
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15 Germany) following manufacturer's instructions, and RNA removed by the addition of excess
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17 RNase A (5 µl of a 10mg/ml solution of ≥70 Kunitz units/mg protein, Sigma-Aldrich, St.
18
19 Louis, MO, USA) into 100 µl of DNA solution followed by incubation at 37 °C for 30 min.
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21 RNA-free genomic DNA was then purified using a QIAquick column (QIAGEN) following
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23 manufacturer's instructions and quantified using a UV spectrophotometer for use in the
24
25 degradation and dilution experiments described below. DNA from a number of buccal swabs
26
27 taken from male and female nestlings by licensed professionals during routine monitoring
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29 exercises at locations across England, Wales, Scotland and the Isle of Man was extracted
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31 using the QIAamp DNA Micro kit optimised for small sample amounts (QIAGEN) following
32
33 manufacturer's instructions and quantified using 1 % ethidium bromide stained agarose gels
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35 containing known concentrations of lambda DNA (Promega, Madison, WI, USA) . Gel based
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37 quantification was used for these samples since they were not used for the degradation and
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39 dilution experiments and were quantified for PCR purposes only.
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44 PCRs were carried out in a 10 µl volume containing 10-20 ng DNA (except dilution series),
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46 3.0 mM MgCl₂, 1X ReddyMix PCR Master Mix (Thermo Fisher Scientific, Waltham, MA,
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48 USA). PCR cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed
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50 by 35 cycles of 94°C for 30 s, 52°C for 60 s (30 s for 212/219 bp products) and 72°C for 90 s
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52 (30 s for 212/219 bp products), followed by a final extension at 72°C for 10 min. Agarose gel
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54 electrophoresis (2 %) and ethidium bromide staining was subsequently carried out to
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56 visualise the products. PCRs were scaled up to 25 µl when purified products were required
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58 for sequencing. Male PCR products were purified using the QIAquick PCR Purification Kit
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3 (QIAGEN) while for female samples, the Z and W amplicons were separated on a 3 %
4 agarose gel followed by ethidium bromide staining, excision of bands, and purification of
5 DNA using the QIAquick Gel Extraction Kit (QIAGEN). Sequencing was carried out using an
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7 ABI3500 using BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems, Carlsbad,
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9 CA, USA).
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15 Quantified DNA from male and female tissue samples was artificially degraded by setting up
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17 20 µl reactions at 37 °C containing ~ 900 ng DNA, 1 unit DNase I (Applied Biosystems) and
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19 1 X DNase I buffer. A time series was carried out for 0, 5, 10, 20, 30, 60, 90 and 180 min (no
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21 DNase added to the 0 min sample). Reactions were terminated by adding 0.1 volume DNase
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23 inactivation reagent (Applied Biosystems), incubated for 5 min at room temperature,
24
25 centrifuged at 10,000 x g for 1.5 min, and the supernatant transferred to a fresh tube.
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27 Degraded samples were then diluted to 20, 10, 5, 2, 1, 0.5, 0.25 and 0.1 ng/µl and used for
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29 triplicate PCRs followed by 2 % agarose gel electrophoresis.
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33 For RFLP analysis, restriction enzyme digestion was carried out using 8 µl PCR product, 1 X
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35 restriction enzyme buffer and 10 units Sfc I enzyme (New England Biolabs, Ipswich, MA,
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37 USA) followed by incubation at 37 °C for 90 min. The enzyme was then heat inactivated at
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39 65 °C for 20 min prior to 2 % agarose gel electrophoresis.
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42 43 44 **Results and Discussion**

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46 Using the Fridolfsson & Ellegren 2550F and 2718R primers, amplification of the 450 bp W
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48 fragment was consistently observed but amplification of the larger 650 bp Z band was highly
49
50 inconsistent. Hen harrier W and Z fragments were consequently sequenced (GenBank
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52 accession numbers FJ415319 and FJ415320) and species specific primers (*HHCHD1FOR*:
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54 5' AGCAGAGTATCTGAAGTATCG 3', *HHCHD1REV*: 5' TCAATCCCCTTTTATTGATCC 3')
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56 designed in exonic regions. Exon/intron boundaries were defined using homologous
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58 sequences from marsh harrier (GenBank accession numbers AB112946 and AB112954).
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3 These new primers, amplifying products of 356 and 565 bp in W and Z genes respectively,
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5 resulted in consistent amplification of both fragments in good quality samples. However, in
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7 forensic samples which have degraded and/or very low amounts of DNA, amplification of
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9 these fragments is likely to be problematic since several studies have shown that there is a
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11 significant negative correlation between maximum amplicon size and the level of
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13 degradation of the sample (e.g. 8,9). Furthermore, Bantock et al. (10) have demonstrated
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15 that reducing fragment size from 300–400 bp using the P2/P8 primer set to 200–250 bp
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17 using new redesigned primers greatly enhanced success rates in sexing museum bird
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19 specimens.
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23 Since redesigning primers in the exonic regions to reduce amplicon size was not possible
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25 due to the large size of the introns in hen harriers (W: 288 bp, Z: 497 bp), homology within
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27 intron sequences was investigated by aligning the Z and W sequences using Bioedit 7.0.5.3
28
29 (11). Sequences were found to be highly divergent except for a region of high homology in
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31 the last ~ 200 nucleotides prior to the binding site of the reverse primer (data not shown). It
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33 was possible to design a new forward primer (*HHCHD1RFLPFOR*: 5'
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35 AGACTGGCAATTACTATATGC 3') within this region which in combination with the
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37 *HHCHD1REV* primer amplified products of 212 and 219 bp in W and Z sequences
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39 respectively. In the *HHCHD1RFLPFOR* primer binding sequence, two substitutions were
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41 observed between the W and Z sequences (G → T at position 7 and A → C at position 13 in
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43 Z sequence, see Fig. 1), but since they are internally situated on the primer, their effects on
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45 PCR amplification are considered minimal, as evidenced in this study and previously
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47 reported by others (12). However, given the intronic location, in order to ensure no additional
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49 substitutions, we sequenced this region in 10 males (ZZ) and 10 females (ZW) from across a
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51 wide geographical range in the UK. Results revealed only these two substitutions across all
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53 individuals, suggesting a highly conserved region (Fig. 1).
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3 To compare amplification of the larger 356 and 565 bp fragments with that of the smaller 212
4 and 219 bp W and Z fragments, a degradation and dilution series of male and female DNA
5 was used for PCR amplification, and results from triplicate PCRs are shown in Table 1. In
6 females, with the *HHCHD1FOR* and *HHCHD1REV* primer set, presence of both 356 and
7 565 bp fragments was scored as a '1' while absence of one or both bands was scored as a
8 '0'. With the *HHRFLPFOR* and *HHCHD1REV* primer set, since the 212 and 219 bp
9 fragments were indistinguishable on a 2 % agarose gel, the presence of a band was scored
10 as a '1' and absence of a band as a '0'. We carried out Mann-Whitney *U*-tests using
11 SPSSv17 (13) to evaluate amplification success between the two primer sets and observed
12 that *HHRFLPFOR* and *HHCHD1REV* performed significantly better than *HHCHD1FOR* and
13 *HHCHD1REV*. The results are summarised in Table 2. When all template amounts and all
14 degradation time points were considered (5 – 180 min i.e. 0 min time point excluded), results
15 were significant when just male data were included, and highly significant when just female
16 or both male and female data were included (test 1). Similar results were obtained when
17 template amounts < 2 ng and all degradation time points (5 – 180 min) were considered (test
18 2). However, when template amounts > 2 ng and all degradation time points (5 – 180 min)
19 were analysed, results were not significant when just male data was included, but significant
20 when just female or both male and female data were included (test 3). This difference
21 between the sexes when higher template amounts are used is most likely because with
22 *HHCHD1FOR* and *HHCHD1REV* primers, amplification of a single band in males is more
23 probable than amplification of two bands in females when DNA is degraded.
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48 Since the 7 bp size difference between the W (212 bp) and the Z (219 bp) fragments is
49 difficult to detect using ordinary agarose gels and any high resolution gel electrophoresis will
50 necessarily require significantly longer running times and/or additional expense, we
51 investigated the possibility of a RFLP within this region using WebCutter 2.0 (© Max Heiman
52 1997). Sfc I enzyme (restriction site: C▼TRYAG) was chosen because it cuts once within
53 the Z sequence resulting in two fragments of 52 and 167 bp, but does not cut within the W
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3 sequence. RFLP patterns should therefore, consist of three bands of 52 bp, 167 bp, and 212
4 bp in females and two bands of 52 and 167 bp in males. In order to confirm that the Sfc I
5 restriction site is conserved across hen harriers from a wide geographic range within the UK,
6 we carried out PCR-RFLP analysis using DNA from 10 male and 10 female individuals (1
7 tissue and 9 buccal swab samples from both sexes) from locations across England, Wales,
8 Scotland and the Isle of Man. RFLP patterns from a few of these individuals are shown in
9 Fig. 2 where three bands of expected sizes in females (lanes 3 & 5) and two bands of
10 expected sizes in males (lanes 7, 9 & 11) are visible. We obtained 100% unambiguous
11 RFLP patterns across all individuals providing good evidence that the Sfc I restriction site is
12 conserved across hen harriers in the UK. Our results also indicate that the two internal
13 substitutions within the *HHRFLPFOR* primer binding sequence do not significantly affect
14 amplification of the 212 and 219 bp W and Z products.
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29 In conclusion, the *HHRFLPFOR* and *HHCHD1REV* primer set described here will be highly
30 useful in sexing hen harriers when samples contain DNA of poor quality and low quantity.
31 Our ultimate objective is to incorporate this primer set into a STR multiplex currently being
32 developed for individualisation but in the meantime, this primer set which allows sexing using
33 either size separation or RFLP, could prove useful for forensic or conservation purposes.
34 This method is also likely to work in the marsh harrier because sequences at primer binding
35 and restriction enzyme recognition sites for both species were identical (data not shown).
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25 Additional information and reprint requests:
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Table 1

Results of the DNA degradation and dilution experiment showing amplification success in triplicate PCRs. '1' indicates successful amplification and '0' indicates lack of amplification.

	Amount of DNA							
	20 ng	10 ng	5 ng	2 ng	1 ng	0.5 ng	0.25 ng	0.1 ng
Degradation time								
Male, <i>HHCHD1FOR</i> + <i>HHCHD1REV</i> (565 bp)								
0 min	111	111	111	111	111	111	111	111
5 min	111	111	011	111	000	101	000	000
10 min	111	111	111	100	100	000	000	011
20 min	111	111	111	111	111	011	010	011
30 min	111	111	111	100	000	000	000	000
60 min	111	111	100	001	000	000	000	000
90 min	111	111	000	100	000	100	000	000
180 min	100	010	000	000	000	000	000	000
Male, <i>HHRFLPFOR</i> + <i>HHCHD1REV</i> (219bp)								
0 min	111	111	111	111	111	111	111	111
5 min	111	111	111	111	111	111	110	110
10 min	111	111	111	111	111	111	111	100
20 min	111	111	111	111	111	111	011	100
30 min	111	111	111	111	110	100	101	000
60 min	111	111	101	001	111	001	000	000
90 min	110	111	000	001	000	100	000	000
180 min	101	010	000	000	000	000	000	000
Female, <i>HHCHD1FOR</i> + <i>HHCHD1REV</i> (356 bp, 565 bp)								
0 min	111	111	111	111	111	111	111	111
5 min	111	111	111	111	000	111	000	000
10 min	111	011	101	010	000	000	000	000
20 min	010	000	000	000	000	000	000	000
30 min	000	100	000	000	000	000	000	000
60 min	000	000	000	000	000	000	000	000
90 min	000	000	000	000	000	000	000	000
180 min	000	000	000	000	000	000	000	000
Female, <i>HHRFLPFOR</i> + <i>HHCHD1REV</i> (212bp , 219 bp)								
0 min	111	111	111	111	111	111	111	111
5 min	111	111	111	111	111	111	111	111
10 min	111	111	111	111	111	111	111	111
20 min	111	101	110	010	000	000	000	001
30 min	110	101	101	001	000	100	000	000
60 min	111	011	000	000	000	001	000	101
90 min	110	001	000	000	000	000	000	000
180 min	000	000	000	000	000	100	000	000

Table 2

Mann-Whitney-*U*-test 2-tailed *p* values obtained in comparisons between the primer sets for various combinations of data (significant values are in bold).

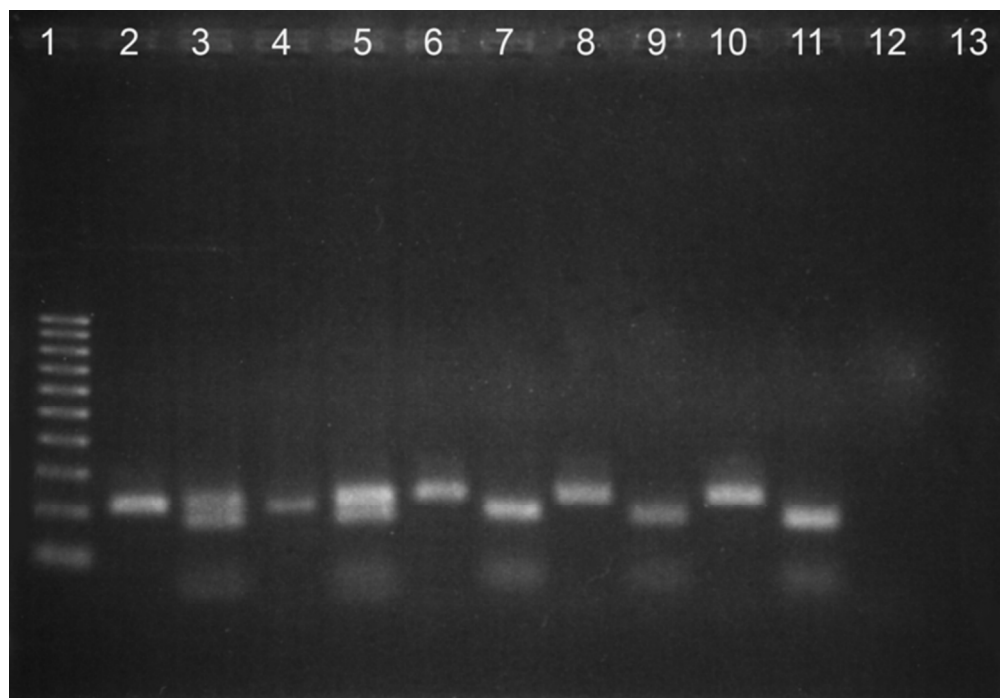
Test	Male	Female	Male and female
1 All template amounts (0.1 ng – 20 ng) All degradation time points (5 min – 180 min)	0.028	0.000	0.000
2 Template amounts < 2 ng All degradation time points (5 min – 180 min)	0.004	0.000	0.000
3 Template amounts > 2 ng All degradation time points (5 min – 180 min)	0.514	0.010	0.039

For Peer Review

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W fragment (female) AATTTTATGTACAGGAAAAGACTGGCAATTAATATGCTAAATACTATTTTGAAA
Z fragment (female) CGCGTCTTTTTCTAGAAAGACTGCAATTAATATGCTAACCAGTATTATGAAG
Z fragment (male) CGCGTCTTTTTCTAGAAAGACTGCAATTAATATGCTAACCAGTATTATGAAG

209x297mm (300 x 300 DPI)



33x23mm (600 x 600 DPI)

Review

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3 Fig 1. Sequence showing the *HHRFLPFOR* primer binding site (highlighted in light grey) in W
4 and Z fragments.
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7 The primer binding site was identical in the W fragment from 10 females (2 from Orkney Islands,
8 Scotland; 2 from SE Highlands, Scotland; 1 from NW Highlands, Scotland, 1 from Galloway,
9 Scotland; 1 from Isle of Man; 2 from Wales; 1 from England), and in the Z fragment from 10
10 females (2 from the Orkney Islands, Scotland; 1 from SE Highlands, Scotland; 1 from NW
11 Highlands, Scotland, 2 from Galloway, Scotland; 1 from the Isle of Man; 2 from Wales; and 1
12 from England) and 10 males (3 from the Orkney Islands, Scotland; 1 from SE Highlands,
13 Scotland; 2 from NW Highlands, Scotland, 1 from Galloway, Scotland; 2 from the Isle of Man;
14 and 1 from Wales). The two nucleotide substitutions between W and Z fragments are
15 highlighted in dark grey.
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32 Fig 2. PCR-RFLP patterns using Sfc I
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34 Lane 1: 100 bp ladder; lanes 2,3: DNA from female tissue undigested, digested (location of
35 sample: England); 4,5: DNA from female buccal swab undigested, digested (location of sample:
36 Scotland, SE Highlands); 6,7: DNA from male tissue undigested, digested (location of sample:
37 England); 8,9: DNA from male buccal swab undigested, digested (location of sample: Scotland,
38 Galloway); 10,11: DNA from male buccal swab undigested, digested (location of sample: Isle of
39 Man); 12,13: negative control.
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