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SkydancerPlex: a novel STR multiplex validated for forensic use in the hen harrier (*Circus cyaneus*)

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ABSTRACT

The hen harrier (*Circus cyaneus*) is a bird of prey which is heavily persecuted in the UK because it preys on the game bird red grouse (*Lagopus lagopus scoticus*). To help investigations into illegal killings of hen harrier, a STR multiplex kit containing eight short tandem repeat (STR) markers and a chromohelicase DNA binding protein 1 (CHD 1) sexing marker was developed. The multiplex kit was tested for species specificity, sensitivity, robustness, precision, accuracy and stability. Full profiles were obtained with as little as 0.25 ng of template DNA. Concurrent development of an allelic ladder to ensure reliable and accurate allele designation across laboratories makes the *SkydancerPlex* the first forensic DNA profiling system in a species of wildlife to be fully validated according to SWGDAM and ISFG recommendations. An average profile frequency of 3.67×10^{-8}, a P_{ID} estimate of 5.3×10^{-9} and a $P_{ID\text{-}SIB}$ estimate of 9.7×10^{-4} make the *SkydancerPlex* an extremely powerful kit for individualisation.

1. INTRODUCTION

The hen harrier is a species of raptor commonly found in Europe, Asia and North America. Although categorised as "Least Concern" on the IUCN Red List of Threatened Species^{TM} due to its extremely large geographical range and fairly large global population size, the hen harrier is facing serious problems in the United Kingdom. Substantial declines in numbers have been observed in the last few decades due to habitat loss [1,2] but also as a direct result of illegal persecution on grouse moors [3-5]. Hen harriers are ground nesting and breed on large open areas with low vegetation such as upland heather moorlands where they prey on small mammals and birds such as red grouse [6]. Large areas of heather moorlands in northern England and parts of southern and eastern Scotland are managed by private landowners for driven red grouse shooting and predation by high numbers of hen harriers has been shown to reduce red grouse density, resulting in smaller numbers of shooting bags [7-9]. Despite being protected by law since 1954 and more recently under the Wildlife and Countryside Act 1981, hen harriers continue to be killed illegally and declining numbers have resulted in the species being included on the red-list of birds of conservation concern in the UK [10]. Due to heavy persecution, the hen harrier is on the brink of extirpation from England, with only four pairs breeding in 2014 [11]. The Royal Society for the Protection of Birds (RSPB) carried out the Skydancer Project between 2011 and 2015 and is currently undertaking the hen harrier LIFE project (running until 2019) in attempts to secure the hen harrier's future in the UK using a host of activities including satellite tagging of birds, nest protection schemes, ground monitoring, liaising with stakeholders and bringing awareness. While 2015 has been the most successful breeding season since 2010 for the hen harrier in England, with 6 successful nests resulting in 18 new fledged chicks, 5 male hen harriers disappeared mysteriously with consequent nest failures [12]. A DNA based tool to identify individual hen harriers would be advantageous in the battle against illegal persecution of hen harriers.

STR loci have become the most commonly used genetic marker for DNA based individualisation. Tetranucleotide STRs are preferred because their stutter percentages are much lower (15%) compared to di- and trinucleotides (30%) [13]. A total of 23 tri and tetranucleotide STR markers have recently been described in the hen harrier [14]. Using a selection of 8 of these markers and a previously described sex identification marker [15], we present here the development of a multiplex kit for the hen harrier validated for forensic use according to the Scientific Working Group for DNA Analysis Methods (SWGDAM) guidelines for DNA analysis methods [16]. An allelic ladder was also developed to assist in the designation of alleles from unknown samples as recommended by the International Society for Forensic Genetics (ISFG) for the use of non-human (animal) DNA in forensic genetic investigations [17].

2. MATERIALS and METHODS

2.1 Marker selection

Eight STR markers with three or more alleles in the screened population sample (n = 63) were incorporated into a multiplex (Table 1). Previously described primers for the chromohelicase DNA binding protein 1 (CHD 1) gene found on avian Z and W sex chromosomes were also added (*HHRFLPFOR* 5′-AGACTGGCAATTACTATATGC-3′ and *HHCHD1REV* 5′-TCAATTCCCCTTTTATTGATCC-3') [15]. In addition, since two substitutions were reported within the *HHRFLPFOR* primer binding site between the Z and W sequences [15], another forward primer *HHRFLPSUBSFOR* 5′-AGACTGTCAATTCCTATATGC-3′ was added to balance amplification of Z and W CHD 1 products.

Table 1. Locus information for the *SkydancerPlex*. Primer sequences for STR loci are detailed in [14].

Locus Name	Repeat Motif	Fluorophore	Allelic size range in bp	Final primer Concentration (µM)
HHBswB220w	$(AAT)_{15}$	6-FAM	86-110	0.1
43895	$(AGAT)_{12}$	6-FAM	148-176	0.2
HH09-C1	$(AAAC)_3$ GAAC $(AAAC)_5$	6-FAM	254-266	0.2
55457	$(AAAC)_8$	HEX	105-113	0.2
HH11-G7	$(CAGCTTTCTTT)_{10}$	HEX	132-199	0.2
CHD 1	-	HEX	212, 219	0.2
22316	$(AAAG)_{10}$	HEX	240-291	0.2
62369	$(AAAC)_{11}$	NED	112-133	0.2
00703	$(AGAT)_{11}$	NED	174-207	0.2

2.2 Samples

DNA from the following types of samples were used for the development and optimisation of the multiplex assay: female and male hen harrier tissue, hen harrier egg shell fragments, hen harrier naturally shed feathers, hen harrier buccal swabs, domestic dog (*Canis lupus familiaris*) buccal swab, pheasant (*Phasianus colchicus*) tissue, blood from two blue tits (*Cyanistes caeruleus*) and commercially available human (*Homo sapiens*) male control 2800 (Promega). DNA from tissue and egg shell fragments was extracted using the DNeasy® Blood and Tissue Kit (Qiagen) as recommended by the manufacturer with the following modification in tissue: 4 µl RNase A (100mg/ml) was added after overnight incubation with proteinase K and prior to column binding and incubated at room temperature for 2 minutes. The hen harrier feather was extracted using the DNeasy® Blood and Tissue Kit (Qiagen) according to the user-developed protocol for the purification of total DNA from feathers with the following modifications: all volumes were doubled except for AW1 and AW2 buffer and 1 µg carrier RNA (Qiagen) was added prior to column binding. DNA from hen harrier and dog buccal swabs was extracted using the QIAamp® DNA Mini Kit (Qiagen) with the following modifications: samples were incubated for 2.5 hours at 56 ºC and 1 µg carrier RNA (Qiagen) was added prior to column binding. DNA from blue tit blood was extracted as part of a separate project (Smith J.A., unpublished).

2.3 Multiplex PCR amplification

The *SkydancerPlex* was optimized and validated in a 12.5 µl reaction volume using 6.25 µl Multiplex PCR Mastermix (Qiagen), 1.25 µl Q-Solution (Qiagen), 1.25 µl of the primer mix (all at 2µM except HHBswB220w at 1 µM , see table 1), 1.0 ng DNA template and PCR-grade H_2O to volume. PCR was carried out on Applied Biosystems 2720 Thermal Cyclers using the following conditions: 15 min activation step at 95 °C followed by 25 cycles of 30 s at 95 °C, 90 s at 55 °C, 30 s at 72 °C and final extension for 1 hour at 60 °C.

2.4 Capillary electrophoresis and data analysis

Fragment analysis of PCR products was performed on an Applied Biosystems 3500 Genetic Analyser using POP-6^{TM} polymer and virtual filter D after spectral calibration using the DS-30 matrix standard (Applied Biosystems). Samples were prepared by adding 9.7 µl Hi-Di^{TM} Formamide (Applied Biosystems) and 0.3 µl GeneScan^{TM} 500 ROX^{TM} Size Standard (Applied Biosystems) to 1 µl PCR product. The allelic ladder (see section 2.6) was prepared by adding 9.7 µl Hi-Di^{TM} Formamide and 0.3 µl GeneScan^{TM} 500 ROX^{TM} Size Standard to 2 µl allelic ladder. Data was analysed using GeneMapper$^{®}$*ID-X* software version 1.2 with a minimum detection threshold of 50 rfu.

2.6 Allele sequencing and allelic ladder construction

For sequencing of alleles, when available, homozygous individuals were used in singleplex PCRs with unlabelled primers. Amplification was carried out in a 20 µl reaction volume containing 10.0 µl ThermoPrime 2x ReddyMix PCR Master Mix (ThermoFisher Scientific), 0.5 µM forward and reverse primer, 1.5 mM $MgCl_2$, 2-20 ng DNA template and PCR-grade H_2O to volume. Cycling parameters were 95 °C for 3 min, 25 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 15 min. Products were purified using the MinElute PCR Purification kit (QIAGEN, Hilden Germany) according to the manufacturer's protocol. Reactions amplifying two alleles from heterozygous individuals were run on large 20 x 20 cm agarose gels for purification. Alleles separated by >20bp were run on a 2.5 % gel at 200 Volts for 2.5 hours while those separated by 11 to 20 bp and <10 bp were run on 3 % and 4 % gels respectively at 165 Volts for 4 hours. Bands were excised from the gel using a sterile scalpel blade and purified using the GenCatch Advanced Gel Extraction Kit (Epoch Life Sciences, Texas, USA) or an EZNA Gel Extraction Kit (Omega Bio-Tek Inc., Georgia, USA) according to manufacturer's protocols. The BigDye$^{®}$ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific) was used for cycle sequencing and products were purified using an ethanol precipitation with 10 mM EDTA, 0.3 M NaOAc (pH 4.6) and 20 µg glycogen. Sequencing was carried out on an ABI3500 genetic analyser (ThermoFisher Scientific) and sequence data analysed using BioEdit software version 7.1.7 [18].

For construction of the allelic ladder, individual alleles were isolated after separate singleplex PCR amplification in 12.5 µl containing 6.25 µl ThermoPrime 2x ReddyMix PCR Master Mix, 0.5 µM of labelled forward and unlabelled reverse primers and 3mM $MgCl_2$. Cycling parameters were 95 °C for 3 min, 25 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by 60 °C for 1 hour. Following capillary electrophoresis of 1 ul PCR product with 12 µl Hi-Di Formamide and 0.3 µl GeneScan^{TM} 500 ROX^{TM} Size Standard (ThermoFisher Scientific), a working stock of each allele was prepared at approximately 500 rfu/µl. Dilutions of 1:10, 1:1000 and 1:100,000 were then prepared from each working stock and 1 µl used in another 12.5 µl singleplex PCR [19]. Capillary electrophoresis was again carried out using 1 µl of the amplified product and the reaction that yielded high peak height intensities and no minus-A products or other artefacts were selected for incorporation into the ladder. For the 00703 locus, a 1:10,000 dilution was found to be optimal. The allelic ladder was finally assembled such that the volume of the allele product added to a 1 ml total volume resulted in approximately 500 rfu across all alleles in 2 µl volume. A total of 55 alleles were combined into the ladder. Sizing data from twenty-four injections of the allelic ladder was collected to determine precision with allele calling. To facilitate inter-laboratory testing, samples of the allelic ladder could be made available to other laboratories (please contact corresponding author).

2.7. Developmental validation

A developmental validation was performed on the *SkydancerPlex* using SWGDAM guidelines [16].

2.7.1 Characterization of STR markers

Mendelian inheritance was examined through parentage verification of one family where a shed feather from an adult female and buccal swabs from four chicks were available. In addition, sibling samples from four nests were genotyped as described in sections 2.3 and 2.4 and genotypes of the parents inferred.

2.7.2 Species Specificity

Primer specificity was determined using 3-10 ng of DNA from two other bird species (pheasant and blue tit) and from the domestic dog. For the human sample, a total of 3 ng DNA was used in the PCR followed by capillary electrophoresis as described in sections 2.3 and 2.4.

2.7.3 Sensitivity

Sensitivity of the STR multiplex was evaluated using DNA from female and male hen harrier tissue. Samples were first diluted to a starting concentration of 1 ng/µl, followed by a serial dilution down to 0.062 ng/µl. Multiplex PCR amplification of 1 µl of diluted sample was carried out using conditions described in sections 2.3 and 2.4.

2.7.4 Stability

Artificially degraded DNA was used to assess stability of the multiplex. Female and male DNA samples degraded using DNAse I for the following times were used: 0 min (no DNAse I added), 5 min, 10 min and 30 min at 20.0 ng/µl, 10.0 ng/µl, 5.0 ng/µl, 2.0 ng/µl, 1.0 ng/µl, 0.5 ng/µl and 0.25 ng/µl were used (see [15] for details). Samples were then amplified using the multiplex PCR and capillary electrophoresis conditions described in sections 2.3 and 2.4.

2.7.5 Precision and accuracy

The precision and accuracy of the multiplex was established through repeatability and reproducibility studies. The multiplex was tested by the same two operators on two separate days using PCR and capillary electrophoresis conditions described in sections 2.3 and 2.4. Three different thermal cyclers were also tested: a 2720 Thermal Cycler (ThermoFisher Scientific), a GeneAmp® PCR System 9700 (ThermoFisher Scientific) and a Veriti® 96-Well Thermal Cycler (ThermoFisher Scientific). Triplicate samples were used for each Thermal Cycler using conditions described in sections 2.3 and 2.4.

2.7.6 Case-type samples

In order to assess samples that may typically be encountered in forensic casework, the multiplex was tested on DNA from a wide range of samples. For tissue and buccal swab DNA, conditions described in section 2.3 were used; for egg shell fragments and feather samples, between 3 and 20 ng DNA template was used with all other conditions as described in sections 2.3 and 2.4.

2.7.7 Population studies

Results from Hardy-Weinberg and linkage disequilibrium tests for the STR markers have been reported in a previous study [14]. Forensic parameters were obtained using PowerStats v1.2 [20]. Probability of Identity (P_{ID}) (unbiased) and Probability of Identify among siblings ($P_{ID-SIB)}$ [21] were obtained using GIMLET v1.3.3 [22] and F_{ST} estimates were obtained using Arlequin v3.5.2.2 [23].

2.7.8 PCR-based studies

A few modifications were made to the cycling parameters recommended in the Qiagen Multiplex PCR Master Mix protocol. Extension time was reduced from 90 sec to 30 sec since maximum amplicon size was <300 bp and the number of cycles was reduced from the recommended 30-45 to 25. As the Qiagen Multiplex PCR Master Mix can be used with or without Q-Solution (Qiagen), a test was carried out. The sample was amplified in duplicate with and without Q-Solution using conditions described in sections 2.3 and 2.4 and the average peak height ratio for heterozygote loci calculated.

Initial tests with the *HHRFLPFOR* and *HHCHD1REV* CHD 1 primers resulted in imbalanced 212 and 219 bp peaks in female individuals, necessitating the addition of an additional forward primer *HHRFLPSUBSFOR* (see section 2.1).

Primer concentrations for the HHBswB220w locus had to be halved in the primer mix in order to balance peak heights across all loci (Table 1).

For the developmental validation, the multiplex was tested on an ABI 2720 Thermal Cycler using two further PCR buffers: AmpliTaq Gold (ThermoFisher Scientific) and 2x Platinum Multiplex PCR Mastermix (ThermoFisher Scientific). Multiplex PCR Mastermix and 2x Platinum Multiplex PCR Mastermix amplification was carried out using the PCR conditions described in section 2.3. AmpliTaq Gold amplification was carried out in a 12.5 µl reaction, using 1x PCR buffer, 1.25 mM dNTPs, 3.0 mM $MgCl_2$, 1 Unit AmpliTaq Gold Polymerase, 1.25 µl of the primer mix, 1.0 ng female DNA and PCR-grade H_2O to volume using cycling conditions described in section 2.3. Capillary electrophoresis was carried out as described in section 2.4.

3. RESULTS

3.1 Selection of STR markers

All selected STR markers (except for HH11-G7) appear within the list of possible tetranucleotide motifs described by Jin *et al*. [24]. HHBswB220w, 43895, 55457, HH11-G7 and 62369 are simple STR loci with no interruptions or substitutions in repeat motifs between the different alleles (Table 2). Allele 6 of marker HH09-C1 contains six consecutive AAAC repeats, while alleles 7, 8 and 9 contain a substitution within the repeat motif as shown in Table 2. This has also been observed in 00703, where the first AGAT repeat in alleles 14 and 15 is AGGT. Genotyping of an adult female and four nestlings and four families of siblings provided support for Mendelian inheritance across all STR loci (data not shown).

Table 2. Repeat motifs in sequenced alleles of eight STR loci. Alleles marked * have not been sequenced.

6-FAM			HEX			NED		
	Size (bp)	Repeat		Size (bp)	Repeat		Size (bp)	Repeat
HHBswB220w			*55457*			*62369*		
9	83	(AAT)9	6*	105	-	9*	112	-
10*	86	-	7	109	(AAAC)7	10	116	(AAAC)10
11*	89	-	8	113	(AAAC)8	11	120	(AAAC)11
12	92	(AAT)12	*HH11-G7*			12	124	(AAAC)12
13	95	(AAT)13	6	132	(CAGCTTTCTTT)6	13	129	(AAAC)13
14	98	(AAT)14	7*	143	-	14*	133	-
15	101	(AAT)15	8	155	(CAGCTTTCTTT)8	*00703*		
16*	104	-	9	166	(CAGCTTTCTTT)9	7*	175	-
17*	107	-	10	177	(CAGCTTTCTTT)10	8	179	(AGAT)8
18*	110	-	11	188	(CAGCTTTCTTT)11	9	183	(AGAT)9
43895			12	199	(CAGCTTTCTTT)12	10	187	(AGAT)10
11	148	(AGAT)11	*22316*			11	191	(AGAT)11
12	152	(AGAT)12	9	240	(AAAG)9	12	195	(AGAT)12
13	156	(AGAT)13	10	244	(AAAG)10	13	199	(AGAT)13
14	160	(AGAT)14	11	248	(AAAG)11	14	203	AGGT (AGAT)13
15	164	(AGAT)15	12	252	(AAAG)12	15	207	AGGT (AGAT)14
18*	176	-	14	260	(AAAG)14			
HH09-C1			15	264	(AAAG)15			
6	254	(AAAC)6	16	268	(AAAG)16			
7	258	(AAAC)3 GAAC (AAAC)3	17	272	(AAAG)17			
8	262	(AAAC)3 GAAC (AAAC)4	17(2)	272	(AAAG)4 AGAG (AAAG)12			
9	266	(AAAC)3 GAAC (AAAC)5	18	275	(AAAG)18			
			19	279	(AAAG)19			
			19(2)	279	(AAAG)4 AGAG (AAAG)14			
			20	283	(AAAG)20			
			21	287	(AAAG)21			
			22*	291	-			

3.2 Species specificity

The primers showed high species specificity since no amplicons were observed using pheasant, blue tit, dog or human DNA (data not shown).

3.3 Sensitivity

A full STR profile was obtained down to 0.125 ng using female DNA (Figure 1). At 0.125 ng, the 212 bp fragment of the sexing marker (CHD 1) dropped just below the 50 rfu threshold but was still clearly present (indicated by a red arrow). DNA from the male hen harrier resulted in a full profile including CHD 1 down to a template concentration of 0.25 ng. At 0.125 ng, peak heights for markers 22316, 62369 and 00703 dropped below the 50 rfu threshold but were still present (indicated by red arrows, Figure 1) but a validated lower limit for a full DNA profile was determined to be 0.25 ng.

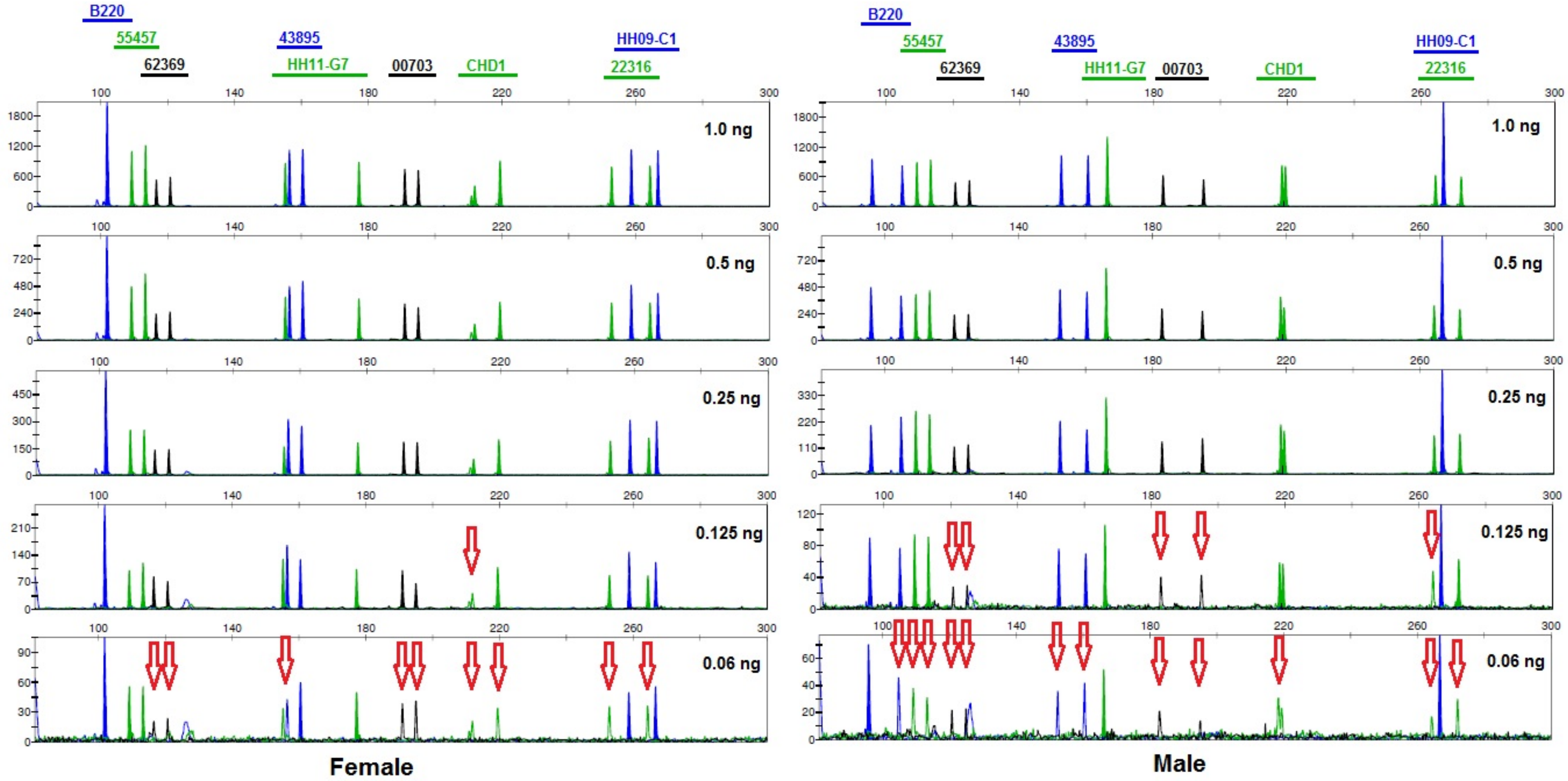

Figure 1. Electropherograms after sensitivity testing of *SkydancerPlex* using a range of template DNA amounts. Peaks indicated by red arrows have dropped below the 50 rfu threshold. Note that panels have different rfu scales.

3.4 Stability

With artificially degraded DNA, full profiles were obtained in both female and male samples with 20.0 ng DNA after 5 and 10 minutes DNase I treatment. With lower amounts of DNA and increasing times of DNase I treatment, peak heights of larger alleles (>240 bp) began to drop below the rfu threshold followed by those of medium sized alleles (132 – 219 bp) (Table 3).

Table 3. Results of stability testing using artificially degraded DNA showing amplification success for alleles across all loci.

	Loci					
Template DNA	B220 55457 62369	HH11-G7 43895 00703 CHD 1	HH09-C1 22316	B220 55457 62369	HH11-G7 43895 00703 CHD 1	HH09-C1 22316
	83 – 133 bp	132 – 219 bp	> 240 bp	83 – 133 bp	132 – 219 bp	> 240 bp
	Female DNA, 0 min			Male DNA, 0 min		
20.0 ng	5/5	8/8	4/4	6/6	6/6	3/3
10.0 ng	5/5	8/8	4/4	6/6	6/6	3/3
5.0 ng	5/5	8/8	4/4	6/6	6/6	3/3
2.0 ng	5/5	8/8	4/4	6/6	6/6	3/3
1.0 ng	5/5	8/8	4/4	6/6	6/6	3/3
0.5 ng	5/5	8/8	4/4	5/6	6/6	3/3
0.25 ng	5/5	8/8	4/4	5/6	6/6	3/3
	Female DNA, 5 min			Male DNA, 5 min		
20.0 ng	5/5	8/8	4/4	6/6	6/6	3/3
10.0 ng	5/5	8/8	4/4	6/6	6/6	3/3
5.0 ng	5/5	7/8	3/4	6/6	3/6	1/3
2.0 ng	5/5	6/8	1/4	6/6	1/6	0/3
1.0 ng	3/5	0/8	0/4	3/6	0/6	0/3
0.5 ng	1/5	0/8	0/4	1/6	0/6	0/3
0.25 ng	0/5	0/8	0/4	0/6	0/6	0/3
	Female DNA, 10 min			Male DNA, 10 min		
20.0 ng	5/5	8/8	4/4	6/6	6/6	3/3
10.0 ng	5/5	6/8	2/4	6/6	6/6	1/3
5.0 ng	5/5	4/8	0/4	6/6	1/6	0/3
2.0 ng	3/5	1/8	0/4	4/6	0/6	0/3
1.0 ng	2/5	0/8	0/4	2/6	0/6	0/3
0.5 ng	0/5	0/8	0/4	0/6	0/6	0/3
0.25 ng	0/5	0/8	0/4	0/6	0/6	0/3
	Female DNA, 30 min			Male DNA, 30 min		
20.0 ng	0/5	0/8	0/4	4/6	0/6	0/3
10.0 ng	0/5	0/8	0/4	3/6	0/6	0/3
5.0 ng	0/5	0/8	0/4	1/6	0/6	0/3
2.0 ng	0/5	0/8	0/4	0/6	0/6	0/3
1.0 ng	0/5	0/8	0/4	0/6	0/6	0/3
0.5 ng	0/5	0/8	0/4	0/6	0/6	0/3
0.25 ng	0/5	0/8	0/4	0/6	0/6	0/3

3.5 Precision and accuracy

The multiplex PCR was carried out by two users on two separate days using the female and male DNA. Full profiles were generated each time and allele designation was consistent using the allelic ladder (data not shown). Three different thermal cyclers and three different mastermixes were tested in triplicate using female DNA and full profiles were obtained in every case with consistent allele designation using the allelic ladder. Mean rfu values across all alleles in triplicate reactions were similar across all mastermixes: 854 rfu (SD 18.5) for the Qiagen multiplex PCR Mastermix, 786 rfu (SD 65.4) for the Platinum Multiplex PCR Mastermix, and 987 (SD 105.7) for AmpliTaq gold. Mean rfu values across all alleles in triplicate reactions across three thermal cyclers using the Qiagen multiplex mastermix were also similar: 854 rfu (SD 18.5) for the 2720 thermal cycler, 1131 rfu (SD 211) for the 9700 thermal cycler, and 1009 rfu (SD 107.4) for the Veriti thermal cycler.

3.6 Case-type samples

Full profiles were obtained for tissue, buccal swab and feather samples although the quality of profiles generated from feathers was found to vary considerably. Out of seventeen feather samples selected for testing, six showed no amplification or partial profiles. Only a partial profile was obtained for 1 out of 4 egg shell fragments, with 12 out of 17 alleles showing peak heights above the 50 rfu threshold. No amplicons were observed for the other 3 egg shell fragments (two from the same egg) (data not shown).

3.7 Population studies

Table 4 shows the allele frequencies observed in 63 individuals from across the UK (England n= 22, Scotland n= 29, Wales n= 8 and Isle of Man n= 4). All individuals were unrelated since buccal swab/feather samples from only one sibling from each nest was used.

Table 4. Allele frequencies for *SkyDancerPlex* STR markers in the UK (n = 63) and descriptive statistics, including the number of alleles (Na), match probability (MP), polymorphism information content (PIC), power of discrimination (PD), power of exclusion (PE), probability of identity (P_{ID}) and probability of identity for siblings ($P_{ID\text{-}SIB}$).

Allele	B220	43895	HH09-C1	55457	HH11-G7	22316	62369	00703
6	-	-	0.016	0.008	0.056	-	-	-
7	-	-	0.347	0.683	0.294	-	-	0.127
8	-	-	0.202	0.310	0.389	-	-	0.032
9	-	-	0.435	-	0.167	0.097	0.048	0.087
10	0.079	-	-	-	0.087	0.056	0.071	0.302
11	0.127	0.016	-	-	-	0.065	0.143	0.032
12	0.103	0.198	-	-	0.008	0.145	0.516	0.175
13	0.119	0.183	-	-	-	-	0.214	0.143
14	0.032	0.492	-	-	-	0.121	0.008	0.095
15	0.198	0.103	-	-	-	0.024	-	0.008
16	0.063	-	-	-	-	0.056	-	-
17	0.262	-	-	-	-	0.129	-	-
18	0.016	0.008	-	-	-	0.032	-	-
19	-	-	-	-	-	0.194	-	-
20	-	-	-	-	-	0.048	-	-
21	-	-	-	-	-	0.024	-	-
22	-	-	-	-	-	0.008	-	-
Na	9	6	4	3	6	13	6	9
MP	0.046	0.152	0.197	0.397	0.122	0.041	0.181	0.074
PIC	0.83	0.63	0.57	0.35	0.68	0.88	0.62	0.80
PD	0.954	0.848	0.803	0.603	0.878	0.959	0.819	0.926
PE	0.482	0.356	0.329	0.122	0.392	0.935	0.433	0.587
P_{ID}	0.038	0.140	0.186	0.397	0.112	0.018	0.146	0.045
$P_{ID\text{-}SIB}$	0.341	0.451	0.474	0.632	0.418	0.313	0.459	0.352

Gene diversity (expected heterozygosity) values ranged from 0.442 to 0.896 [14]. Levels of polymorphism and power of discrimination were high and match probability was low across all loci (Table 4). Calculated as the product of MP values across all 8 loci, the average profile frequency was 3.67×10^{-8} (1 in 27.3 million). Overall P_{ID} and $P_{ID\text{-}SIB}$ estimates were 5.3×10^{-9} and 9.7×10^{-4} respectively. An F_{ST} of 0.022 was estimated when individuals from England, Wales and Isle of Man (n=34) were compared to individuals from Scotland (n=29). Profile frequencies calculated using the Balding & Nichols formula [25] using an F_{ST} of 0.03 were 1 in 644 million for the female DNA profile and 1 in 396 million for the male DNA profile respectively.

3.8 Allelic ladder

An allelic ladder comprising 95 % of all alleles i.e. 55 alleles out of 58 observed alleles was developed (Figure 2). Allele 11 of marker 43895, allele 6 of marker 55457 and allele 12 of marker HH11-G7 were not included in the allelic ladder due to unavailability of suitable individuals to isolate the alleles from. A total of 80 % of the alleles (44/55) in the allelic ladder have been sequenced. The remainder (11) were not sequenced since no suitable individuals were available for isolation (heterozygous genotypes had alleles too close in size for successful separation) or small size of amplicon fell within size exclusion range of columns used for purification.

Alleles were consistently and accurately called across all STR loci with the use of the allelic ladder. Precision testing using 24 separate injections of the allelic ladder resulted in all allele sizes within three standard deviations of the mean (SD was no more than 0.036 and 3xSD was no more than 0.11) making allele bins of mean±0.5 bp highly conservative.

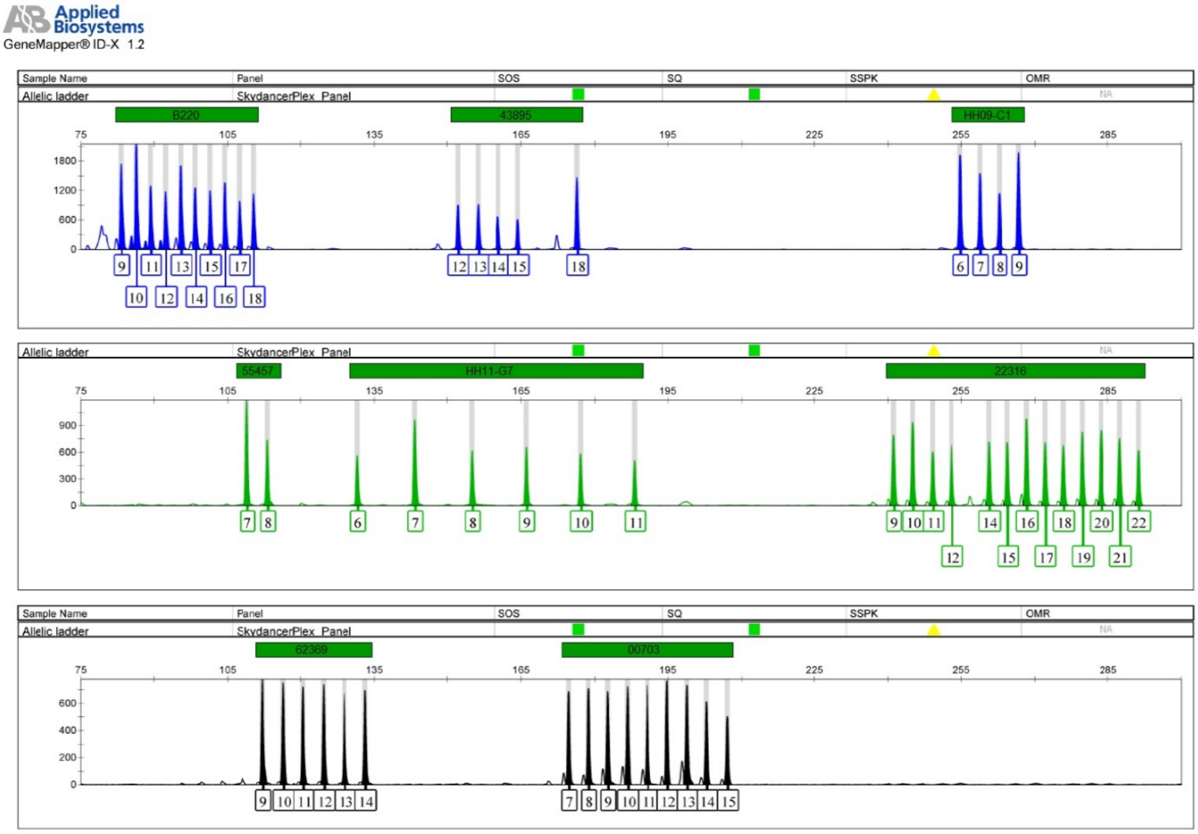

Figure 2. Allelic ladder for the *SkydancerPlex***.** Contains 55 out of 58 observed alleles across 8 STR markers.

3.9 PCR based studies

During initial tests with all primer concentrations at 0.2 µM, a peak height of 1700 rfu was observed for the homozygous allele at HHBswB220w compared to an average of 570 rfu for heterozygous alleles in the other 6-FAM labelled loci. The primer concentration for this locus was consequently reduced to 0.1 µM. After this adjustment, well balanced profiles were obtained at all 8 STR markers using 1 ng template DNA (Figure 3 and 4 show profiles from female and male individuals respectively). Incorporation of the additional *HHRFLPSUBSFOR* primer for the CHD-1 marker resulted in more balanced Z and W products, although the 219 bp Z band in males occasionally results in split peaks (Figure 4).

As part of the optimisation process, the Multiplex PCR Master Mix (Qiagen) was tested with and without the addition of Q-solution (Qiagen). All peak height ratios were well above the 70 % [26] threshold (88-99%), but the average peak height ratio with the addition of Q-solution was 95.3 % compared to 94.5 % without Q-solution (data not shown).

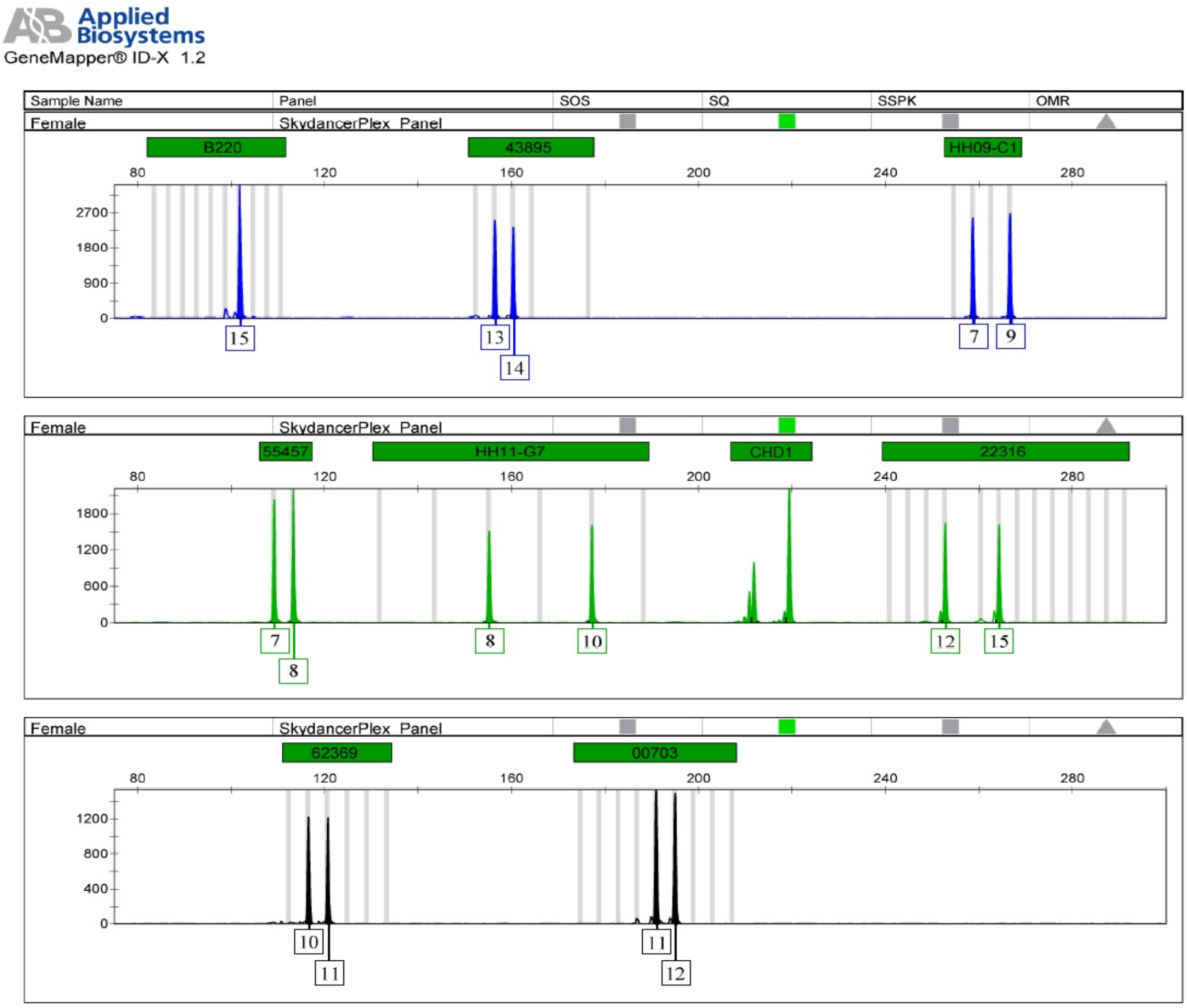

Figure 3. Profile consisting of 8 STR markers and sexing marker CHD 1 generated using *SkydancerPlex* for the female hen harrier. The sexing marker CHD1 is present as two peaks at 212 and 219 bp in the green panel.

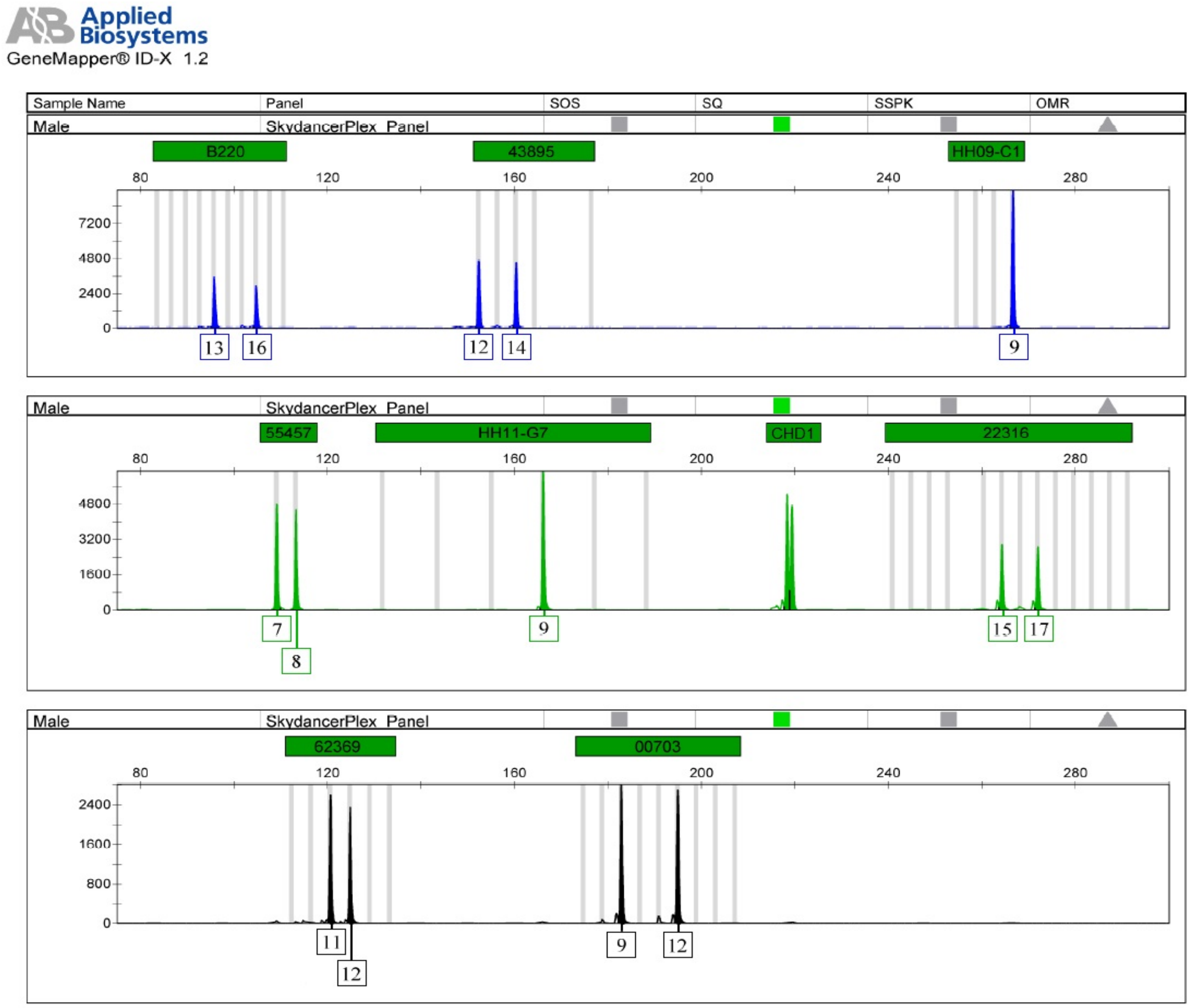

Figure 4. Profile consisting of 8 STR markers and sexing marker CHD 1 generated using *SkydancerPlex* for the male hen harrier. The sexing marker CHD1 is present as a split peak at 219 bp in the green panel.

Stutter percentages varied from 1.7 % to 15.6 %. The highest stutter percentages (3.1 % - 15.6 %) were found for marker B220, a trinucleotide repeat, with an average of 8.7 %. The average stutter percentage for the tetranucleotide repeats was 4.9 %. No stutter was observed for marker HH11-G7 with a repeat motif of 11 nucleotides. When comparing the stutter percentages for B220 with the three different PCR buffers, the lowest was observed using Platinum Mastermix (8.4 %), followed by Multiplex PCR Mastermix (8.6 %) and AmpliTaq Gold (11.9 %). AmpliTaq Gold also showed stutter products at markers 22316, 62369 and 00703 (6.6 % - 14.7 %) whereas the other two PCR mastermixes did not. No significant difference in stutter percentage for marker B220 was observed when using different thermal cyclers (8.6 % - 8.8 %).

4. DISCUSSION

Most animal STR multiplexes validated for forensic use to date have been for domesticated species such as cats [27], dogs [19] and pigs [28]. Although there are increasing numbers of STR multiplexes being reported for wildlife species e.g. brown bears [29], red deer [30], elephant [31] and tigers [32], they often lack full forensic validation and/or the incorporation of an allelic ladder. The *SkydancerPlex* is to our knowledge the first STR multiplex for a species of wildlife that includes an allelic ladder and has been fully validated for forensic use using SWGDAM guidelines. It shows high levels of species specificity since no amplicons were observed for any of the tested species. However, since cross-species amplification of STR markers in closely related birds is frequently observed [14, 33-36], it is possible that amplification will be seen in other closely related species such as the Montagu's harrier (*Circus pygargus*) or marsh harrier (*Circus aeruginosus*). STR markers incorporated within *SkydancerPlex* exhibit high power of discrimination, with an average profile frequency of 3.67 x 10^{-8} (1 in 27.3 million) and probability of identity (P_{ID}) of 5.3 x 10^{-9}. Even the highly conservative estimate of probability of identity among siblings (P_{ID-SIB}) was only 9.7 x 10^{-4}, making the *SkydancerPlex* a highly useful tool for investigations into cases of illegal persecution of hen harriers. Estimates of profile frequencies for the female and male DNA samples using a conservative 0.03 estimate for F_{ST} in the Balding & Nichols' formula were reassuringly low at 1 in 644 million and 1 in 396 million respectively.

SkydancerPlex showed good sensitivity, with full profiles being obtained down to 0.125 ng for both the female and male DNA although rfu levels fell below the threshold of 50 for several alleles. However, using the allelic ladder, all these allele peaks fell clearly within the respective allelic bins, enabling allelic designation. Further enhancement in sensitivity could be obtained by increasing primer concentrations for HEX and NED labelled primers which showed lower rfu values in comparison to FAM labelled primers [37]. All peak height ratios were well above the 70 % threshold across all loci. In the case of the sexing marker, incorporation of an additional forward primer for CHD 1 improved peak height for the 219 bp amplicon. However, the 212 bp and 219 bp amplicons remain unbalanced and further enhancement could be achieved by reducing the *HHRFLPSUBSFOR* primer concentration. The split peaks seen with the 212 and 219 bp CHD 1 Z chromosome amplicons are unlikely to be the result of incomplete adenylation since full adenylation was obtained for all STR markers with a final extension time of one hour. Addition of a GTTTCTT or ‘PIG-tail’ to the 5'-end of the reverse primer [38] or a complementary oligonucleotide to the unlabelled primer to prevent secondary structure between the labelled single strand of amplicon and unlabelled primer post amplification [39] might resolve this issue.

All STR markers within *SkydancerPlex* have an amplicon size lower than 300 bp and should be successfully amplified in degraded DNA samples. Most of the human STR kits designed specifically for degraded samples such as AmpFISTR® MiniFiler^{TM} contain STR loci with amplicon sizes of ≤ 250 bp [40]. The stability study showed that full profiles could still be obtained after 5 minutes of DNase I treatment using 20.0 and 10.0 ng DNA and after 10 minutes of DNase I treatment using 20.0 ng DNA. With lower amounts of DNA, allele dropout was seen with larger loci e.g. with 5 mins DNase I treatment and 5.0 ng DNA, all smaller alleles (83-133 bp) were successfully amplified while allele dropout was seen in larger alleles (132-219 bp and >240 bp). No amplicons were observed after 5 mins DNase I treatment using <0.5 ng DNA and after 10 mins DNase I treatment using <1.0 ng DNA. After 30 mins DNase I treatment, only a few small amplicons (83-133 bp) were detected in the male DNA using high amounts of DNA.

The *SkydancerPlex* is very robust producing full profiles using three different PCR mastermixes and thermal cyclers, making it suitable for use in any laboratory. Allele calling can also be standardised across laboratories using the allelic ladder developed during this study. The *SkydancerPlex* allelic ladder includes 55/58 alleles detected and shows very well balanced peaks. Furthermore, it demonstrates high levels of precision and accuracy with 3xSD consistently <0.12 which is well within the standard 0.5bp used for allelic bins. The percentages of stutter observed are very low, with a maximum of 15.6 % observed for a trinucleotide repeat and an average of 4.9 % (range 1.7 % - 14.7%) for tetranucleotide repeats. This is comparable to stutter percentages for the AmpFlSTR® Identifiler® Plus kit which vary between 4.0 and 13.6 % [41].

Several case type samples were successfully used. However, feather samples were variable in terms of DNA profile generated. Higher DNA yields and better PCR amplification success has previously been reported from plucked feathers compared to moulted feathers [42,43]. Feathers also contain the pigment melanin which inhibits PCR amplification [44]. For the egg shell fragment samples, only 1 out of 4 samples produced a partial PCR profile (12 out of 17 alleles at rfu above 50, but 14 out of 17 alleles could be called overall). Egg shell contains calcium which is also known to be a PCR inhibitor [44], so DNA profiling of egg shell fragments could be improved if the egg shell is powdered and decalcified with EDTA prior to DNA extraction [45].

5. CONCLUSION

The *SkydancerPlex* is the first STR multiplex for a species of wildlife that is fully validated according to the ISFG and SWGDAM guidelines. It has high species specificity and sensitivity, is highly robust providing full profiles using several different PCR buffers and thermal cyclers, and the availability of an allelic ladder makes usage across laboratories easier and ensures accurate and consistent allele designation. Furthermore, the *SkydancerPlex* is highly discriminatory, with an average profile frequency of 3.67 x 10^{-8} (1 in 27.3 million) and P_{ID} and $P_{ID\text{-}SIB}$ estimates of 5.3 x 10^{-9} and 9.7 x 10^{-4} respectively, highlighting its potential use in forensic investigations.

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