Forensic Science International: Genetics 22 (2016) 100–109

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Research paper

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

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ARTICLE INFO

Article history:
Received 8 December 2015
Received in revised form 2 February 2016
Accepted 3 February 2016
Available online 8 February 2016

Keywords:
Hen harrier
Non-human DNA
Validation
STR multiplex kit
SWGDAM
ISFG

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™ 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].

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http://dx.doi.org/10.1016/j.fsigen.2016.02.003
1872-4973/

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.

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 (100 mg/ml) was added after overnight incubation with proteinase K and prior to column binding and incubated at room temperature for 2 min. 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 h at 56 °C 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 2 μM 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 h 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™ 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™ Formamide (Applied Biosystems) and 0.3 μl GeneScan™ 500 ROX™ Size Standard (Applied Biosystems) to 1 μl PCR product. The allelic ladder (see Section 2.5) was prepared by adding 9.7 μl Hi-Di™ Formamide and 0.3 μl GeneScan™ 500 ROX™ 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.

2.5. 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 × 20 cm agarose gels for purification. Alleles separated by >20 bp were run on a 2.5% gel at 200 Volts for 2.5 h while those separated by 11 to 20 bp and <10 bp were run on 3% and 4% gels respectively at 165 Volts for 4 h. 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 v3.1Cycle 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 3 mM $MgCl_2$. 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 h. Following capillary electrophoresis of 1 ul PCR product with 12 μl Hi-Di Formamide and 0.3 μl GeneScan™ 500 ROX™ Size Standard (ThermoFisher Scientific), a working stock of each allele was prepared at approximately 500 rfu/μl.

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