Forensic Science International: Animals and Environments 5 (2024) 100084

Contents lists available at ScienceDirect

Forensic Science International: Animals and Environments

journal homepage: www.sciencedirect.com/journal/forensic-science-international-animals-and-environments

Original research

Joint detection of microsatellites and flanking sequences – SNPSTR markers for *Athene noctua* to fight illegal wildlife trade

Annika Mozer [a,*], Albia Consul [a], Bernhard Misof [a], Richard Jäger [b], Klaus Olek [c], Jonas J. Astrin [a]

[a] Leibniz Institute for the Analysis of Biodiversity Change, Museum Koenig, Adenauerallee 127, 53113 Bonn, Germany
[b] Department of Natural Sciences & Institute for Functional Gene Analytics, Bonn-Rhein-Sieg University of Applied Sciences, von-Liebig Str. 20, 53359 Rheinbach, Germany
[c] Labor für forensische Analytik, Friedrich-Ebert-Str. 17, 51373 Leverkusen, Germany

ARTICLE INFO

Keywords:
SNPSTR
Illegal Wildlife Trade
Wildlife Forensics

ABSTRACT

Trade of wild-caught animals is illegal for many taxa and in many countries. Common regulatory procedures involve documentation and marking techniques. However, these procedures are subject to fraud and thus should be complemented by routine genetic testing in order to authenticate the captive-bred origin of animals intended for trade. A suitable class of genetic markers are SNPSTRs that combine a short tandem repeat (STR) and single nucleotide polymorphisms (SNPs) within one amplicon. This combined marker type can be used for genetic identification and for parentage analyses and in addition, provides insight into haplotype history. As a proof of principle, this study establishes a set of 20 SNPSTR markers for *Athene noctua,* one of the most trafficked owls in CITES Appendix II. These markers can be coamplified in a single multiplex reaction. Based on population data, the percentage of observed and expected heterozygosities of the markers ranged from 0.400 to 1.000 and 0.545 to 0.850, respectively. A combined probability of identity of $5.3 * 10^{-23}$ was achieved with the whole set, and combined parentage exclusion probabilities reached over 99.99%, even if the genotype of one parent was missing. A direct comparison of an owl family and an unrelated owl demonstrated the applicability of the SNPSTR set in parentage testing. The established SNPSTR set thus proved to be highly useful for identifying individuals and analysing parentage to determine wild or captive origin. We propose to implement SNPSTR-based routine certification in wildlife trade as a way to reveal animal laundering and misdeclaration of wild-caught animals.

1. Introduction

The annual revenue from illegal wildlife trade (IWT) has been estimated at up to 23 billion dollars, which is even higher than the revenue from the illegal trade in light weapons [1]. Prices paid for wildlife contraband are extraordinary, e.g. rhino horn is traded at a higher price than gold [2] and exotic birds are worth more than the same weight of drugs [3]. At the same time, the high profits are coupled with a low risk of prosecution and even if prosecuted, low sentencing. For example, Kitade and Naruse analysed juridical outcomes of wildlife crimes and show that the fines for trafficking wildlife were only 13–29% of the estimated respective market values [4].

However, the consequences of IWT on biodiversity are severe. Direct exploitation of species is a major cause of biodiversity loss, leaving the planet more vulnerable to climate change [5]. One of the taxa strongly affected by IWT are birds. Approximately 45% of all bird species worldwide are exploited by humans [6]. Solely in the Mediterranean area every year 11–36 million birds are captured or killed illegally, mostly to be used as food, pets, or in sports [7]. Laws forbidding the trade of wild-caught birds are applied by several countries. For example, the EU has a permanent ban on the import of all wild birds, regardless of their conservation status [8] and in the US, exporting wild-caught native

Abbreviations: BOLD, Barcode of Life Data System; CE, Capillary Electrophoresis; CITES, Convention on International Trade in Endangered Species of Wild Fauna and Flora; DNA, Deoxyribonucleic acid; HWE, Hardy-Weinberg Equilibrium; InDel, Insertion or Deletion; ISFG, International Society for Forensic Genetics; IUCN, International Union for Conservation of Nature; IWT, Illegal Wildlife Trade; MPS, Massive Parallel Sequencing; POE, Probability of Exclusion; POI, Probability of Identification; PCR, Polymerase Chain Reaction; SNP, Single Nucleotide Polymorphism; STR, Short Tandem Repeats; SSR, Simple Sequence Repeat.

* Correspondence to: Leibniz Institute for the Analysis of Biodiversity Change (LIB), Adenauerallee 127, 53113 Bonn, Germany.
E-mail addresses: A.Mozer@leibniz-lib.de (A. Mozer), a.consul@leibniz-lib.de (A. Consul), b.misof@leibniz-lib.de (B. Misof), richard.jaeger@h-brs.de (R. Jäger), k.olek@t-online.de (K. Olek), j.astrin@leibniz-lib.de (J.J. Astrin).

https://doi.org/10.1016/j.fsiae.2024.100084
Received 10 November 2023; Received in revised form 15 January 2024; Accepted 1 February 2024
Available online 6 February 2024

birds from the country or importing exotic birds is prohibited, unless these are captive-bred [9]. However, several studies showed that frequently animals asserted being captive-bred are actually taken from the wild (e.g. [10,11]). A study by Shepherd et al. found that the high volume of captive-bred, CITES-listed bird species, mostly exported from the Solomon Islands, would suggest several thousand breeding pairs at captive-breeding facilities. However, not a single captive-breeding facility for birds is officially registered on the Solomon Islands, indicating that most, if not all, allegedly captive-bred birds must, in fact, be wild-caught [12].

For owls, an increasing trend of trafficking has been recorded [13,14]. These animals are to a great extent traded for pet keeping [14,15] as well as for other purposes, such as food in restaurants [16] or as traditional medicine for the treatment of weakening eyesight [17]. Furthermore, in Japan, captive-bred owls are kept in "bird cafes" for entertainment [14]. Currently, individually numbered bird rings and documents are used to certify a bird as captive-bred. However, cases of laundering wild-caught birds as supposedly captive-bred have been recorded, with the bird rings being falsified [18] and documents being forged [10]. Therefore, this issue remains of forensic relevance and calls for a molecular tool for revealing IWT.

This study proposes the use of SNPSTR markers to authenticate the origin of traded animals. They were first described by Mountain et al. [19]. A SNPSTR is a combined marker that contains a short tandem repeat (STR; also known as simple sequence repeat, SSR, or microsatellite) as well as a single nucleotide polymorphism (SNP) in close proximity to the STR, usually within less than 400 bp [19]. Thus, both variants can be PCR-amplified with one primer set. Furthermore, spatial proximity renders the probability of interchromosomal recombination within a SNPSTR marker negligible [19]. The usability of other types of compound markers has been successfully demonstrated, e.g. STRs combined with Alu deletions [20] or STRs combined with deletion–insertion polymorphisms [21]. SNPSTRs have been used in human forensic science e.g. to resolve unbalanced DNA mixtures [22]. In the context of wildlife conservation, Farke et al. analysed STRs and flanking SNPs in eastern Hermann's tortoises (*Testudo hermanni boettgeri*) [23]. Thus, in wildlife forensics, SNPSTRs are promising markers and might provide the basis of a molecular approach to effectively reveal IWT. To present a case study for the application of SNPSTR markers, the current study focuses on the little owl *Athene noctua* (Scopoli, 1769). *A. noctua* belongs to the family Strigidae and 12 subspecies are recognised [24]. The species is characterized by its compact size of around 19–25 cm while weighing usually 160–250 g [24]. *A. noctua* is native to a variety of habitats in Europe, North Africa, and Asia and has been introduced in England and New Zealand [24,25]. Although *A. noctua* is classified as "Least concern" by the IUCN red list [25], several countries have recorded a significant decline in population sizes (e.g. [26,27]). *A. noctua* is listed in CITES Appendix II [28]. Therefore, trade is regulated to prevent species extinction in the future due to overexploitation. This listing seems reasonable considering that *A. noctua* is among the three most traded, CITES-listed owl species [13]. Poaching [29] and illegal capture of little owls [30], probably to be kept as pets [31], and illegal trade [32] have been recorded in many countries. So far, separate STR (e.g. [33,34]) and SNP markers (e.g. [35]) have been developed for *A. noctua*. However, these markers have mainly been used for genetic assessment of populations.

According to the National Research Council, research should be directed towards forensic marker systems that make each profile unique [36]. While the emerging technologies of Massive Parallel Sequencing (MPS) entail higher costs as compared to current standard capillary electrophoresis systems, the ISFG highlights the advantages of MPS (higher resolution; easier analysis of mixtures, degraded samples, stutter or artifacts; no need to develop an allelic ladder, etc.) and sees a shift towards increased use of MPS methods in the future [37]. By inevitably including SNPs in the STR amplicons, MPS suggests the analysis of a forensic marker system, SNPSTR, that combines a short tandem repeat (STR) and single nucleotide polymorphisms (SNPs) within one amplicon and has a higher discriminatory power than either marker type in isolation. As a proof of concept, this study establishes a SNPSTR set for *A. noctua* and demonstrates its usefulness for individual identification and for assessing parentage in a wildlife forensic framework.

2. Materials and methods

2.1. DNA samples

Little owl samples (tissues; n = 14; 10 individuals sampled to establish the SNPSTR marker set, and additional four samples for family testing (two parents, one offspring, and one unrelated individual)) were obtained from the Biobank at the Leibniz Institute for the Analysis of Biodiversity Change. Sampling covers the species' distribution range in Germany and Italy and includes a family from a raptor center in Germany.

DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) or BioSprint 96 Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Species confirmation and control for contamination was ensured in accordance with recommendations of the International Society for Forensic Genetics (ISFG) regarding the use of non-human (animal) DNA in forensic genetic investigations [38] by DNA barcoding [39], i.e. sequencing a 658 bp fragment of the cytochrome c oxidase subunit 1 gene with primers LCO1490-JJ (5′-CHACWAAYCATAAAGATATYGG-3′) and HCO2198-JJ (5′-AWACTTCVGGRTGVCCAAARAATCA-3′) and respective PCR conditions from Astrin and Stüben [40] and comparison to the Barcode of Life Data System (BOLD; [41]).

Concentrations of the extracts were quantified by the QuantiFlour ONE dsDNA System on a Quantus Fluorometer (Promega, Fitchburg, United States). If concentrations were below 10 ng/μL, 100 μL of the sample were concentrated on a Savant Speed Vac SPD111V (Thermo Scientific, Waltham, United States) at 35 °C for 30 min

2.2. Selection of STR Candidate Loci

Whole genome sequencing was performed on one sample after TruSeq DNA PCR-free library preparation on a NovaSeq 6000 (150 bp, paired-end; Illumina, San Diego, United States; Macrogen, Amsterdam, The Netherlands). Prior to filtering, reads were trimmed using fastp ([42]; minimum length requirement: 100 bp; phred quality: ≥ Q15) and assembled using SPAdes ([43]; -isolate paired-end). Scaffolds were filtered for specified candidate STRs (tetranucleotides, with 11–20 repeats, with motifs of at least three different bases, and with no further repetitive sequence 170 bp up- or downstream of the STR) using PERF [44], CD-HIT [45], and BEDTools [46].

2.3. Multiplex PCR

Primer pairs for 30 suitable STR candidates were designed using PrimerPlex (v. 2.76, Premier Biosoft, San Francisco, United States). Primers were synthesised by Metabion (Planegg, Germany). PCRs were carried out with the Multiplex PCR kit (Qiagen; primer concentration 0.2 μM) on a Biometra TGradient Thermocycler (Analytik Jena, Jena, Germany) using at least 20 ng of genomic DNA as template and including a blank sample to check for possible contamination. A 12-step temperature gradient of 60 ± 3 °C was applied to the PCR block and the optimal annealing temperature was determined visually based on fluorescence after an agarose gel electrophoresis (1.5%, 100 V, 60 min; stained with GelRed, Biotium, San Francisco, United States). The PCR was carried out according to the manufacturer's protocol with a modified temperature cycle (95 °C for 15 min, (95 °C for 30 s, 63 °C for 1.5 min, and 72 °C for 1.5 min) for 35 cycles, 72 °C for 10 min, 4 °C for ∞), again, a blank was used to check for possible contamination prior to library preparation (agarose gel electrophoresis; same conditions as

described above).

2.4. MPS analysis

Before library preparation, PCR products were purified with AMPure XP beads (Beckman Coulter, Indianapolis, United States). Libraries were prepared with the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, United States). Samples were sequenced on a MiSeq using a Nano v2 kit (Illumina). Reads were merged, trimmed, mapped, and analysed using Geneious Prime (v. 2022.1.1, plugin BBDuk, Auckland, New Zealand). Minimum coverage per called allele was 10x. Heterozygosity was determined by $\geq$ 0.15 of the reads, but flanking regions were also considered to identify unbalanced alleles and artifacts. The STR sequence and allele nomenclature followed the DNA Recommendations 1997 of the ISFG [47], while SNP nomenclature followed the Human Genome Variation Society (HGVS) Recommendations [48]. A locus was considered a SNPSTR if the STR was variable and at least one SNP has been found among the 10 samples.

Sequencing data were uploaded to NCBI (BioProject ID: PRJNA954578; SRA Runs: 31663787- 31663773) and primer sequences, as well as the full reference sequence of all SNPSTR markers (as per Recommendation #6 of the ISFG regarding the use of non-human (animal) DNA in forensic genetic investigations [38] and Consideration 6 of the DNA commission of the ISFG on minimal nomenclature requirements [37]), can be found in Appendix Table A1.

2.5. Statistical analysis

Due to the absence of a chromosome-level genome assembly of a strigiform species, candidates could not be mapped on chromosomes to avoid linkage. Therefore, linkage disequilibrium was accounted for by avoiding complementary STR motifs (implemented in PERF; [44]) and using only one candidate per assembled scaffold.

Relevant parameters (Recommendation #10 of the ISFG: Recommended parameters regarding the use of non-human (animal) DNA in forensic genetic investigations [38]), such as the number of alleles (N_a), allele frequencies, observed heterozygosity (H_o), expected heterozygosity (H_e), unbiased heterozygosity expected (uHe), Chi-Square test for Hardy-Weinberg-Equilibrium (HWE), fixation index and probability of identity (POI), were calculated using GenAIEx software [49,50] based on the 10 sampled individuals. The network for AthNoc29 was generated with Network 10 software (v. 10.2, Fluxus Technology, Colchester, England).

According to the ISFG: Recommendations regarding the use of non-human (animal) DNA in forensic genetic investigations [38], specificity should also be demonstrated. We therefore tested our SNPSTR set also with another owl species (*Bubo bubo*), as well as a member of the Piciformes and a member of the Psittaciformes.

2.6. Parentage Testing

The probability of exclusion (POE) for the SNPSTR marker set was computed using GenAIEx software [49,50]. A direct comparison of the genotypes of two parents, an offspring, and an unrelated individual was also analysed to determine the alleged parentage of parent 1 and parent 2 to the offspring and the unrelated individual.

3. Results

3.1. Establishing a SNPSTR set for Athene noctua

In order to find variable SNPSTR markers we chose to sequence STRs and analyse the candidate loci for flanking SNPs (see Fig. 1). Overall, a set of 20 polymorphic SNPSTR markers was established which can be amplified in a single PCR reaction (Fig. 1 and Table 1). The number of alleles (N_a), observed (H_o), and expected heterozygosities (H_e), the

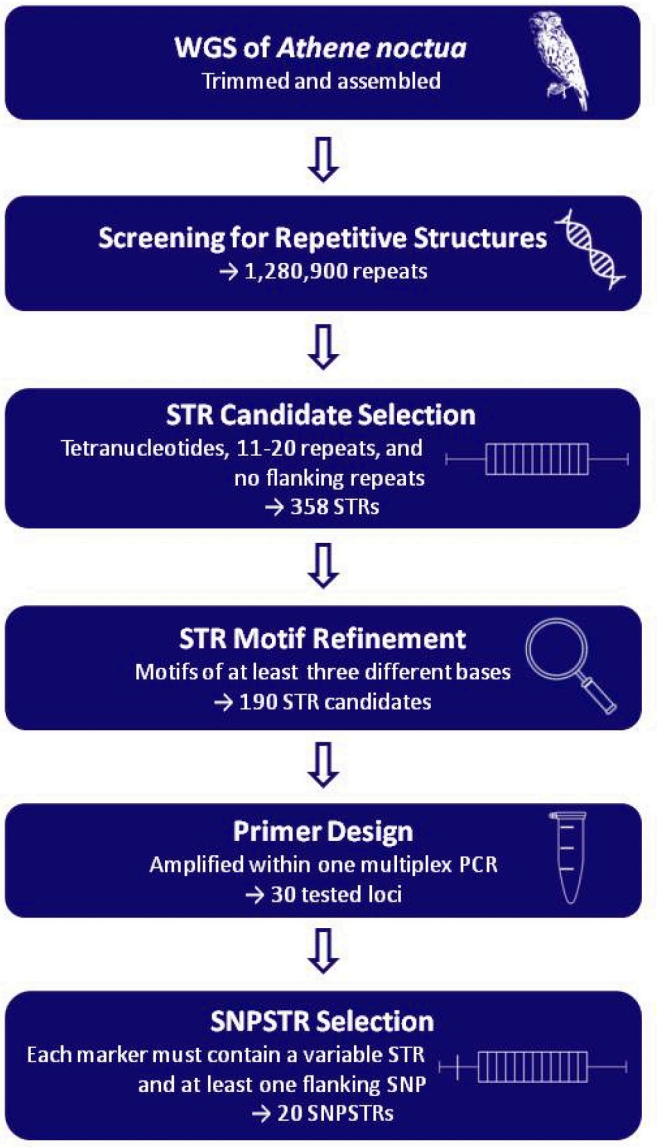

Fig. 1. Workflow and results of SNPSTR marker development for *Athene noctua*. First, a whole genome sequencing (WGS) run was performed. After trimming and assembling the obtained reads, the sequences were screened for repetitive structures. These repetitive structures were filtered for tetranucleotide short tandem repeats (STRs) with 11–20 repeats and no other flanking repetitive structures in close proximity to the STR. STRs with motifs consisting of at least three different bases (e.g. AGAT) were then selected. From these candidates, a multiplex PCR was designed that encompassed 30 candidate loci. These were then used to amplify and sequence 10 samples. Each locus was analysed for STR variability and the presence of at least one flanking single nucleotide polymorphism (SNP). If both were confirmed, a so-called SNPSTR marker was described. In total, 20 SNPSTR markers for *Athene noctua* were identified in this study.

reference STR motif, and the SNPs found for each SNPSTR marker are stated in Table 1.

Identified alleles per sample, allele frequencies, and deviations from HWE of the described SNPSTR markers are listed in Appendix Table A2, A3, and A4, respectively. The majority of markers conformed to the HWE, while four markers (AthNoc5, AthNoc7, AthNoc24 and AthNoc29) did not. The total number of observed alleles per marker ranged from 5 to 11, averaging 7.6 $\pm$ 2.0 alleles per marker. Observed heterozygosities (H_o) ranged from 0.400 up to 1.0 (mean = 0.715 $\pm$ 0.187), while expected heterozygosities (H_e) ranged from 0.545 to 0.850 (mean

Table 1
Set of 20 SNPSTR markers for *Athene noctua*. For each SNPSTR, the number of identified alleles (N_a), the observed (H_o) and expected (H_e) heterozygosity, the STR motif of the reference and the SNPs are stated. SNPs printed in grey were only found during parentage testing and not included in the statistical analysis.

Locus	N_a	H_o	H_e	STR motif	SNPs
AthNoc1	6	0.700	0.770	$[ATAG]_{12}$	122 A>G, 126 G>A, 242 C>T
AthNoc2	8	0.900	0.820	$[ATAG]_{12}[AT]$	41 G>C, 297 T > G
AthNoc5	9	0.500	0.840	$[TAGA]_{12}[TA]$	61 G>A, 78 G>C, 136 G>A, 279_280insT, 284 A>G
AthNoc7	11	0.800	0.840	$[CAGA]_2$ $[TAGA]_{12}$	80 G>T, 187 G>A
AthNoc8	7	0.800	0.700	TCCC TTCT TCCT $[TCCA]_{11}[TCC]$	175 G>A, 241 A>G
AthNoc9	9	1.000	0.827	$[AGAT]_{13}$ $[GATA]_2[GAT]$ $[GATA]_2[GA]$	43 C>A, 254 G>T, 260 T > C
AthNoc11	5	0.600	0.765	$[ATCC]_{11}$	205 C>T, 264 T > C
AthNoc12	5	0.500	0.545	$[AGAT]_{11}[A]$	270 G>A
AthNoc13	6	0.800	0.795	$[ATCC]_{11}$	86 A>G
AthNoc14	8	0.700	0.800	$[CATC]_{12}[CT]_2$	43 G>C
AthNoc16	5	0.500	0.585	$[CATC]_{11}[C]$	227 G>A, 253 G>A, 288 A>G
AthNoc17	5	0.800	0.720	$[GAAT]_{13}[G]$	78 C>A, 129 C>T, 207 G>T
AthNoc21	9	0.900	0.850	$[GATA]_{11}[GAT]$	145 G>A, 255 C>A, 261 G>A, 290 T > C
AthNoc23	7	0.400	0.780	$[ATCC]_{11}$	32 A>T, 32delA, 213 T > C
AthNoc24	6	0.400	0.640	$[TCCA]_{11}[TC]$	105 A>G, 170 T > A
AthNoc25	8	0.700	0.705	$[TCCA]_{11}[TCC]$	35delG
AthNoc26	8	1.000	0.825	$[TCCA]_{13}$ CCCA	97 C>A, 106 T > C, 215 C>T, 271 T > G
AthNoc28	7	0.900	0.780	$[ATCC]_{12}[A]$	105 A>C, 229 C>T
AthNoc29	11	0.600	0.840	$[ATAG]_{11}[ATA]$	146 A>G, 150 C>T, 162 G>A, 236 T > C, 244 G>A, 272 T > C
AthNoc30	11	0.800	0.840	$[GATA]_{13}$ GATG	106 C>T, 210_213delAATG, 265 G>A

$= 0.763 \pm 0.089$). Half of the found SNPSTRs consisted of a compound STR motif, nine SNPSTRs had a simple STR motif (for all 10 tested samples) and one showed a complex STR motif. In 50% of the loci, the STR motif was ATCC (including variations thereof, e.g. TCCA or CATC), followed by AGAT (and variations hereof; 45% of the loci) and AATG (5% of the loci). Overall 49 SNPs were recorded, on average 2.45 ± 1.31 SNPs per marker were found. The transition/transversion ratio was 0.484.

Although InDels were not specifically sought, they were recorded when observed (found in four markers). Furthermore, variable STR markers with no flanking SNP were recorded as well (AthNoc3, AthNoc4, AthNoc6, AthNoc10, AthNoc15, AthNoc19, AthNoc20, and AthNoc22; primer and STR information can be found in Appendix Table A1 and A2, respectively) as these still contain forensically valuable information, although not used in this study.

The combined marker set achieved a probability of identity (POI) of $5.3 * 10^{-23}$ for non-related individuals and a POI of $5.7 * 10^{-9}$ for siblings. The fixation index of the tested individuals was 0.066.

To test the species specificity of the primer set, the MPS assay was also applied to DNA from the owl species *Bubo bubo*, as well as from a member of the Piciformes and a member of the Psittaciformes, respectively. The results of the specificity can be found in Appendix Figure A1. While products within the expected size ranges were observed for *B. bubo,* no amplifications could be detected for Australaves.

To demonstrate the potential of the SNPSTR markers to delineate evolutionary relationships of haplotypes of one locus, an evolutionary network was established for the observed SNPSTR haplotypes for AthNoc29 (Fig. 2).

3.2. Parentage testing

Probabilities of exclusion (POE) for various constellations, as calculated for the combined marker set from the population data (Appendix Table A3), are presented in Table 2 and show that the marker set is suitable for parentage analysis. In Table 3, the identified genotypes of a family (two parents, one offspring) and an unrelated individual are listed. If the unrelated individual were sold as an alleged offspring of parent 1 and parent 2, genetic analysis with the SNPSTR set could exclude that this owl is an offspring of the tested parents.

Table 2
Probability of exclusion (POE) using 20 SNPSTR markers.

	Combination of 20 SNPSTR
Probability of Exclusion (POE 1 - When the other parent is known)	0.999999982583489
Probability of Exclusion (POE 2 - When the genotype of one parent is missing)	0.999976703958464
Probability of Exclusion (POE 3 - Excluding a putative parent pair)	0.999999999999944

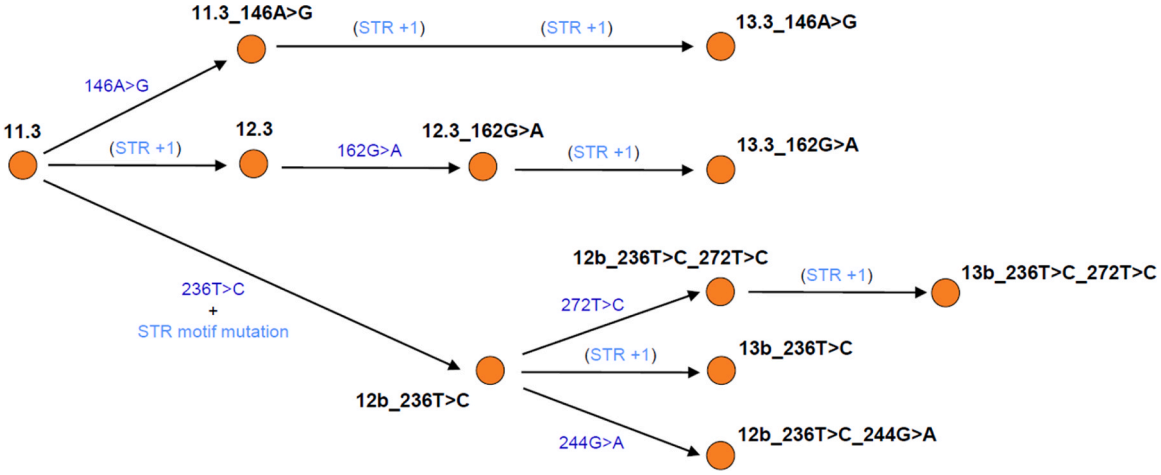

Fig. 2. Network of observed SNPSTR haplotypes for AthNoc29. The orange dots represent sampled haplotypes, changes in dark blue show SNP events while light blue ones indicate a mutation of the STR (repeat unit +1). Due to the different mutation rates of SNPs and STRs, the length of the branches does not reflect time. Created with Network (v. 10.2, Fluxus Technology, Colchester, England).

Table 3
Genotypes of a family of *Athene noctua* and an unrelated individual obtained from 20 SNPSTR markers. Alleles passed on from the parents to the offspring are highlighted in bold letters.

Locus	Parent 1	Parent 2	Offspring	Unrelated Individual
AthNoc1	**10** | 11	**10** | 12	**10**	11 | 12
AthNoc2	**41 G>C_11.2**	**41 G>C_9.2** | 41 G>C_10.2	**41 G>C_9.2** | **41 G>C_11.2**	11.2_297T>G | 41 G>C_12.2
AthNoc5	**13.2_279_280insT**	**78 G>C_13.2**	**78 G>C_13.2** | **13.2_279_280insT**	12.2 | 12.2_279_280insT
AthNoc7	**11c** | 11d_18	**13b** | 14b	**11c** | **13b**	12 | 13
AthNoc8	10.3 | **10.3_241A>G**	**10.3**	**10.3** | **10.3_241A>G**	10.3 | 11.3
AthNoc9	**16** | 17_260T>C	**15**	**15** | **16**	17
AthNoc11	**11_205C>T** | 11_264T>C	**10** | 11	**10** | **11_205C>T**	11
AthNoc12	12.1 | **15.1b**	**11.1** | 12.1	**11.1** | **15.1b**	11.1
AthNoc13	7 | **86 A>G_9b**	9 | **86 A>G_10b**	**86 A>G_9b** | **86 A>G_10b**	11 | 12
AthNoc14	11 | **13**	9 | **11c**	**11c** | **13**	12 | 13
AthNoc16	10.1 | **11.1_253G>A_288A>G**	9.1 | **12.1**	**11.1_253G>A_288A>G** | **12.1**	11.1
AthNoc17	**12.1**	10.1 | **12.1**	**12.1**	13.1
AthNoc21	**8.3_255C>A_290T>C** | 11.3_255C>A_290T>C	9.3_255C>A_290T>C | **11.3_255C>A_290T>C**	**8.3_255C>A_290T>C** | **11.3_255C>A_290T>C**	10.3 | 11.3_255C>A_290T>C
AthNoc23	**32 A>T_9_213T>C**	**32 A>T_9_213T>C** | 12	**32 A>T_9_213T>C**	11
AthNoc24	**12.2** | 105 A>G_12.2	**12.2** | 13.2	**12.2**	11.2 | 105 A>G_13.2
AthNoc25	**10.3**	7.3 | **10.3**	**10.3**	11.3
AthNoc26	106 T > C_11 | **12**	12b | **106 T > C_12_215C>T_271T>G**	**12** | **106 T > C_12_215C>T_271T>G**	106 T > C_11 | 13
AthNoc28	**9.1** | 12.1	11.1 | **12.1**	**9.1** | **12.1**	10.1 | 12.1
AthNoc29	**150 C>T_12d_236T>C** | 14c_236T>C_272T>C	11.3 | **146 A>G_12.3**	**150 C>T_12d_236T>C** | **146 A>G_12.3**	11.3 | 146 A>G_11.3
AthNoc30	**12**	**12**	**12**	13 | 14

4. Discussion

4.1. Establishing a SNPSTR set

Given the ongoing trend of owl trafficking [13,14], the development of a molecular tool is urgently needed to help combat illegal wildlife trade before it severely and irreversibly affects the populations of *A. noctua*, as it already occurred in many other species. In this proof of concept study, we have established a set of 20 unlinked SNPSTR loci that can simultaneously be PCR-amplified and analysed by massive parallel sequencing (MPS) in order to reveal identity and family relationships of small owls. However, the population data used in our study were derived from only ten individuals, and several SNPSTR alleles were observed only once. Therefore, it should be kept in mind that true allele frequencies may be under- or overestimated and our population data are not ready to use.

This study proves that one of the major advantages of SNPSTR markers is the higher power of discrimination of SNPSTRs compared to the more commonly used STR markers because STR isoalleles (having the same amplicon size) can be resolved by SNPs. Using conventional capillary electrophoresis (CE), for AthNoc29 only three STR alleles (11.3, 12.3, 13.3) would have been identified, in contrast to the 11 haplotypes that can be resolved through SNPSTR analysis and MPS (see Fig. 2 and Appendix Table A2). This analysis allows for discrimination of isoalleles bearing sequence variants in the repeat units (e.g. 13 and 13c) and of isoalleles bearing SNPs in the flanking regions that are part of the amplicon (e.g. for 11.3 and 11.3_146A>G). For the whole SNPSTR set, the expected heterozygosity (H_e) for all markers was ≥ 0.7, except for three (AthNoc12 with H_e of 0.545, AthNoc16 with H_e of 0.585, and AthNoc24 with H_e of 0.640). Two markers (AthNoc9 and AthNoc26) even reached a H_o of 1.00 (see Table 1). In human forensics, the heterozygosity of a suitable marker should be at least 0.7 [51]. However, as this study uses a small sample size (with individuals from two different countries), this could have a strong effect on these tested parameters and could also explain why several markers are not in HWE. Nevertheless, for most loci, allele frequency distributions were relatively balanced (Appendix Table A4), and only four loci displayed statistically significant deviations from HWE (Appendix Table A3). We assume that with increasing sample size, the markers with a H_e below 0.7 would likely also reach higher heterozygosity given the fact that the analysis of the four additional samples for parentage testing revealed additional alleles already (Table 1 in grey; these were not considered for the statistic calculations of allele frequencies, POI, and POE as the three individuals were related to each other). Additionally, it is possible that an increased sample size might reveal SNPs in the STRs proper described here (AthNoc3, AthNoc4, AthNoc6, AthNoc10, AthNoc15, AthNoc19, AthNoc20, and AthNoc22).

The balance of heterozygous alleles per SNPSTR marker is 0.44 $\pm$ 0.02, while an average of 0.04 $\pm$ 0.01% of the reads per marker were identified as stutters (−8 bp, −4 bp, +4 bp, and +8 bp combined; Appendix Table A4). Thus, another advantage of the SNPSTR markers is their ability to help resolve unbalanced alleles and identify possible stutters.

Although the percentage of reads for each marker is relatively evenly balanced (0.04 $\pm$ 0.02), we recommend increasing the primer concentrations for AthNoc2, AthNoc9, and AthNoc12 in future use, as these concentrations, although still analysable, were below the range of the standard deviation (Appendix Table A4).

Although it is generally assumed that as a standard for a representative database, 200 unrelated samples should be included (e.g. [52]), in wildlife forensics this often is not feasible, especially in rare or critically endangered species. Nevertheless, in the past, wildlife forensic prosecutions have been conducted based on significantly smaller databases. For example, poaching of a red-tailed black cockatoo (*Calyptorhynchus banksii*) has been proven and led to a conviction based on a database with 30 individuals [53]. However, sample sizes should reflect the population(s) in question.

As mentioned, the sample size in this study was very limited and thus the population data are hardly representative of a native population. As *A. noctua* is a sedentary bird [54] and does not show strong dispersal behaviour [26,27], European populations form two genetically distinct population clusters [34]. However, strong genetic admixture has been detected in northern and central Europe [34]. Future studies may determine whether a single database for *A. noctua* is statistically valid or whether multiple databases, depending on population clusters, are needed. In comparison to other STR studies in birds (e.g. [53,55]), the POI using all 20 SNPSTR markers ($5.3 * 10^{-23}$) was several orders of magnitude higher. If considering only allele lengths, the 20 markers of the present study resulted in a POI of $7.8 * 10^{-18}$ which is in the range of the aforementioned STR studies in birds [53,55] thus demonstrating the gain in POI by analysing the combined markers. Even if considering only the 16 SNPSTR markers conforming to HWE, a POI of $4.4 * 10^{-18}$ was

achieved. Although this study comprised only 10 individuals, for most loci more than 7 alleles were present. This is likely due to the higher variability of SNPSTR markers as compared to STRs. The fixation index (0.066) indicates no inbreeding between individuals. Given that the number of mature individuals of *A. noctua* amounts to five to ten million globally [25], the attained POIs will be more than sufficient, both for the SNPSTRs and the length-based analysis. However, the improved POI of SNPSTRs as compared to length-based analysis may be beneficial in cases of partial profiles e.g. due to sample degradation. Our study thus shows that using SNPSTR markers for *A. noctua* even a small population database can provide a sufficient POI. Thanks to the high POI, the SNPSTR set can also be used as a tool for forensic identity testing, e.g. in casework on thefts from zoos and animal parks. Such thefts have been recorded with increasing frequency in recent years [56]. Required reference samples can usually be obtained from materials (such as feathers) provided by the animals' caretakers or from previous veterinary examinations. Apart from zoo thefts, SNPSTR analysis can be applied to effectively connect crime scenes (e.g. a poached nest) to seized animals, as well as in other casework scenarios involving identity proving in IWT.

It could be argued that AthNoc25 does not constitute a SNPSTR marker proper since the SNP consists of a deletion rather than a substitution. However, some sources also consider single base pair insertions and deletions as SNPs (e.g. [57]) because their information value is comparable and relevant for forensic analysis. Therefore, we chose to include AthNoc25 in our set of SNPSTR markers. Furthermore, the relatively high presence of InDels (found in a fifth of our analysed markers), indicates another advantage of our method over traditional non-sequencing detection techniques: InDels in close proximity to the STRs can easily confound results in purely fragment length-based analysis. For example, the 4 bp InDel (equal in length to the STR repeat) in AthNoc30 (see Appendix Table A2) is located outside the repeat region. Several studies have also found such types of InDels in forensic human STRs (e.g. [37,58,59]).

The underlying dataset of this study is too small to assess the relationship between the STR motifs and flanking SNPs, but previous studies analysing flanking regions of STRs state that the mutation rate of the flanking region depends on the STR motif (e.g. [60–62]) as well as the STR length (e.g. [62,63]).

As can be seen in Fig. 2, analytically combining SNPs and STRs provides insight into the possible temporal order of mutation events and can be used to infer the relative age of SNPSTR haplotypes, based on the much higher propensity for replication slippage mutations of STRs than point mutations. For example for AthNoc29 haplotype 13b_236T>C_272 T > C seems to be younger than 12b_236 T > C since the haplotype 12b_236T>C_272T>C was also identified. Therefore, the SNP event likely occurred earlier than the STR mutation. The same might be true for 11.3_146A>G and 13.3_146A>G where the latter seems to be younger since 11.3 was also found. Here, a double-step mutation from 11.3_146A>G to 13.3_146A>G would also be a possible explanation; however, over 90% of the recorded STR mutations are single-step slippage events [64,65]. STR repeat unit loss is also an explanation model: It is possible that the 272 T > C SNP occurred after 13b_236T>C since haplotype 13b_236T>C_272T>C was identified and then a mutation with the loss of one repeat could have occurred resulting in 12b_236T>C_272T>C. In the event of 12.3_162G>A likely the STR mutation occurred before the SNP mutation, as the haplotype 12.3 was also sampled. However, more sampling would be required to be able to delineate which of the changes, the STR motif mutation or the 236 T > C occurred earlier. Nevertheless, it can be stated that overall, even the relatively small number of only 11 sampled haplotypes gave a promising insight into the genetic history of AthNoc29. The fact that a SNP can provide insights into the evolutionary history of a linked STR and vice versa has also been demonstrated by Ramakrishnan and Mountain. Their study for the estimation of divergence time showed that the analysis of 20 SNPSTR markers was more accurate and less biased than that of 100 STR loci [66]. Therefore, Fig. 2 not only demonstrates the higher power of resolution of SNPSTR markers compared to STRs alone, but also the potential of SNPSTR markers for genetic history applications in wildlife forensics. It is conceivable that SNPSTR markers could be used to distinguish populations more accurately and, conversely, to assign unknown samples to their population origin (e.g. [67]). This would be particularly interesting if all the markers could be analysed in combination using appropriate software. In addition, populations and species might be analysed and assessed using SNPSTR markers, thus providing also the basis for conservation legislation.

Klein et al. identified 15 polymorphic STR markers in barn owls (*Tyto alba*). Seven of these markers also showed polymorphy in *A. noctua* [68]. Here, amplification success of the *A. noctua* markers has been observed for another owl species but not for Australaves (Appendix Figure A1). It therefore appears that at least some of the SNPSTR markers we developed for *A. noctua* will also amplify in other Strigidae or even Strigiformes species and may show variation, potentially even species-specific SNP or STR alleles. However, due to e.g. primer binding site mutations undetected null alleles might occur, therefore validation studies have to be done before transfering the SNPSTR markers to another species. Nevertheless, the transferability of an established molecular marker set is particularly important in wildlife forensics. Since 1999, more than 5000 animal species have been seized worldwide in connection with IWT [69]. However, genetic tools exist for only a fraction of these species, so the transferability of established markers would save valuable time and resources in the fight against IWT. Future studies may shed light on the applicability of the same SNPSTR markers in related species, the possibility of species identification using SNPSTR marker sets, and the possibility of population-specific and therefore population analyses based on SNPSTR markers. However, further validation studies and, in particular, larger data sets are needed for this.

In summary, despite focusing on a small population cohort, this study identified a versatile SNPSTR marker set for *A. noctua*. While for forensic purposes, larger sample sizes seem generally preferable, in wildlife forensic practice, it is often impossible to compile large datasets. Our study thus proves that even only ten samples can be sufficient to identify variable SNPSTR markers for forensic testing.

4.2. Parentage testing

As the probability of parentage exclusion with 20 SNPSTR markers reached > 0.9999 (Table 2), the presented SNPSTR set can be used for parentage or kinship testing and thus reveal captive-bred vs. wild origin of traded owls. This is also shown exemplarily in the comparison of the genotypes of a family and an unrelated individual (Table 3). Here, the unrelated individual shows it cannot be an offspring of the tested parents in accordance with the Mendelian inheritance rules due to alleles of 15 markers that cannot be found in the respective genotypes (all except AthNoc8, AthNoc12, AthNoc14, AthNoc21, and AthNoc28). Again, the higher resolution of SNPSTRs becomes apparent, as a pure STR analysis would yield only 10 markers that were inconsistent with parentage. This would still be enough for exclusion but in challenging cases, like degraded material (leading to loss of certain alleles or loci), absence of one parent, or other scenarios the higher power of the SNPSTRs could make a significant difference. The combined likelihood ratios for the parents were in the order of 10^4-10^5 when the other parent was not available and 10^5-10^7 when the other parent was available (Appendix Table A5). For comparison, the combined likelihood ratios using only the length-based alleles of the loci considered were in the order of 10^4 when the other parent was not available and 10^5 when the other parent was available (Appendix Table A5). Although these pure length-based values would already be convincing, the SNPSTR-based values provide greater certainty, which could be particularly important if individual loci fail, e.g. in the case of degraded DNA. However, for these calculations several SNPSTR loci could not be taken into account, because their alleles were not recorded in the ten samples used to establish the

database and thus respective allele frequencies were unknown. Therefore, we also chose to calculate these specific loci on the basis of fragment length allele frequencies and yielded combined likelihood ratios in magnitudes of 10^5 and 10^7-10^8, respectively. Even when only length-based alleles were considered for all loci, the combined likelihood ratios were in the order of 10^5 and 10^6-10^7 (Appendix Table A5). Therefore, although more loci were available for the pure length-based calculation, the magnitude was the same or lower than for calculations including SNPSTR and length-based alleles, again demonstrating the greater certainty provided by SNPSTRs. However, the inclusion of more SNPSTR alleles (through SNPSTR allele frequencies due to more sampling) would certainly increase the likelihood ratios. For comparison, in human paternity testing, combined likelihood ratios of $> 10^4$ are typicaly considered as supporting an alleged paternity [70]. As the assessment of origin (captive-bred or wild-caught) is a major issue for authorities and experts in illegal bird trade [71], SNPSTR sets provide a viable solution to this challenge.

According to CITES, only second-generation and subsequent offspring from breeding facilities can be traded under the term "captive-bred" [72]. A certification based on SNPSTR markers could therefore effectively prove parentage in breeding lines. This would establish confidence in lawful breeders and would routinely detect wild-caught animals, which are usually poached opportunistically, implying that their genotypes will not be directly related to alleged parents. An additional advantage would be that routine genetic testing allows for early detection of signs of inbreeding and could thus reduce legal takings from the wild to augment breeding populations [73].

Although not observed within the data presented in this study, it is possible that a germline mutation occurs, especially given the high slippage mutation rate of STRs. For example, SE33, one of the most informative STR markers in human forensics [74], has a mutation rate of 1.7% [64]. In this context, SNPSTR markers bear a further advantage: In case of a slippage mutation of the STR, the SNP will still remain unchanged and vice versa, therefore the combination of both within one marker can be used to reveal a genealogical relationship even in case of a germline mutation of the STR part. This could also simplify the practice of Recommendation #9 of the ISFG regarding the use of non-human (animal) DNA in forensic genetic investigations [38] due to the verification by the other, combined variation.

4.3. Outlook on the usage of SNPSTR markers in wildlife forensics

Besides the advantages of SNPSTRs already mentioned, there are several others, such as the analysis of DNA mixtures. As SNPSTRs combine SNPs with STRs, mixture analysis would still be possible in contrast to biallelic SNPs where this is not the case. A study conducted by Tan et al. showed that the analysis of extremely unbalanced DNA mixtures is immensely improved by SNPSTR markers [22]. Moreover, SNPSTR markers would enhance the existing STR databases with additional information. One concern against a shift in a marker system is that prior investments into databases might be rendered obsolete, as would be the case for a shift from STRs to SNPs [75]. However, if future SNPSTR studies, like the current study, follow ISFG recommendations on minimal nomenclature requirements to ensure backward compatibility and comparison with existing STRs [37], SNPSTR markers can be used on existing STR databases. Furthermore, with the increasing use of MPS, many standard STRs will possibly turn out to actually represent SNPSTRs. Previous studies estimated that 25–50% of all human STRs bear the possibility to be SNPSTRs [19,66], and Agrafioti and Stumpf found bioinformatically over 600,000 SNPSTRs in humans, more than 800,000 in mice, and 250,000 in dogs [76].

Although the full extent of the SNPSTR markers used in this study can only be achieved by sequencing, SNPSTR markers can also be analysed by CE. CE is currently the standard method in forensic laboratories. SNP-specific primers as shown in Mountain et al. would be a compromise between MPS and CE. However, allelic ladders would be required for a valid CE analysis, and product sizes need to be considered, as multiplexing in CE is more limited than in MPS. Nevertheless, approximately 46% of European forensic genetics laboratories already possess a MPS system and over 26% plan to install one in their laboratory [77]. Despite the higher costs, it is therefore likely that the use of MPS systems will increase in the future, also as predicted by the ISFG [37], not least because the cost of sequencing has fallen significantly over the last two decades [78]. However, during the transition phase, SNPSTRs make sense in light of the compatibility between length-based STR databases and sequenced alleles, thus forensic practitioners working exclusively with STRs can still use SNPSTRs for their STR workflow.

5. Conclusion

Shepherd et al. [12] proposed owls should be ranked with a higher protection status, which would also direct more attention from law enforcement agencies. As demonstrated in this proof of concept study, a versatile tool for authorities to reveal IWT in *A. noctua* now exists. Using our set of SNPSTRs, individual identification, as well as parentage exclusion, can be proven with high levels of confidence despite a small dataset. Moreover, a deeper insight into the evolutionary history of a marker is also possible due to the combination of the two variation types within a SNPSTR. Given the fact that MPS technologies are on the rise, SNPSTR markers might be the tool of the future as they provide more information but still ensure backward compatibility with possibly existing STRs.

Within the FOGS project (Forensic Genetics for Species Protection; https://fogs-portal.de/en), we are establishing a database of SNPSTR sets for dozens of vertebrate species relevant to IWT. The database will be freely available to authorities, laboratories, and institutions and thus can be used e.g. to confirm or refute the legal origin of traded animals and should, we hope, help to prevent IWT.

Funding

This work is funded through the FOGS project by the German Federal Ministry of Education and Research under grant number 01LC1801A.

Ethical statement

All samples were obtained from the Biobank of the Leibniz Institute for the Analysis of Biodiversity Change, therefore no animals were harmed for this study.

CRediT authorship contribution statement

Mozer Annika: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Consul Albia:** Funding acquisition, Project administration, Writing – review & editing. **Misof Bernhard:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Jäger Richard:** Resources, Writing – review & editing. **Olek Klaus:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Astrin Jonas J:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Acknowledgments

The authors would like to thank Laura von der Mark, Bruno Hüttel, Christian Woehle, Julia Holtel, Camilla Bruno Di Nizo, France Gimnich,

Sarah Knieps, and Sophia Forat for their valuable help as well as all sample donators (Raptor Center Hellenthal, Zoo Wuppertal, Zoo Krefeld, and the Italian League for Bird Protection).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsiae.2024.100084.

References

[1] C. Nellemann, R. Henriksen, P. Raxter, N. Ash, E. Mrema (Eds.), The Environmental Crime Crisis: Threats to Sustainable Development from Illegal Exploitation and Trade in Wildlife and Forest Resources, United Nations Environment Programme, Nairobi, Kenya, Arendal, Norway, 2014.

[2] D.H. Barron, How the illegal wildlife trade is fueling armed conflict, Georget. J. Int. Aff. 16 (2015) 217–227.

[3] M.E. Zimmerman, The black market for wildlife: combating transnational organized crime in the illegal wildlife trade, Vanderbilt J. Transnatl. Law 36 (2003) 1657–1689.

[4] T. Kitade, Y. Naruse, Crossing the Red Line: Japan's Exotic Pet Trade, TRAFFIC, Japan Office, Tokyo, Japan, 2020.

[5] IPBES, Summary for Policymakers of the Global Assessment Report on Biodiversity and Ecosystem Services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, IPBES Secretariat, Bonn, Germany, 2019.

[6] BirdLife International, State of the World's Birds 2022: Insights and solutions for the biodiversity crisis, BirdLife International, Cambridge, UK, 2022.

[7] A. Brochet, W. van den Bossche, S. Jbour, P.K. Ndang'ang'a, V.R. Jones, W.A.L. I. Abdou, A.R. Al-Hmoud, N.G. Asswad, J.C. Atienza, I. Atrash, N. Barbara, K. Bensusan, T. Bino, C. Celada, S.I. Cherkaoui, J. Costa, B. Deceuninck, K. S. Etayeb, C. Feltrup-Azafzaf, J. FigeljI, M. Gustin, P. Kmecl, V. Kocevski, M. Korbeti, D. Kotrošan, J. Mula Laguna, M. Lattuada, D. Leitão, P. Lopes, N. López-Jiménez, V. Lucić, T. Micol, A. Moali, Y. Perlman, N. Piludu, D. Portolou, K. Putilin, G. Quaintenne, G. Ramadan-Jaradi, M. Ružić, A. Sandor, N. Sarajli, D. Saveljić, R.D. Sheldon, T. Shialis, N. Tsiopelas, F. Vargas, C. Thompson, A. Brunner, R. Grimmett, S. Butchart, Preliminary assessment of the scope and scale of illegal killing and taking of birds in the Mediterranean, Bird. Conserv. Int. 26 (2016) 1–28, https://doi.org/10.1017/s0959270915000416.

[8] Commission regulation (EC) of 23 March 2007 laying down animal health conditions for imports of certain birds into the community and the …, Off. J. Eur. Union (No 318/2007) (2007).

[9] Wild Bird Population Act of 1992: 16 U.S.C. §§ 4901–4916, 1992.

[10] D. van Uhm, Wildlife and laundering: interaction between the under and upper world, in: T. Spapens, R.D. White, D. van Uhm (Eds.), Green Crimes and Dirty Money, Routledge, Taylor and Francis Group, New York, NY, 2018, pp. 197–214.

[11] V. Nijman, C.R. Shepherd, Wildlife trade from ASEAN to the EU Issues With the Trade in Captive-bred Reptiles from Indonesia, TRAFFIC Europe,, Cambridge, UK, 2009.

[12] C.R. Shepherd, C.J. Stengel, V. Nijman, The Export and Re-export of CITES-listed Birds from the Solomon Islands, TRAFFIC, Southeast Asia, Petaling Jaya, Selangor, Malaysia, 2012.

[13] C.T. Panter, E.D. Atkinson, R.L. White, Quantifying the global legal trade in live CITES listed raptors and owls for commercial purposes over a 40-year period, Avocetta 43 (2019) 23–36, https://doi.org/10.30456/AVO.2019104.

[14] M. Vall-llosera, S. Su, Trends and characteristics of imports of live CITES-listed bird species into Japan, Ibis 161 (2019) 590–604, https://doi.org/10.1111/ibi.12653.

[15] S. Chng, J.A. Eaton, Snapshot of an on-going trade: an inventory of birds for sale in Chatuchak weekend market, Bangkok, Thailand, BirdingASIA 25 (2016) 24–29.

[16] C.R. Shepherd, L.A. Shepherd, An emerging Asian taste for owls? Enforcement agency seizes 1,236 owls and other wildlife in Malaysia, BirdingASIA 11 (2009) 85–86.

[17] M.A. Webster, Hong Kong's trade in wildlife, Biol. Conserv. 8 (1975) 203–211, https://doi.org/10.1016/0006-3207(75)90064-6.

[18] R.Rd.N. Alves, E.E.G. Nogueira, H.F.P. Araujo, S.E. Brooks, Bird-keeping in the Caatinga, NE Brazil, Hum. Ecol. 38 (2010) 147–156, https://doi.org/10.1007/s10745-009-9295-5.

[19] J.L. Mountain, A. Knight, M. Jobin, C. Gignoux, A. Miller, A.A. Lin, P.A. Underhill, SNPSTRs: empirically derived, rapidly typed, autosomal haplotypes for inference of population history and mutational processes, Genome Res. 12 (2002) 1766–1772, https://doi.org/10.1101/gr.238602.

[20] S.A. Tishkoff, E. Dietzsch, W. Speed, A.J. Pakstis, J.R. Kidd, K. Cheung, B. Bonné-Tamir, A.S. Santachiara-Benerecetti, P. Moral, M. Krings, Global patterns of linkage disequilibrium at the CD4 locus and modern human origins, Science 271 (1996) 1380–1387, https://doi.org/10.1126/science.271.5254.1380.

[21] V. Castella, J. Gervaix, D. Hall, DIP-STR: highly sensitive markers for the analysis of unbalanced genomic mixtures, Hum. Mutat. 34 (2013) 644–654, https://doi.org/10.1002/humu.22280.

[22] Y. Tan, P. Bai, L. Wang, H. Wang, H. Tian, H. Jian, R. Zhang, Y. Liu, W. Liang, L. Zhang, Two-person DNA mixture interpretation based on a novel set of SNP-STR markers, Forensic Sci. Int. Genet. 37 (2018) 37–45, https://doi.org/10.1016/j.fsigen.2018.07.021.

[23] C.M. Farke, K. Olek, W.M. Gerding, C. Distler, Multiple paternity and sperm storage in captive Hermann's tortoises, Testudo hermanni boettgeri determined from amniotic fluid adhering to the eggshell, Mol. Cell. Probes 29 (2015) 254–257, https://doi.org/10.1016/j.mcp.2015.05.009.

[24] D. van Nieuwenhuyse, J.-C. Génot, D.H. Johnson, The Little Owl: Conservation, Ecology and Behavior of Athene noctua, firstst pbk. edition, Cambridge University Press; Lightning Source UK Ltd,, Cambridge, New York, Milton Keynes UK, 2010.

[25] BirdLife International, Athene noctua, The IUCN Red List of Threatened Species (2019). ⟨https://doi.org/10.2305/IUCN.UK.2019–3.RLTS.T22689328A155470112.en⟩.

[26] M. Żmihorski, D. Altenburg-Bacia, J. Romanowski, M. Kowalski, G. Osojca, Long-term decline of the little owl (Athene noctua Scop. 1769) in Central Poland, Pol. J. Ecol. 54 (2006) 321–324.

[27] M. Šálek, L. Schröpfer, Population decline of the little owl (Athene noctua Scop.) in the Czech Republic, Pol. J. Ecol. 56 (2008) 527–534.

[28] CITES, Athene noctua, 2022. ⟨https://cites.org/eng/taxonomy/term/3299⟩ (accessed 3 December 2022).

[29] C.R. Shepherd, The owl trade in Jakarta, Indonesia: a spot check on the largest bird markets, in: BirdingASIA, 18, 2012, pp. 58–59.

[30] R.A. Molina-López, L. Darwich, Causes of admission of little owl (Athene noctua) at a wildlife rehabilitation centre in Catalonia (Spain) from 1995 to 2010, Anim. Biodivers. Conserv. 34 (2011) 401–405.

[31] D. Morris, Owl, Reaktion Books,, London, UK, 2009.

[32] C.T. Panter, R.L. White, Insights from social media into the illegal trade of wild raptors in Thailand. Traffic Bull. 32 (2020) 5–12.

[33] C. Pertoldi, I. Pellegrino, M. Cucco, N. Mucci, E. Randi, J. Terp Laursen, P. Sunde, Loeschcke, Volker, Nygaard Kristensen, Torsten, genetic consequences of population decline in the Danish population of the little owl (Athene noctua), Evolut. Ecol. Res. 14 (2012) 921–932.

[34] I. Pellegrino, A. Negri, G. Boano, M. Cucco, T.N. Kristensen, C. Pertoldi, E. Randi, M. Šálek, N. Mucci, Evidence for strong genetic structure in European populations of the little owl Athene noctua, J. Avian Biol. 46 (2015) 462–475, https://doi.org/10.1111/jav.00679.

[35] I. Pellegrino, L. Boatti, M. Cucco, F. Mignone, T.N. Kristensen, N. Mucci, E. Randi, A. Ruiz-Gonzalez, C. Pertoldi, Development of SNP markers for population structure and phylogeography characterization in little owl (Athene noctua) using a genotyping- by-sequencing approach, Conserv. Genet Resour. 8 (2016) 13–16, https://doi.org/10.1007/s12686-015-0513-8.

[36] National Research Council, The Evaluation of Forensic DNA Evidence: Committee on DNA Forensic Science: An Update, Commission on DNA Forensic Science: An Update, National Research Council, National Academy Press, Washington, D.C., 1996.

[37] W. Parson, D. Ballard, B. Budowle, J.M. Butler, K.B. Gettings, P. Gill, L. Gusmão, D. R. Hares, J.A. Irwin, J.L. King, P. de Knijff, N. Morling, M. Prinz, P.M. Schneider, C. van Neste, S. Willuweit, C. Phillips, Massively parallel sequencing of forensic STRs: considerations of the DNA commission of the International Society for Forensic Genetics (ISFG) on minimal nomenclature requirements, Forensic Sci. Int. Genet. 22 (2016) 54–63, https://doi.org/10.1016/j.fsigen.2016.01.009.

[38] A. Linacre, L. Gusmão, W. Hecht, A.P. Hellmann, W.R. Mayr, W. Parson, M. Prinz, P.M. Schneider, N. Morling, ISFG: recommendations regarding the use of non-human (animal) DNA in forensic genetic investigations, Forensic Sci. Int. Genet. 5 (2011) 501–505, https://doi.org/10.1016/j.fsigen.2010.10.017.

[39] P.D.N. Hebert, S. Ratnasingham, J.R. deWaard, Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species, Proc. Biol. Sci. 270 (Suppl 1) (2003) S96–S99, https://doi.org/10.1098/rsbl.2003.0025.

[40] J.J. Astrin, P.E. Stüben, Phylogeny in cryptic weevils: molecules, morphology and new genera of western Palaearctic Cryptorhynchinae (Coleoptera:Curculionidae), Invert. Syst. 22 (2008) 503, https://doi.org/10.1071/IS07057.

[41] S. Ratnasingham, P.D.N. Hebert, bold: the barcode of life data system (http://www.barcodinglife.org), Mol. Ecol. Notes 7 (2007) 355–364, https://doi.org/10.1111/j.1471-8286.2007.01678.x.

[42] S. Chen, Y. Zhou, Y. Chen, J. Gu, fastp an ultra-fast all-in-one FASTQ preprocessor, Bioinformatics 34 (2018) i884–i890, https://doi.org/10.1101/274100.

[43] A. Prjibelski, D. Antipov, D. Meleshko, A. Lapidus, A. Korobeynikov, Using SPAdes De Novo assembler, Curr. Protoc. Bioinforma. 70 (2020) e102, https://doi.org/10.1002/cpbi.102.

[44] A.K. Avvaru, D.T. Sowpati, R.K. Mishra, PERF: an exhaustive algorithm for ultra-fast and efficient identification of microsatellites from large DNA sequences, Bioinformatics 34 (2018) 943–948, https://doi.org/10.1093/bioinformatics/btx721.

[45] L. Fu, B. Niu, Z. Zhu, S. Wu, W. Li, CD-HIT: accelerated for clustering the next-generation sequencing data, Bioinformatics 28 (2012) 3150–3152, https://doi.org/10.1093/bioinformatics/bts565.

[46] A.R. Quinlan, I.M. Hall, BEDTools: a flexible suite of utilities for comparing genomic features, Bioinformatics 26 (2010) 841–842, https://doi.org/10.1093/bioinformatics/btq033.

[47] B. Olaisen, W. Bär, B. Brinkmann, B. Budowle, A. Carracedo, P. Gill, P. Lincoln, W. R. Mayr, S. Rand, DNA Recommendations 1997 of the International Society for Forensic Genetics, Vox Sang. 74 (1998) 61–63, https://doi.org/10.1159/000030907.

[48] J.T. den Dunnen, R. Dalgleish, D.R. Maglott, R.K. Hart, M.S. Greenblatt, J. McGowan-Jordan, A.-F. Roux, T. Smith, S.E. Antonarakis, P.E.M. Taschner, HGVS Recommendations for the Description of Sequence Variants: 2016 Update, Hum. Mutat. 37 (2016) 564–569, https://doi.org/10.1002/humu.22981.

[49] R. Peakall, P.E. Smouse, Genalex 6: genetic analysis in Excel. Population genetic software for teaching and research, Mol. Ecol. Notes 6 (2006) 288–295, https://doi.org/10.1111/j.1471-8286.2005.01155.x.

[50] R. Peakall, P.E. Smouse, GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update, Bioinformatics 28 (2012) 2537–2539, https://doi.org/10.1093/bioinformatics/bts460.
[51] A. Barbaro, STR Typing and available multiplex kits including validation methods, in: P. Shrivastava, H.R. Dash, J.A. Lorente, J. Imam (Eds.), Forensic DNA Typing: Principles, Applications and Advancements, firstst ed. twentiethtwentieth.,, Springer Singapore, Singapore, 2020, pp. 27–43.
[52] J.S. Buckleton, J.-A. Bright, D. Taylor, Forensic DNA Evidence Interpretation, Second edition, CRC Press, Boca Raton, FL, 2016.
[53] N.E. White, R. Dawson, M.L. Coghlan, S.R. Tridico, P.R. Mawson, J. Haile, M. Bunce, Application of STR markers in wildlife forensic casework involving Australian black-cockatoos (Calyptorhynchus spp.), Forensic Sci. Int. Genet. 6 (2012) 664–670, https://doi.org/10.1016/j.fsigen.2011.10.003.
[54] W. Hagemeijer, M.J. Blair, The EBCC Atlas of European Breeding Birds: Their Distribution and Abundance, T & A D Poyser, London, UK, 1997.
[55] C. Jan, L. Fumagalli, Polymorphic DNA microsatellite markers for forensic individual identification and parentage analyses of seven threatened species of parrots (family Psittacidae), PeerJ 4 (2016) e2416, https://doi.org/10.7717/peerj.2416.
[56] D.P. van Uhm, T. Spapens, Illegal trade in protected birds in the Netherlands, Crim. defiance Eur. Beyond.: Organ. Crime. Crime. -Terror Nexus (2020) 217–243.
[57] K. Vajpayee, D.C. Sagar, H.R. Dash, Forensic DNA typing: inception, methodology, and technical advancements, in: P. Shrivastava, H.R. Dash, J.A. Lorente, J. Imam (Eds.), Forensic DNA Typing: Principles, Applications and Advancements, firstst ed. twentiethtwentieth, Springer Singapore, Singapore, 2020, pp. 3–26.
[58] K.B. Gettings, R.A. Aponte, P.M. Vallone, J.M. Butler, STR allele sequence variation: current knowledge and future issues, Forensic Sci. Int. Genet. 18 (2015) 118–130, https://doi.org/10.1016/j.fsigen.2015.06.005.
[59] I. Gomes, A. Brehm, L. Gusmão, P.M. Schneider, New sequence variants detected at DXS10148, DXS10074 and DXS10134 loci, Forensic Sci. Int. Genet. 20 (2016) 112–116, https://doi.org/10.1016/j.fsigen.2015.10.005.
[60] E.J. Vowles, W. Amos, Evidence for widespread convergent evolution around human microsatellites, PLoS Biol. 2 (2004) E199, https://doi.org/10.1371/journal.pbio.0020199.
[61] M. Brandström, H. Ellegren, Genome-wide analysis of microsatellite polymorphism in chicken circumventing the ascertainment bias, Genome Res 18 (2008) 881–887, https://doi.org/10.1101/gr.075242.107.
[62] M.A. Varela, W. Amos, Heterogeneous distribution of SNPs in the human genome: Microsatellites as predictors of nucleotide diversity and divergence, Genomics 95 (2010) 151–159, https://doi.org/10.1016/j.ygeno.2009.12.003.
[63] M.F. Santibáñez-Koref, R. Gangeswaran, J.M. Hancock, A relationship between lengths of microsatellites and nearby substitution rates in mammalian genomes, Mol. Biol. Evol. 18 (2001) 2119–2123, https://doi.org/10.1093/oxfordjournals.molbev.a003753.
[64] D. Chandra, V.C. Mishra, A. Raina, V. Raina, Mutation rate evaluation at 21 autosomal STR loci: Paternity testing experience, Leg. Med. (Tokyo) 58 (2022) 102080, https://doi.org/10.1016/j.legalmed.2022.102080.
[65] B. Brinkmann, M. Klintschar, F. Neuhuber, J. Hühne, B. Rolf, Mutation rate in human microsatellites: influence of the structure and length of the tandem repeat, Am. J. Hum. Genet. 62 (1998) 1408–1415, https://doi.org/10.1086/301869.
[66] U. Ramakrishnan, J.L. Mountain, Precision and accuracy of divergence time estimates from STR and SNPSTR variation, Mol. Biol. Evol. 21 (2004) 1960–1971, https://doi.org/10.1093/molbev/msh212.
[67] R. Ogden, A. Linacre, Wildlife forensic science: a review of genetic geographic origin assignment, Forensic Sci. Int. Genet. 18 (2015) 152–159, https://doi.org/10.1016/j.fsigen.2015.02.008.
[68] A. Klein, G.J. Horsburgh, C. Küpper, A. Major, P.L.M. Lee, G. Hoffmann, R. Mátics, D.A. Dawson, Microsatellite markers characterized in the barn owl (Tyto alba) and of high utility in other owls (Strigiformes: AVES), Mol. Ecol. Resour. 9 (2009) 1512–1519, https://doi.org/10.1111/j.1755-0998.2009.02715.x.
[69] UNODC, World Wildlife Crime Report: Trafficking in Protected Species, United Nations, Vienna, 2020.
[70] A.R. Thomsen, C. Hallenberg, B.T. Simonsen, R.B. Langkjaer, N. Morling, A report of the 2002-2008 paternity testing workshops of the English speaking working group of the International Society for Forensic Genetics, Forensic Sci. Int. Genet. 3 (2009) 214–221, https://doi.org/10.1016/j.fsigen.2009.01.016.
[71] J. Ribeiro, L. Reino, S. Schindler, D. Strubbe, M. Vall-llosera, M.B. Araújo, C. Capinha, M. Carrete, S. Mazzoni, M. Monteiro, F. Moreira, R. Rocha, J.L. Tella, A.S. Vaz, J. Vicente, A. Nuno, Trends in legal and illegal trade of wild birds: a global assessment based on expert knowledge, Biodivers. Conserv 28 (2019) 3343–3369, https://doi.org/10.1007/s10531-019-01825-5.
[72] CITES, Resolution Conf. 10.16 (Rev.): Specimens of animal species bred in captivity, 2022. ⟨https://cites.org/sites/default/files/document/E-Res-10–16-R11_0.pdf⟩ (accessed 17 March 2023).
[73] CITES, Resolution Conf. 12.10 (Rev.): Registration of operations that breed Appendix I animal species in captivity for commercial purposes (2010).
[74] Z. Grubic, K. Stingl, N. Martinez, B. Palfi, V. Brkljacic-Kerhin, A. Kastelan, STR and HLA analysis in paternity testing, Int. Congr. Ser. 1261 (2004) 535–537, https://doi.org/10.1016/s0531-5131(03)01654-6.
[75] J.M. Butler, M.D. Coble, P.M. Vallone, STRs vs. SNPs: thoughts on the future of forensic DNA testing, Forensic Sci. Med. Pathol. 3 (2007) 200–205, https://doi.org/10.1007/s12024-007-0018-1.
[76] I. Agrafioti, M.P.H. Stumpf, SNPSTR: a database of compound microsatellite-SNP markers, Nucleic Acids Res. 35 (2007) D71–D75, https://doi.org/10.1093/nar/gkl806.
[77] T.E. Gross, J. Fleckhaus, P.M. Schneider, Progress in the implementation of massively parallel sequencing for forensic genetics: results of a European-wide survey among professional users, Int. J. Leg. Med. 135 (2021) 1425–1432, https://doi.org/10.1007/s00414-021-02569-0.
[78] K.A. Wetterstrand, DNA Sequencing Costs: Data from the NHGRI Genome Sequencing Program (GSP), 2021. ⟨https://www.genome.gov/sequencingcostsdata⟩ (accessed 14 January 2024).