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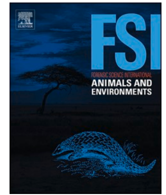

Original Research

STRoe deer: A validated forensic STR profiling system for the European roe deer (*Capreolus Capreolus*)

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A B S T R A C T

European roe deer (*Capreolus capreolus* L.) are the most common game species in Europe, hunted for meat and trophies. Forensic investigations involving roe deer poaching may often benefit from an individual identification method to link a suspect to a specific incident. The current paper presents a forensically validated DNA profiling system for European roe deer called "STRoe deer". This DNA profiling system consists of 12 novel unlinked tetranucleotide short tandem repeat (STR) loci and two sexing markers, with an allelic ladder to facilitate accurate genotyping. Validation results using 513 European roe deer samples collected from a single population from the Swiss Plateau demonstrated successful amplification of all 14 loci with as little as 0.05 ng of European roe deer DNA. Species-specificity tests showed that other members of the Cervidae family exhibited partial profiles and non-specific peaks, whereas most members of the Bovidae family showed just non-specific cross-species amplification products. Three different methods to calculate match probabilities for randomly sampled European roe deer genotypes resulted in median match probabilities ranging from 1.4×10^{-13} to 2.5×10^{-5}. These methods accounted for possible population structure, occurrence of null alleles and individual relatedness. Based on these results, we conclude that STRoe deer is a robust genotyping system that should prove a valuable tool for individual identification and sexing of European roe deer to support criminal investigations.

1. Introduction

It has been nearly 30 years since the first use of non-human DNA fingerprinting led to convictions for laundering wild birds of prey into captive falconry by refuting parentage claims [1]. Since then, the assignment of non-human DNA samples to a particular individual has been routinely used [2]. Reported forensic casework involving individual identification of animals includes many types of investigation, ranging from poaching of a wild boar [3] to armed robbery [4]. Unlike taxonomic identification of evidence, which can be performed based on morphological analysis or through DNA sequencing of universal barcoding genes, individual identification requires genetic analyses of multiple, taxon-specific, polymorphic DNA markers such as short tandem repeat (STR) or single nucleotide polymorphism (SNP) loci [5]. Besides individual identification, STR analyses have assisted forensic investigations regarding a specimen's population of origin [e.g. 6,7] or its wild or captive bred source, via parentage testing and population assignment tests. Despite the wide range of applications to criminal casework, the availability of published, forensically validated non-human DNA profiling systems complying with the current standards from organizations such as the International Society of Forensic Genetics (ISFG) [5] or the Society for Wildlife Forensic Sciences (SWFS) [8], has been limited to only a few species, such as dogs (*Canis lupus familiaris*), carpet pythons (*Morelia spilota*), Eurasian badgers (*Meles meles*), and tigers (*Panthera tigris*) [2,9–12]. Current standards regarding the use of non-human DNA in identity testing include, among others,

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recommendations for the selection of reference material, the primer design, the repeat motif, the nomenclature of STR alleles, and estimation of the relevant population and forensic genetic parameters [5,8].

The informative value of a possible match between the field evidence and a sample associated with a possible suspect has to be evaluated when applying STR profiling systems in forensic casework [5]. Therefore statistics such as match probabilities (MP) or likelihood ratios (LR) should be reported [8]. MP is the probability that another individual of the same population shares the DNA profile with the donor of the field evidence. LRs are a measure of how much more probable one proposition is relative to an alternative [13]. The formulas to calculate MP and LR can be adapted, depending for example on the information available about the donor of the profile (e.g. breed, relatedness, (sub)population of origin), the structure of the applied reference dataset (e.g. inbreeding, subpopulations), or the markers used to genotype a sample. One method, based on the recommendation 4.2 of the US National Research Council (NRCII) [14], requires the inclusion of an estimate of population subdivision (e.g. F_{ST}) which is especially important when calculating MPs for animal species. F_{ST} values among animal populations are often higher (e.g. 0.12 for Eurasian badgers [11] and 0.16 for dogs [15] than the values reported for human populations (0.01−0.03) [14] due to more pronounced sub-structure. Inserting zero for the F_{ST} value in the sampling formula [16] equates to the use of the simple product rule and leads to the lowest and hence least conservative MP.

The European roe deer (*Capreolus capreolus,* L.) is widespread in Europe; it is hunted both as a trophy and for its meat. In many countries, the roe deer hunting season is restricted to a few weeks each year, requires a permit, and is subject to a quota system set by the authorities [e.g. 17–19]. Successful prosecution of hunting regulation violations, i.e. poaching, often requires individual identification to link evidence collected at a potential crime scene (e.g. kill site) back to biological material (e.g. antlers, meat, trace blood samples) associated with the suspect who might have committed the offence [e.g. 20]. The number of deer taken illegally each year might be substantial, but reliable numbers are difficult to obtain; the act of poaching happens in secrecy. Therefore, validated tools such as STR profiling systems are invaluable to shed light on illegal kills [1,2]. Most STR markers currently applied to European roe deer, red deer (*Cervus elaphus*), or fallow deer (*Dama dama*), for example, were cross-amplified from loci developed for reindeer (*Rangifer tarandus*), mule deer (*Odocoileus hemionus*), tule elk (*Cervus canadensis nannodes*), or sika deer (*Cervus nippon*) [21–23], bearing the risk of ascertainment bias [24]. Additionally, some of these STRs are di-nucleotide repeats, which do not comply with current international forensic recommendations [5,8] due to a higher tendency to produce stutter artefacts and heterozygote imbalance compared to STR markers with longer repeat units [5].

In this paper we describe the development and validation of "STRoe deer", a European roe deer-specific STR profiling system created to shed light on suspected poaching cases. STRoe deer was designed following current ISFG and SWFS standards and recommendations regarding the use of non-human (animal) DNA [5,8] and validated on samples from a Swiss roe deer population. To assess STRoe deer's performance, we calculated MPs based on three different, widely used methods. As elsewhere, it is difficult to obtain reliable numbers for the actual level of roe deer poaching in Switzerland. Nonetheless, limited information are available regarding poaching cases for some of the Swiss cantons. The canton of Grisons reported 10–20 poaching incidents in 2016 [25], whereas in 2020, the canton of Valais reported a single poaching case involving 26 illegally killed animals (5 red deer, 12 roe deer, 6 chamois, and 3 hares) [26]. While the focus of the current study was to design a tool to address such issues in Switzerland, the broader aim of STRoe deer is to enable forensic practitioners to process cases requiring individual identification of European roe deer and facilitate standardization of this method across European wildlife forensic laboratories.

2. Material & methods

2.1. Sample collection and DNA extraction

Successful individual identification relies on a representative reference dataset of allele frequencies of the species (preferably for each population) of interest [5], in order to estimate relevant parameters needed to calculate the significance of a potential DNA profile match. To generate such a dataset, roe deer tissue (muscle), blood samples (collected with cotton swabs), and buccal swabs were collected from licensed hunters, butchers, and suspected poaching cases reported to the police. A total of 513 samples consisting of 253 tissue samples, 251 blood samples and 9 buccal swabs were collected between 2015 and 2017, originating from the Swiss Plateau. Tissue samples were stored at -20 °C until DNA extraction with the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's instructions. Blood and buccal swabs were dried and stored at room temperature before applying a Chelex DNA extraction method [27]. In brief, this method adds 1 mL of 5 % Chelex 100 (Bio-Rad) to each tube containing a piece of a blood or buccal swab, followed by vortexing, incubation for 1 h at 56 °C followed by incubation of 8 min at 100 °C. Finally, the tubes were centrifuged for 5 min at 13′600 x g before transferring 200 μL of the supernatant to a new tube for use as template DNA. The DNA extracts were quantified using a Quantus™ fluorometer (Promega).

2.2. Development of STRs and polymorphism screening

A roe deer reference genome was downloaded from GenBank (US National Center of Biotechnology Information) [https://www.ncbi.nlm.nih.gov/genome/17154 and 28] and screened for tetra-nucleotide STRs with QDD Version 3 [29]. Primers were designed with Primer 3 (integrated into QDD [30]). STRs for the initial polymorphism screening were selected based on the following criteria: tetra-nucleotide repeat motif; PCR-product length between 60 and 400 base pairs (bp); pure repeats with ≥8 repeat units in the representative genome; no mono- or dinucleotide repeats in the flanking regions; and only a single STR-motif per contig (to minimize the possibility of pairwise linkage between loci). To assess variability of 40 selected STRs, one of four universal primers (M13) was added to each forward primer as described by Kopps et al. [31]. Between two and four STR loci were combined into a multiplex PCR for screening. The 20 μL PCR reaction volume consisted of 10 μL 2x Qiagen Multiplex Master mix (Qiagen), primers (forward, reverse, fluorescently-labeled M13 tag; each 100–300 nM), 1 ng template genomic DNA and RNase-free water (Qiagen). Due to the lower annealing temperatures of the fluorescently labelled M13 universal primers compared to the sequence-specific primers, a touch-down PCR was run [32] on a GeneAmp 9700 Thermocycler gold (Thermo Fisher Scientific) instrument. PCR amplification was performed with the following thermocycling conditions: 15 min initial activation at 95 °C; 5 x [30 s at 94 °C, 90 s at 62 °C and 90 s at 72 °C]; 5 x [30 s at 94 °C, 90 s at 58 °C and 90 s at 72 °C]; 5 x [30 s at 94 °C, 90 s at 55 °C and 90 s at 72 °C]; 20 x [30 s at 94 °C, 90 s at 50 °C and 90 s at 72 °C]; with a final extension step of 10 min at 72 °C. The denatured PCR products were run on an ABI 3500 DNA Analyzer and evaluated with GeneMapper *ID-X* v1.4 software (both Thermo Fisher Scientific) using GS-600 (Liz) as a size standard (Thermo Fisher Scientific). We genotyped 200 European roe deer samples for the 40 STRs selected for screening. The number of alleles and the observed and expected heterozygosity of each locus were calculated in GenAlEx v6.5 [33,34]. After the initial screening, 13 autosomal STR loci were selected based on degree of polymorphism (≥ 4 alleles), absence of null alleles, and desired PCR-product length (60 to 355 bp).

2.3. Development of sexing markers

DNA sequence alignments of ZFX and SRY gene regions (located on

Table 1
Details of the optimized STRoe deer multiplex PCR: primer sequences for 13 autosomal STRs and two sexing loci, associated GenBank accession number, repeat motif, size range, number of alleles (in 513 individuals) and primer concentrations (equal amounts for forward and reverse primer). Added pigtails are underlined in the corresponding primer sequences. Capcap3.1 and Capcap37 were significantly linked, laboratories implementing STRoe deer can therefore decide which marker to keep in the multiplex reaction.

Locus name	GenBank accession number	Repeat motif	Primer sequence (5′-3′)	Size range (bp)	No. alleles	Dye	Primer concentration
Capcap1	MW800113	(CTAT)n	F: AGGATGGACAGGACCTTCTGA R: GATCCTCTCGCTTCTTCCTACT	65–100	7	NED	200 nM
Capcap2	MW800114	(GATA)n	F: TGGGTTCCCATTGCCTTCTC R: GTTTTCATGTGCTTATTGACCATCTGT	70–125	12	6-FAM	200 nM
Capcap3.1	MW800115	(TCTA)n	F: TGTCTTTCCTTGTCTATCTCTCTGT R: GTTTGTCAGAGAAACAGAACCAGCAG	80–110	6	VIC	200 nM
Capcap5	MW800116	(ATAG)n	F: ACCAGCTCTCATACAACACCC R: ACGTGGGTTAATGGTTCAAGGT	95–130	8	PET	400 nM
Capcap10	MW800117	(GATA)n*	F: GCACCTCACTGCAATGGAAAG R: GGAGTGGGAAGGTTGCTCTAG	185–230	9	6-FAM	200 nM
Capcap15	MW800118	(CTAT)n	F: GAATGCTGTGGTGTTCTCTGC R: GTTTCTTGGCTGGATTTCTGTTAGGACA	120–135	4	NED	500 nM
Capcap17	MW800119	(CATA)n**	F: TCCATTTGGTTGGTACACGGA R: GGAAGTCCCACTAGAGGCTCT	140–155	4	6-FAM	200 nM
Capcap25	MW800120	(TCCA)n	F: CAGATAACACGGTGGCTCTGA R: CATGGTGCCTGGTACATAGCT	155–175	5	VIC	400 nM
Capcap29	MW800121	(AGAT)n	F: AAGCCCATGACCTGAAACCAA R: GCTTCCAGCAGGAGGGTATAT	175–210	9	NED	400 nM
Capcap31	MW800122	(CCTA)n	F: ATTACACCACCAGCTTCCCTG R: GAGGCCCAGCATCTGAGTAAA	215–240	6	VIC	200 nM
Capcap35	MW800123	(ATCT)n***	F: ACCCAAACCAAACACCCTGAA R: GTTTCTCCCTCGGGATAATCAAGTATT	285–315	6	VIC	200 nM
Capcap36	MW800124	(TATC)n	F: GGCTCAGGGCTAAGATGTGAT R: GTTTTTGACCAGGAAGAAAGCCACA	300–350	12	6-FAM	300 nM
Capcap37	MW800125	(AGAT)n****	F: TTGTAACTCTAATAGACTTGAAGGGCA R: TTTGGCCTAAGTGCTTGAGCT	275–300	6	NED	500 nM
Deer_Sry	MW800126	n/a	F: TCACAGACAATCGCAGCGCA R: ACCCCAGCTGCTTGCTGATCT	180	1	PET	400 nM
Deer_Zfx234	MW803144	n/a	F: ACAGTTTCTGTGGTCTTAGGTTTCAC R: CCCCAAACTGTAAAGTCATGGGCCAC	235	1	PET	700 nM

[*] Allele 4 has a deletion (AGAT) between the primer binding site and the repeat tract.
[**] Allele 9′s last repeat is CATA > CATG.
[***] Allele 7 has a deletion (CTCT) after the repeat tract.
[****] Allele 7.2 has an insertion of $(AT)_5$AGTT in front of the actual repeat tract $(AGAT)_5$.

the X and Y chromosome respectively) were constructed using publicly available sequences for European roe deer and red deer (ZFX: DQ415950-1, SRY: DQ888700, GQ272386, DQ888691-2) in Geneious 5.1 (http://www.geneious.com/), hence they were not intended to be roe deer specific. Primers were designed for both genes using the Primer 3 [30] functionality within Geneious to amplify short regions of each, ensuring the control X-specific amplicon was longer than the Y-specific amplicon (Table 1). Sex determination was made by the observation of peaks at the ZFX and SRY fragment lengths, with females having only a ZFX fragment and males having both a ZFX and a SRY fragment. The shorter Y-specific amplicon minimizes the risk of observing only an X peak in male individuals due to DNA degradation.

2.4. Multiplex design and PCR amplification

The 13 newly developed STR loci and the two sexing markers were combined into one multiplex PCR assay hereafter referred to as "STRoe deer". To facilitate multiplex genotyping and to adjust fragment lengths, pigtails [35] were added to some of the reverse primers, and additionally, the forward primers were fluorescently labeled (for primer sequences see Table 1). To avoid overlapping marker ranges, the 15 selected loci were arranged in a way that the distance between marker size ranges with the same dye-label was ≥ 20 bp. All available 513 samples were genotyped using STRoe deer. The 20 μL reaction volume consisted of 10 μL 2x Qiagen Multiplex Master mix (Qiagen), 2 μL of 10x Primermix (primers with varying concentrations, see Table 1), template DNA (0.4–0.6 ng) and RNase-free water (Qiagen). PCR-conditions: 15 min initial activation at 95 °C, followed by 30 cycles of 30 s denaturation at 94 °C, 90 s annealing at 63 °C and 60 s extension at 72 °C, followed by a final extension step of 30 min at 60 °C. PCR reactions were run on a GeneAmp 9700 Thermocycler gold instrument, the denatured PCR products were run on an ABI 3500 DNA Analyzer and scored with GeneMapper *ID-X* 1.4 software using GS-600 (Liz) as a size standard.

2.5. Repeat motif sequence determination and construction of an allelic ladder

To establish allelic ladders for the markers included in STRoe deer, we selected homozygous individuals from the 513 roe deer samples. Per allele, two homozygous individuals (if available) were randomly chosen to amplify (in a singleplex reaction with unlabeled primers, see PCR conditions in section 2.4) and sequence the homozygote allele to be included in the allelic ladder. The AMPure bead system (Beckman Coulter) was used to purify the PCR products, which were then quantified with a Qubit 4 Fluorometer (Thermo Fisher Scientific). Cycle sequencing was conducted with the BigDye™ Terminator v1.1 Cycle Sequencing Kit according to the manufacturer's protocol. The BigDye XTerminator™ Purification Kit was used to clean up the cycle sequencing products and the sequences were then run on an ABI 3130 DNA Analyzer (all Thermo Fisher Scientific). Each allele included in the allelic ladder was sequenced to ensure a correct allele nomenclature, based on the number of repeats [5]. Sequence alignment and editing was performed using BioEdit version 7.0.5.3 [36]. To create an allelic ladder for each STR locus, the respective amplicons were reamplified with fluorescent primers (in a singleplex reaction, see PCR conditions in section 2.4) and combined into a tube and run on an ABI 3500 DNA Analyzer. Peak heights were then equalized by adding different volumes of the singleplex amplicons to the pool. To replicate the equalized

singleplex mixtures, they were diluted to 1/1000 – 1/16'000, and then reamplified in a singleplex reaction with 20 cycles. To build the final allelic ladder, including all STRoe deer loci, the reamplified singleplex pools were combined.

2.6. Validation of STRoe deer

2.6.1. Sensitivity, and peak height ratio and stutter ratio

To assess the sensitivity of STRoe deer, we used different numbers of PCR cycles (28, 30, and 32) and different amounts of DNA template from one male sample (1, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025 und 0.0125 ng). Each dilution was run in triplicate. To describe the peak height ratio, the same data as for the sensitivity analysis were used (except for 0.025 and 0.0125 ng template DNA). The peak height ratios were calculated in heterozygotes by dividing the peak height of the shorter peak by the peak height of the longer peak [8]. The stutter ratio was calculated using DNA profiles of 89 randomly chosen individuals from the representative roe deer data set: the height of the stutter-peak was divided by the height of the actual peak and multiplied by 100.

2.6.2. Stability

To assess the effects of degraded target DNA on STRoe deer, TURBO™ DNase (Thermo Fisher Scientific) was used to degrade DNA of a male European roe deer sample ("E"). The DNA of sample E (50 μL of 10 ng/μL) was digested for different periods (0 s, 5 s, 10 s, 30 s, 60 s, 90 s and 120 s) using 0.2 U of TURBO™ DNase, 10 μL 10x DNase buffer (Thermo Fisher Scientific) and RNase-free water, for a total volume of 100 μL. To stop the digestion process, aliquots of 10 μL of the digested DNA-solution were transferred to 490 μL TLE buffer (including 10 μg glycogen (Thermo Fisher Scientific)) and incubated at 95 °C for 10 min. From each of the DNA/TLE/glycogen mixture 5 μL were then analyzed with STRoe deer (for PCR conditions see section 2.4). Only alleles with rfu values above 100 were scored, alleles with rfu values below 100 were considered as dropouts.

2.6.3. Species-specificity

For the species-specificity testing, we collected samples from different species (Fig. 3) from various sources (i.e. zoos, farms, butchers) and analyzed them with STRoe deer (30 cycles, 0.4 ng input DNA). The species tested included individuals from the deer family (Cervidae) and the bovid family (Bovidae), which were selected due to their close evolutionary relatedness to the European roe deer. Additionally, individuals from the felid and canid families (Felidae and Canidae) and a human were tested as they could act as potential contamination sources. To assess the species specificity of STRoe deer per species and marker tested, observed peaks were grouped into five categories: (1) peak in bin, (2) peak outside of bins (within marker range), (3) out of marker range peak, (4) non-specific peak, (5) no peak. Peaks from the categories 1, 2, and 3 are usually accompanied by STR characteristic stutter and/or shoulder peaks. Category 4 peaks do not show these characteristics and are therefore considered as artefacts. Since we did not test each marker with a singleplex reaction, the defined marker range per locus (based on 513 European roe deer) was used to assign peaks to a marker. If a peak was found outside of any marker range, the peak was assigned to the closest marker; however, without sequencing the PCR product, the genomic origin cannot be determined with certainty.

2.6.4. Repeatability and robustness

To test for repeatability, three female samples (A, B and C, one PCR product each), three male samples (D, E and F, one PCR product each) and the final allelic ladder were run eleven times each on an ABI 3500 DNA Analyzer and the standard deviation of the obtained fragment lengths was calculated. Because the bin-width used in this study was 1 bp, the threefold standard deviation should be < 0.5 bp to allow for consistent allele calling. The positions of the bins were defined based on the allelic ladder, which was included in every run. To examine the robustness of STRoe deer, the female samples (A, B and C) and the male samples (D, E and F) were amplified ten times and analyzed over a period of two weeks, using two different PCR machines (both GeneAmp 9700 Thermocycler gold) and two different genetic analyzers (both ABI 3500 DNA Analyzer). To demonstrate the robustness of STRoe deer, the allele calls of these six samples had to be consistent over all ten analyses.

2.6.5. Mixtures

In forensic casework, field evidence can be a mixture from more than one individual. We analyzed different mixtures, consisting of a male and female European roe deer, to assess the effects of the mixture ratio on the interpretation of the obtained DNA profile. The mixture ratios were the following: 1:1, 1:2, 1:3, 1:4, 1:5, 1:7, 1:15, and 1:31, with the male sample being the minor component. The total amount of input DNA was 2 ng (for PCR conditions see 2.4). Each mixture was analyzed in triplicates. The ratio for which all of the unique alleles of the minor component could be scored was determined. Only alleles with rfu values above 100 were scored.

2.7. Population analysis

To assess the level of genetic structure in our model population, we analyzed 513 European roe deer samples from the Swiss Plateau with STRoe deer. GenAlEx v6.5 and GENEPOP 4.2 [37] were used to calculate population genetic parameters, including allele frequencies, F- statistics and probability of identity P(ID) and to test for Hardy-Weinberg equilibrium and pairwise linkage disequilibrium. To calculate the polymorphism information content (PIC), the formula of Botstein et al. [38]. was used. Mendelian inheritance of the markers was not examined, due to the absence of known parent-offspring pairs within our reference dataset. However the Hardy-Weinberg equilibrium results strongly suggest that none of the loci are allosomal (Table 3). Population genotypes were used as a reference dataset to calculate MPs relevant to forensic casework.

2.8. Methods to calculate the match probability of two profiles

To assess the discrimination power of STRoe deer in our study population, 10^6 genotypes were randomly drawn from the allele frequency distribution and used to calculate the MP between two samples. The allele frequencies of the 513 genotyped European roe deer samples were used as reference dataset, but a conservative minimum allele frequency threshold of $5/(2n) = 0.0049$ was applied when calculating MPs [14]. We compared three different methods to calculate the MP: The first method was based on the recommendation 4.2 of the NRCII [14], which requires the inclusion of an estimate of population subdivision (θ), to account for differences in allele frequencies between sub-populations. To explore the influence of θ, the MP was calculated using four different values (0, 0.0275, 0.15 and 0.25). For the second method, we calculated the MP using a variation of the product rule: frequencies of heterozygotes were calculated as $f = 2pq$, whereas the frequency of a homozygote was set as the frequency of the observed allele ($f = p$) instead of $f = p^2$. This altered calculation for homozygotes was applied to account for the effects of possible null alleles [23,39]. The third method incorporated the possibility that the sample associated with the possible suspect originates from a full or half sibling of the donor of the field evidence. For each of the three methods, we calculated minimum, maximum, median, mean, and standard deviation of the distribution of MPs across the 10^6 randomly sampled genotype combinations. Additionally, the frequencies of the two most common alleles for each locus were used to calculate the MP and LR for the theoretical most common genotype [40]. This conservative approach to calculate the MP, whereby a match seems more common than it actually is, is appropriate for use where a profile match is observed, but specific information about the relevant population(s) or specific allele identities are not available. Under this approach, instead of reporting the actual MP (or its LR) for

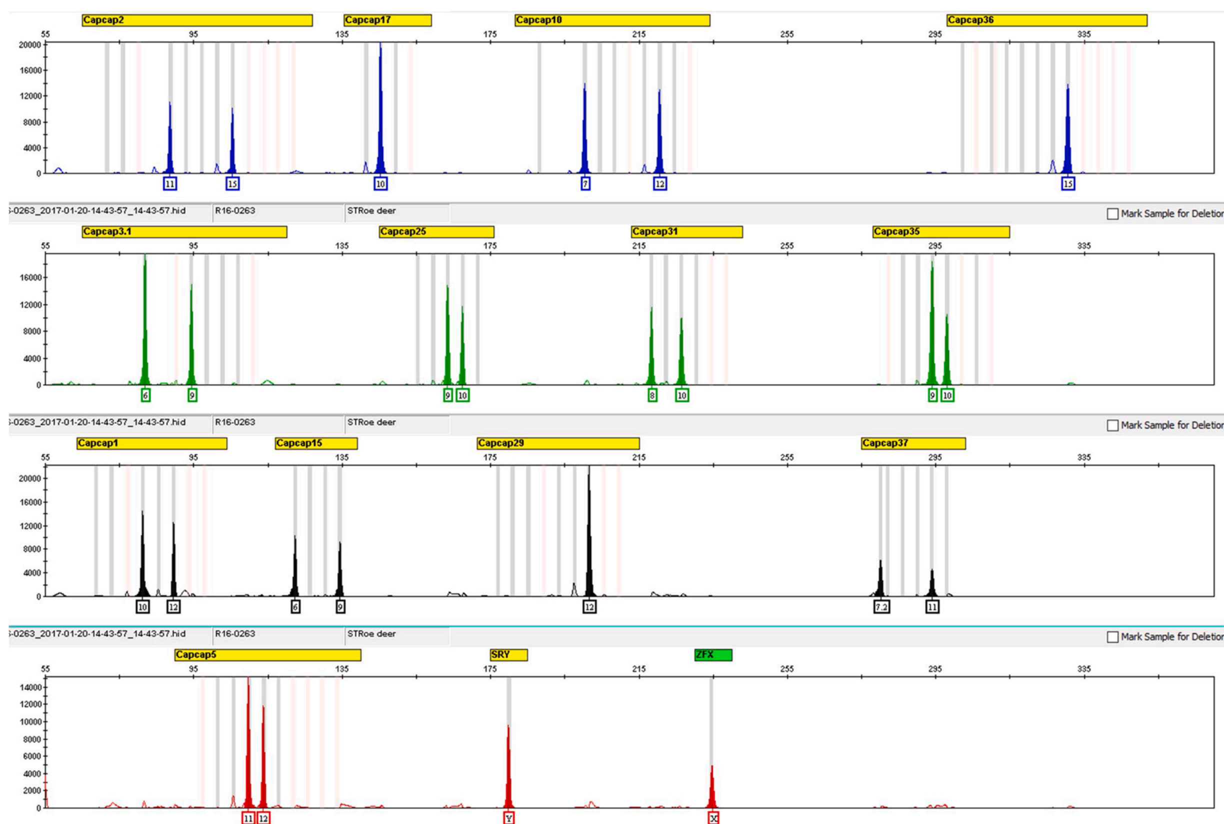

Fig. 1. GeneMapper® ID-X Software plot of a male European roe deer individual analyzed with STRoe deer.

Table 2
Composition of the allelic ladder of STRoe deer.

Locus	Number of alleles incl. in the allelic ladder/ total number of alleles in the reference population	Alleles incl. in the allelic ladder
Capcap1	5/7	7, 8, 10, 11, 12
Capcap2	7/12	7, 8, 11, 12, 13, 14, 15
Capcap3.1	5/6	6, 9, 10, 11, 12
Capcap5	5/8	9, 10, 11, 12, 13
Capcap10	7/9	4, 7, 8, 9, 11, 12, 13
Capcap15	4/4	6, 7, 8, 9
Capcap17	3/4	9, 10, 11
Capcap25	5/5	7, 8, 9, 10, 11
Capcap29	6/9	6, 7, 8, 10, 11, 12
Capcap31	4/6	8, 9, 10, 11
Capcap35	5/6	7, 8, 9, 10, 12
Capcap36	7/12	8, 10, 11, 12, 13, 14, 15
Capcap37	6/6	7.2, 8, 9, 10, 11, 12
Deer_Sry	1/1	Y
Deer_Zfx234	1/1	X

the specific, matching genotype, simply the MP or LR of the theoretical most common genotype is reported to make sure that the informative value of the actual match is not overestimated.

3. Results and discussion

From the initial 40 screened STRs, down to 13 that entered the validation phase, testing for pairwise linkage disequilibrium (including Bonferroni Correction [41]) left 12 unlinked STR markers in STRoe deer. Capcap3.1 and Capcap37 were significantly linked (p-value: 0.00041). Since linkage violates assumptions required for calculations relating to matching profiles, we decided to exclude Capcap37 from further calculations. This exclusion was based on the lower PIC value of Capcap37 compared to Capcap3.1 (see Table 3) and its larger allele size, as shorter amplicon length facilitates genotyping of degraded samples. Nevertheless, we included Capcap37 in the validation process and maintained its alleles in the allelic ladder, since in other European populations, Capcap37 may be more informative than Capcap3.1, and laboratories implementing STRoe deer can therefore decide which marker to keep in the multiplex reaction.

3.1. Multiplex design and allelic ladder

The 12 unlinked autosomal STR loci included in STRoe deer were selected based on number of alleles (≥ 4), no evidence of null alleles, and desired PCR-product length. The amplicon length of the selected markers ranged from 65 to 350 bp (Table 1 and Fig. 1). The total number of alleles identified across all 15 loci was 96, 71 were included in the allelic ladder (including 6 alleles for Capcap37). These 71 alleles consisted of 69 STR alleles (3–7 per locus) and the two sexing markers (Table 2 and Fig. 2). To facilitate the implementation of STRoe deer in other non-human DNA laboratories, aliquots of the allelic ladder described in this publication are available on request. Sequencing the STR alleles included in the allelic ladder showed that all loci exhibited a pure repeat tract and that four of the 69 sequenced alleles exhibited variants (SNPs/INDELS) compared to the representative genome. The variants were located between the primer binding sites and the actual tetra-nucleotide repeat tract (Table 1). The nomenclature of the STR alleles was established based on the number of repeat motifs [42].

3.2. Validation

3.2.1. Sensitivity, and peak height ratio and stutter ratio

The sensitivity of STRoe deer was influenced by the number of PCR

Table 3
Allele frequencies, observed heterozygosity (H_o), expected heterozygosity (H_{exp}) and Polymorphism Information Content (PIC) of the 13 STRoe deer loci analyzed in 513 European roe deer. In bold: the two most common alleles per locus. None of the loci showed a significant deviation from Hardy-Weinberg-Equilibrium after Bonferroni Correction.

Allele	Marker Capcap1	Capcap2	Capcap3.1	Capcap5	Capcap10	Capcap15	Capcap17	Capcap25	Capcap29	Capcap31	Capcap35	Capcap36	Capcap37
4	–	–	–	–	0.16179	–	–	–	–	–	–	–	–
6	–	–	0.24366	–	–	**0.49415**	–	–	0.03216	–	–	–	–
7	**0.24561**	0.07407	–	–	**0.24561**	**0.18616**	–	0.00195	0.05458	–	0.03509	–	–
7.2	–	–	–	–	–	–	–	–	–	–	–	–	0.06043
8	0.23489	**0.14425**	0.04483	0.01852	0.05750	0.14912	–	**0.40741**	**0.34795**	**0.55945**	0.08577	0.05945	0.09747
9	–	0.05556	**0.25439**	0.01754	0.02827	0.17057	0.07505	0.12865	0.05556	0.11988	**0.48830**	0.00292	**0.15984**
10	0.09844	–	**0.36647**	**0.28655**	0.00585	–	**0.58967**	**0.41228**	**0.31481**	**0.15984**	**0.27973**	0.13060	0.07602
10.1	–	–	–	–	–	–	–	–	–	–	–	0.00097	–
11	0.14327	**0.25341**	0.06920	0.21540	0.19201	–	**0.31774**	0.04971	0.09552	0.11111	0.01657	0.03996	**0.54873**
12	**0.26803**	0.08772	0.02144	**0.41131**	**0.20663**	–	0.01754	–	0.08967	0.02924	0.09454	0.19493	0.05750
13	0.00292	0.11209	–	0.03411	0.08285	–	–	–	0.00585	0.02047	–	**0.20858**	–
14	0.00682	0.12963	–	0.00195	0.01949	–	–	–	0.00390	–	–	0.10526	–
15	–	0.11306	–	0.01462	–	–	–	–	–	–	–	**0.23879**	–
16	–	0.01754	–	–	–	–	–	–	–	–	–	0.01170	–
17	–	0.00097	–	–	–	–	–	–	–	–	–	–	–
18	–	0.00097	–	–	–	–	–	–	–	–	–	0.00487	–
19	–	0.01072	–	–	–	–	–	–	–	–	–	0.00195	–
H_o	0.79532	0.82846	0.73294	0.70175	0.75828	0.63353	0.52632	0.62573	0.73879	0.59259	0.66667	0.80117	0.64133
H_{exp}	0.78239	0.85613	0.73436	0.70029	0.82255	0.66983	0.54539	0.64502	0.75551	0.63347	0.66551	0.82803	0.65110
PIC	0.74740	0.84069	0.68957	0.64797	0.79863	0.62298	0.46985	0.57573	0.72038	0.59811	0.61674	0.80548	0.62085

cycles. Using 28 cycles, allelic dropout was observed for ≤ 0.05 ng template DNA, whereas with 30 PCR cycles, allelic dropout was observed for ≤ 0.025 ng template DNA. Applying 32 PCR cycles led to pull-ups or spectral saturation for ≥ 0.5 ng template DNA. Such artefacts can impact the interpretation of DNA profiles; therefore, 30 cycles were set as the standard cycle number. Applying 30 PCR cycles and using ≥ 0.05 ng template DNA, STRoe deer successfully amplified all 14 loci. The mean peak height ratio over all loci was 0.79, with a minimum of 0.74 for locus Capcap25 and a maximum of 0.81 for locus Capcap15. Stutter ratios ranged from 1.9 % (Capcap15) to 10.7 % (Capcap5), which is comparable with stutter ratios exhibited by human-specific tetra-nucleotide STRs [43].

3.2.2. *Stability*

After a DNA digestion period of 5 s with TURBO™ DNase (Thermo Fisher Scientific), sample E showed clearly reduced rfu values compared to the undigested control, but no allelic dropout was observed. Digesting sample E for 10 s resulted in complete absence of PCR product for markers > 230 bp in length (Capcap35, Capcap36, Capcap37 and ZFX) and a further reduction of the measured rfu values for shorter markers. Extending the digestion time to 30 s led to a complete allelic dropout in 11 markers, whereas in Capcap15 just the longer allele dropped out, and only three markers (Capcap1, Capcap3.1 and Capcap5) did not experience any allelic dropouts. After a digestion time of 60 s only two alleles (allele 10 (99 bp) and allele 12 (114 bp), Capcap3.1 and Capcap5) persisted. After 90 s of digestion, allele 12 (107 bp) of Capcap3.1 was the only allele that was still observed. Prolonging the digestion process to 120 s led to a complete allelic dropout in all 15 markers. In summary, the process of DNA degradation reduced the observed rfu values of all the markers included in STRoe deer, and as expected, the markers with the larger allele sizes were the first ones to experience allelic dropout. In case of a partial profile, we strongly suggest applying a species-identification-test [e.g. 44,45] to confirm that the observed partial profile is caused by degraded DNA, rather than DNA amplification of a non-target species (see section 3.2.3). Field evidence containing PCR-amplification-inhibitors (e.g. soil), can also result in partial profiles and therefore limit the accurate interpretation of STR loci. To minimize carry-over of inhibitors to the DNA extracts, samples can be extracted using a silica-membrane-based (e.g. QIAamp DNA Mini Kit, QIAGEN) or magnetic-bead-based extraction kit (e.g. PrepFiler™ Forensic DNA Extraction Kit, Thermo Fisher Scientific).

3.2.3. *Species-specificity*

Of the 23 tested species, a complete DNA-profile (peaks of all 15 markers in category 1) was only obtained for samples from the European roe deer. Other members of the Cervidae family exhibited on average peaks for seven STR loci in categories 1 and 2, whereas members of the Bovidae family showed on average peaks for two STR loci in category 1 and 2 (Fig. 3). This distribution reflects the phylogeny of the European roe deer, as it is more closely related to other members of the Cervidae than to members of the Bovidae [e.g. 46]. Since all tested non-target species exhibited five or more peaks in the categories 3–5, a profile of a non-target species can be differentiated from a profile originating from a European roe deer. To determine the species of a non-target DNA contributor, a species-identification-test [e.g. 44,45] is recommended, performed either routinely prior to applying STRoe deer, or after encountering a STRoe deer profile exhibiting peaks from the categories 3–5. The ZFX marker was not designed to be species-specific, and our study produced a signal in most of the deer and bovid species. The species specificity of the SRY marker could not be assessed systematically because the sexes of the tested specimens were unknown. If a mixture of a European roe deer and a non-target species is encountered in a casework sample and the SRY amplicon is observed, the sex of the European roe deer cannot be determined with certainty; hence, the sex should be reported as undetermined. STRoe deer did not produce any peaks when applied with feline, canine or human DNA, therefore

Fig. 2. GeneMapper® ID-X Software plot of the STRoe deer allelic ladder.

Species	Number	Family
Roe deer (*Capreolus capreolus*)	513	Cervidae
Reindeer (*Rangifer tarandus*)	1	
Elk (*Alces alces*)	3	
Red deer (*Cervus elaphus*)	3	
Sika deer (*Cervus nippon*)	3	
Fallow deer (*Dama dama*)	3	
Eld's deer (*Panolia eldii*)	1	
The chamois (*Rupicapra rupicapra*)	1	Bovidae
Alpine ibex (*Capra ibex*)	3	
Goat (*Capra hircus*)	1	
Sheep (Ovis aries)	2	
Springbok (*Antidorcas marsupialis*)	1	
Blackbuck (*Antilope cervicapra*)	1	
Sable antelope (*Hippotragus niger*)	1	
Arabian oryx (*Oryx leucoryx*)	1	
Lesser kudu (*Tragelaphus imberbis*)	1	
Bison (*Bison bison*)	2	
European cattle (*Bos taurus*)	2	
Eurasian lynx (*Lynx lynx*)	1	Felidae
Red fox (*Vulpes vulpes*)	1	Canidae
Domestic dog (*Canis lupus familiaris*)	1	
Eurasian wolf (*Canis lupus lupus*)	3	
Human (*Homo sapiens*)	1	Hominidae

Capcap-marker: 1, 2, 3.1, 5, 10, 15, 17, 25, 29, 31, 35, 36, 37, SRY, ZFX

Category 1 (peak in bin)
Category 2 (off ladder peak)
Category 3 (out of marker range peak)
Category 4 (non-specific peak)
Category 5 (no peak)
NA

Fig. 3. Cross-amplification of STRoe deer markers showing their species-specific amplification and the numbers of tested individuals. The species specificity of the SRY marker could not be assessed systematically because the sexes of the tested specimens were not known.

potential contamination with such DNA should not interfere with the genotyping of European roe deer using this method.

3.2.4. Repeatability and robustness

After running the samples A – F (one PCR product each) and the allelic ladder eleven times on the genetic analyzer (ABI 3500 DNA Analyzer), the threefold standard deviation (including 99.73 % of all

Table 4
To describe the informative power of a match of two DNA profiles, three different statistical methods were compared (LR: likelihood ratio of direct match vs. unrelated, if not stated differently). Using the allele frequencies of 513 analyzed European roe deer samples at 12 loci as reference dataset, 10^6 genotypes were randomly sampled to calculate match probabilities (MP).

		Minimum	Maximum	Median	Mean	SD	Most common expected genotype
NRCII, recommendation 4.2							
$\theta = 0$	MP	1.4×10^{-23}	1.5×10^{-9}	1.4×10^{-13}	3.1×10^{-12}	1.6×10^{-11}	2.5×10^{-9}
	LR	6.6×10^{8}	7.2×10^{22}	7.0×10^{12}	9.7×10^{16}	7.2×10^{19}	4.1×10^{8}
$\theta = 0.0275$	MP	3.6×10^{-19}	3.8×10^{-9}	2.4×10^{-12}	1.8×10^{-11}	6.4×10^{-11}	5.3×10^{-9}
	LR	2.6×10^{8}	2.8×10^{18}	4.2×10^{11}	2.1×10^{13}	2.9×10^{15}	1.9×10^{8}
$\theta = 0.15$	MP	5.1×10^{-13}	4.9×10^{-7}	1.7×10^{-9}	4.3×10^{-9}	8.6×10^{-9}	6.2×10^{-8}
	LR	2.0×10^{6}	2.0×10^{12}	6.1×10^{8}	2.1×10^{9}	7.9×10^{9}	1.6×10^{7}
$\theta = 0.25$	MP	4.1×10^{-11}	1.2×10^{-5}	3.0×10^{-8}	7.4×10^{-8}	1.6×10^{-7}	2.4×10^{-7}
	LR	8.0×10^{4}	2.5×10^{10}	3.4×10^{7}	8.4×10^{7}	1.9×10^{8}	4.2×10^{6}
Variation of the product rule							
Accounting for possible null alleles	MP	9.7×10^{-22}	1.2×10^{-6}	5.6×10^{-12}	3.5×10^{-10}	4.0×10^{-9}	8.9×10^{-9}
	LR	8.4×10^{5}	1.0×10^{21}	1.8×10^{11}	3.5×10^{15}	1.5×10^{18}	1.1×10^{8}
Direct match vs. sibling							
Direct match vs. full sibling	MP	1.3×10^{-6}	1.5×10^{-4}	2.5×10^{-5}	2.8×10^{-5}	1.4×10^{-5}	1.2×10^{-4}
	LR	6.6×10^{3}	7.6×10^{5}	3.9×10^{4}	4.6×10^{4}	2.7×10^{4}	8.0×10^{3}
Direct match vs. half sibling	MP	1.2×10^{-14}	1.2×10^{-7}	7.5×10^{-10}	2.1×10^{-9}	3.9×10^{-9}	1.1×10^{-7}
	LR	8.4×10^{6}	8.5×10^{13}	1.3×10^{9}	9.4×10^{9}	1.5×10^{11}	8.8×10^{6}

values based on the definition of the normal distribution) of the allele sizes of the samples (A – F) and the allelic ladder ranged from 0 to 0.2478 (min: allelic ladder, Capcap5, allele 13, max: sample E, Capcap26, allele 16). Therefore, using the bin width of 1 bp allowed consistent calling of each allele. The test for robustness showed that the allele calls of samples A – F matched in all ten runs. However, regardless how robust a method is, there is always a possibility that external factors lead to a larger migration variability than expected and therefore an allelic ladder should be included with every run [5,47].

3.2.5. Mixtures

In our mixture study of two individuals, the male sample exhibited 17 unique alleles in its genotype (including the Y allele). These 17 unique alleles allowed us to assess the minor component of the mixture, and these were unambiguously identified up to a mixture ratio of 1:7 (0.25 ng male DNA vs. 1.75 ng female DNA). In the ratios 1:15 and 1:31 all the unique peaks of the minor component were still scorable (> 100 rfu), but for some markers the strength of the major component amplification made the distinction between pull ups, stutter peaks, and the actual peaks of the minor component ambiguous. This is particularly the case for markers exhibiting larger allele sizes (e.g. Capcap36 and Capcap37) and/or higher stutter ratios (e.g. Capcap5 and Capcap36). Identifying alleles of the minor component unambiguously may be difficult if they coincide with stutter peaks of the major component. However, identifying such alleles is possible as long as the height ratio of the minor peak to the major peak is higher than the stutter ratio of that particular marker. The stutter ratios of STRoe deer ranged from 1.9 % (Capcap15) to 10.7 % (Capcap5). To score a mixture profile accurately, the relative positions of major and minor component peaks, a rfu threshold evaluated through a sensitivity study, and marker specific stutter ratios should be taken into account. However, samples including DNA of more than one European roe deer individual are unlikely to be encountered in deer poaching cases.

3.3. Population analysis

The 513 genotyped individuals showed 88 alleles across the 12 unlinked STR loci and the number of alleles per locus varied from 4 to 12 (Table 1, allele frequencies see Table 3). P(ID) and $P(ID)_{sib}$ were 3.1×10^{-12} and 2.8×10^{-5} respectively. Given that the census population size of European roe deer in Switzerland (as of 2019) was estimated at 142’000 individuals [48], the power of discrimination provided by the STR loci included in STRoe deer is adequate for forensically relevant analyses.

The per STR locus calculated PIC values ranged from 0.470 (Capcap17) to 0.841 (Capcap2) and the observed heterozygosity values ranged from 0.526 (Capcap17) to 0.828 (Capcap2). The expected heterozygosity ranged from 0.545 (Capcap17) to 0.856 (Capcap2) (Table 3). After Bonferroni Correction [41], none of the loci deviated significantly from Hardy-Weinberg expectations, indicating that there is no strong effect of null alleles, population structure, or inbreeding within this population. The Fixation Index (F_{IS}) was 0.0275 ± 0.0083 (mean ± SE) indicating that in the population of the Swiss Plateau, the level of individual inbreeding is low. Evidence for population substructure was examined using software STRUCTURE [49], but no significant population subdivision was observed (data not shown). Rivers and fenced highways cutting through the Swiss distribution range of the European roe deer may have an effect on the dispersal of these animals [50,51], but the existence of over 300 wildlife corridors throughout Switzerland appears to facilitate gene flow between different areas inhabited by European roe deer [52,53].

3.4. Methods to calculate the match probability of two profiles

These calculations involved data from the 12 unlinked STR markers. For the first method, we used θ values ranging from 0 to 0.25 to simulate the effect of substructure commonly encountered in a variety of animal populations [11,54,55]. Given the observed lack of substructure in our Swiss European roe deer data set, we used the observed level of individual inbreeding (mean $F_{IS} = 0.0275$) as a conservative estimate of θ. On this basis the median value of the MP was one order of magnitude higher than assuming no inbreeding when using the formula recommended by the NRCII [14] (1.4×10^{-13} vs. 2.4×10^{-12}, see Table 4). When assuming significant population structure ($\theta = 0.15$ and 0.25), we obtain median values of 1.7×10^{-9} and 3.0×10^{-8}, respectively. Dealing with a structured population and including an underestimated θ correction will lead to underestimation of the MP, making a match between two profiles seem rarer than it actually is and over valuing the significance of the MP calculation. Calculating the MP using the modified product rule, the second method, accounting for the possible presence of null alleles, resulted in a median value for the MP of 5.6×10^{-12} (ranging from 9.7×10^{-22} to 1.2×10^{-6}). However, the actual effect of this method on the MP depends clearly on the number of homozygote loci that a profile exhibits and can, therefore, vary considerably. The third method, calculating the MP considering the possibility that the matching sample associated with a possible suspect, originates from a full or half sibling of the individual from which the field evidence is derived, resulted in median values for the MPs of 2.5×10^{-5} (1.3×10^{-6}

- 1.5×10^{-4}) and 7.5×10^{-10} (1.2×10^{-14} - 1.2×10^{-7}), respectively. This method is highly conservative and may be applicable to casework involving individuals from natural populations, where it is a reasonable proposition that relatives from the donor of the field evidence could be involved. Our final approach, calculating the MP for the theoretically most common genotype, revealed that in this study the allele frequencies of two of the markers (Capcap15 and Capcap31) were distributed so unevenly, that the probability of a homozygous genotype was higher than the probability of the heterozygous genotype with the two most common alleles. Incorporating this into our calculations, the MPs of the most common expected genotype (for this reference dataset) ranged between 1.2×10^{-4} and 8.9×10^{-9}.

The third method we examined (direct match vs. full sibling), which turns out to be the most conservative method for our data, has been adopted in our laboratory for forensic casework involving European roe deer. Given that we found neither strong substructure nor inbreeding in the European roe deer population spanning the Swiss Plateau, we do not consider it necessary to include any estimates for F_{ST} or the inbreeding coefficient for MP calculations for this population. However, practitioners wishing to use STRoe deer for other regions of Europe would need to establish their own population data set and consider which method to calculate MP is most appropriate for their region.

4. Conclusions

STRoe deer includes 12 unlinked forensically informative STR loci and two sex specific markers that allow for individual identification of European roe deer. We applied a set of common validation experiments to a population of European roe deer from Switzerland, demonstrating the reliability of STRoe deer in this population and showed that this STR profiling system is sufficiently robust to use in forensic casework.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Lucy Webster and Rob Ogden are guest editors of this special issue, but they did not handle this paper and were not involved in its peer review.

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