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Molecular traceability of red deer meat products using microsatellite markers

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Summary
Traceability systems help guarantee that products bought from stores exactly correspond to the description on the label. Traceability maps the journey of the food, from the store, throughout processing, right back to the original livestock. DNA can be used as a natural barcode, as it carries unique genetic information and can be used to identify individual animals. Genetic variability of markers was analysed with the aim of confirmation of the available set of 13 microsatellites. With the addition of five polymorphic markers, the probability of finding two animals sharing the same profile was, on average, four in ten million for the entire dataset. In Slovakia in 2015, 65 126 red deers were recorded. Using only the five extra polymorphic markers, it was possible to obtain a reliable individual genetic traceability system.

Keywords
genetic traceability; red deer; individual identification; microsatellite

In the last few years, the consumption of game meat has gained increasing preference among consumers, who appreciate its texture and flavour as well as the low fat and cholesterol contents, together with the absence of anabolic steroids or other drugs. The increasing importance and high commercial value of game meat in many parts of the world have led to frequent fraudulent practices, such as mislabelling or selling less valuable meat instead of the meat of more higher valued species [1].

Game meat authenticity not only relates to the industrial economic profit resulting from illegal trading, handling or substitution of species, but also to public health risks such as zoonosis or even allergies to a particular meat protein. In this context, although wild game meats may originate from farms having regulated hygienic standards and fair commercial practices, many industries worldwide export big amounts of wild game meats that lack safety and traceability controls throughout the food and feed processing chain [2].

The term "traceability" was defined by the European Regulation 178/2002 [3] as "the ability to trace and follow a food, feeds, food producing animal or ingredients, through all stages of production and distribution". Traceability has driven many issues related to food crisis management, traceability of bulk products, quality and identity preservation concerns, fraud prevention, anticounterfeiting [4], and minimizes food adulteration [5]. Considering all the mentioned aspects, enforcement of legislation on meat safety, traceability and authenticity is needed to achieve an active control on the commerce of game animals and their products.

To fulfil this demand, adoption of precise and efficient methodologies to assess meat sources and verify the authenticity of game meat products is of prime importance for the meat industry sector [6]. In order to control game meat products, the methods for a growing number of game species authentication have been developed [2]. Traditional traceability focuses on the labelling system

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and on the management of processed food batches [7]. Genetic traceability is based on the identification of animals and their products through the study of DNA [8]. Molecule of DNA is useful for that purpose because of its relatively high heat stability compared with proteins and other cell components, which denature during mechanical and thermal processing [9]. Moreover, owing to numerous mitochondria per cell and their greater copy numbers than nuclear genes, mitochondrial gene targets provide a more intense signal in molecular-based techniques such as polymerase chain reaction (PCR) and downstream applications [10]. In addition to species traceability, DNA molecule has the feature to be enormously variable among individuals allowing for individual traceability [8]. Therefore, molecular techniques are being increasingly utilized in food forensic analysis not only to combat fraud, verify and authenticate ingredients, distinguish between closely related species, but also to improve the traceability [8–10].

Primary prerequisite of successful detection of species is to choose adequate genetic markers [2]. The most widely used are microsatellites also known as short tandem repeats (STRs) and single nucleotide polymorphism [8]. Microsatellites (1 to 10 nucleotides long) and minisatellites (> 10 nucleotides long) are subcategories of tandem repeats that, together with the predominant interspersed repeats (or remnants of transposable elements), make up genomic repetitive regions [11]. Molecular-genetic methods are also suitable for the genetic identification of plant foodstuffs [12, 13]. Microsatellite markers were used in many applications, such as assessment of parentage, breed assignment tests and traceability [14–16].

The possibility of using microsatellite primers developed in *Bovidae* to amplify microsatellite markers in *Cervidae* was surveyed by using 75 microsatellite primer sets of bovine, ovine or caprine origin to analyse DNA from moose, red deer, reindeer and roe deer from Scandinavia. On average for the four cervids, approximately 50% of the ovine/caprine primer pairs amplified a specific PCR product, compared to only 16% of the bovine primers. Approximately 50% of both ovine/caprine and bovine primers that amplified a specific product were polymorphic, giving 15 polymorphic microsatellite markers in moose, 11 in red deer, 21 in reindeer and 10 in roe deer. Reindeer had a higher proportion of polymorphic loci, more alleles per locus and a higher mean heterozygosity than the other cervids [17]. For cervids, microsatellites have been characterized among others in reindeer [18]. RADKO et al. [19] investigated genetic differentiation among six populations of red deer in Poland based on microsatellite DNA polymorphism. Their analysis involved 12 STR markers (BM1818, OarAE129, OarFCB5, OarFCB304, RM188, RT1, RT13, T26, T156, T193, T501, TGLA53), for which conditions for simultaneous amplification were established. Based on their study, it can be concluded that the chosen set of 12 microsatellite markers could be used to evaluate the genetic structure and to monitor changes in Poland's red deer population.

In this study we analysed the possibilities of genetic identification of red deer meat products by using 13 microsatellites.

MATERIALS AND METHODS

Animals and sample collection

The set of samples consisted of 90 items belonging to three groups: raw meat (RM) samples (n = 30), cured meat (CM) samples (n = 30) and the group of randomly collected (RC) samples (n = 30) from the market in Slovakia. Samples of *semitendinosus* muscle and *triceps brachii* muscle were collected at the deer slaughter plant from 60 *Cervus elaphus* males (< 1.5 years old). These animals had grazed on summer pasture in the western part of Slovakia. The chill after slaughter started at 6–8 °C with a reduction to a temperature of < 2 °C overnight, approximately 9–15 h after dressing of the carcass. After deboning on the following day, samples were vacuum-packed and stored at 2–3 °C for 7 days post mortem and then frozen at approximately –20 °C. Samples were thawed at 3–4 °C for approximately 15 h and used for all procedures. No pedigree data were recorded and samples were collected randomly.

Curing

Curing of 30 red deer meat samples with brine was realized by adding a concentration of 10% NaCl to 10% nitrate salt. The ratio of brine and meat was 3:1 in order to ensure the desired diffusion of salt into each sample. Thus, samples were loaded into the cold brine stored at 4 °C for 7 days. After 7 days of cold storage, samples were desalted in lukewarm water for 30 min and then put into the smoking chamber, where they were dried at approximately 24 °C for 6 h. After sufficient drying, the samples were cold-smoked three times in 2 h. Samples processed in this way were subsequently placed in a climatic chamber where the climatic conditions during storage were gradually adjusted until the temperature reached 15 °C, relative humidity was 75% and air flow of 0.3 $m \cdot s^{-1}$.

Genotyping

Genomic DNA was obtained as a whole-cell lysate from meat tissues (2 g) using lysis buffer containing Tris-HCl 1 mol·l^{-1}, $MgCl_2$ 2 mol·l^{-1}, KCl 1 mol·l^{-1}, Tween 20, MilliQH_2O (Sigma-Aldrich, St. Louis, Missouri, USA). Each sample was mixed with 1 000 μl of lysis buffer and 3 μl of Proteinase K (Sigma-Aldrich) and homogenized by SHM2 Homogenizer (Stuart, Stone, United Kingdom). Samples were subsequently incubated in HS61A sterilizer (Chirana, Brno, Czech Republic) for 90 min at 65 °C. To inactivate proteinase K, the lysate was heated to 95 °C for 15 min and chilled on ice for 1 min. Finally, the lysate was centrifuged (Microspin 12; Biosan, Riga, Latvia) for 1 min at 10 000 ×*g*.

Thirteen microsatellite markers were used in two optimized multiplex PCR reactions. The eight microsatellite markers of mixture (Tab. 1) were amplified in a modified multiplex PCR, according to previous methods [24–26] using fluorescently labelled primers. Additional five microsatellite markers were amplified in the second multiplex PCR (Tab. 2). PCR was carried out in 10-μl volumes consisting of 1 μl of whole-cell lysate containing DNA, 1.2× Colorless GoTaq Flexi Buffer (Promega, Madison, Wisconsin, USA), 1.8 mmol·l^{-1} $MgCl_2$ (Promega), 0.34 mmol·l^{-1} dNTP (PE Applied Biosystems, Foster City, California, USA), 80–400 nmol·l^{-1} of primers, and 0.5 U of GoTaq Hot Start Polymerase (Promega). Samples were initially heated to 95 °C for 5 min and then subjected to 30 cycles of PCR amplification at 95 °C for 30 s, 59 °C for 90 s and 72 °C for 90 s, followed by a final extension at 72 °C for 90 s in PTC-150 Minicycler (MJ Research, St. Bruno, Quebec, Canada). Sequence analysis was performed with the ABI Prism 310 Genetic Analyzer (PE Applied Biosystems) and genotypes of individuals were evaluated by GeneMapper (PE Applied Biosystems) software.

Statistical analysis

The genetic variability of markers was analysed with the aim of validating the chosen STR set. Allelic frequencies, observed and expected

Tab. 1. Investigated short tandem repeats from the first multiplex, primer sequence and fluorescent label.

Locus	Primer sequences	Label	Reference
BM888	5'-ACTAGGAGGCCATATAGGAGGC-3' 5'-AGCTCAAAACGAGGGACAGGG-3'	VIC	[20]
0arFCB5	5'-AAGTTAATTTTCTGGCTGGAAAACCCCAG-3' 5'-ACCTGACCCTTACTCTCTTCACTC-3'	FAM	[21]
RM 188	5'-GCACTATTGGGCTGGTGATT-3' 5'-GGTTCACAAAGAGCTGGAC-3'	VIC	[22]
RT1	5'-CATATGGCTAACTACCTAGCTTGCC-3' 5'-GAGTCCCAAAGATTTCAGCCCTAC-3'	VIC	[23]
RT13	5'-GCCCAGTGTTAGGAAAGAAGA-3' 5'-CATCCCAGAACAGGAGTGAG-3'	NED	[23]
T26	5'-TGCCATAGTTTTTCCTACCTTC-3' 5'-GAAGTTCCAATAGACACGCTC-3'	FAM	[23]
T156	5'-ATGAATACCCAGTCTTGTCTG-3' 5'-TCTTCCTGACCTGTGTCTTG-3'	FAM	[23]
T501	5'-CTCCTCATTATTACCCTGTGA-3' 5'-ACATGCTTTGACCAAGACCC-3'	PET	[23]

Tab. 2. Investigated short tandem repeats from the second multiplex, primer sequence and fluorescent label.

Locus	Primer sequences	Label	Reference
IOBT965	5'-GGGGTTGTGGGTAAGCGGAGTT-3' 5'-GATCTAGCGCCAGACAGACGTGTCAT-3'	FAM	[27]
BM1818	5'-AGTGCTTTCAAGGTCCATGC-3' 5'-AGCTGGGAATATAACCAAAGG-3'	VIC	[28]
ETH225	5'-ACATGACAGCCAGCTGCTACT-3' 5'- GATCACCTTGCCACTATTTCCT-3'	NED	[27, 29]
Haut14	5'-CCAGGGAAGATGAAGTGACC-3' 5'-TGACCTTCACTCATGTTATTAA-3'	VIC	[27]
CSSM19	5'-TTGTGAGCAACTTGTTTT-3'GTATCT 5'-TGTTTTAAGCCACCCAATTATTTG-3'	PET	[27]

Tab. 3. Summary of statistics for investigated microsatellite markers.

Locus	*k*	H_{obs}	H_{exp}	*PIC*
OarFCB5	14	0.800	0.852	0.832
T156	14	0.478	0.893	0.877
BM888	17	0.678	0.852	0.833
RT1	9	0.667	0.713	0.683
RT13	14	0.522	0.887	0.871
T501	12	0.800	0.874	0.855
T26	12	0.800	0.856	0.836
RM188	12	0.689	0.815	0.787
IOBT965	8	0.511	0.742	0.703
BM1818	10	0.533	0.702	0.673
ETH225	13	0.611	0.872	0.854
CSSM19	16	0.311	0.884	0.867
Haut14	14	0.633	0.786	0.754
Mean ± *SD*	12.69 ± 2.49	0.62 ± 0.14	0.83 ± 0.07	0.80 ± 0.07

k – number of observed alleles per locus, H_{obs} – observed heterozygosity, H_{exp} – expected heterozygosity, *PIC* – polymorphism information content, *SD* – standard deviation.

heterozygosity, and polymorphism information content (*PIC*) per each locus were calculated with Cervus 3.0.7 software (Field Genetics, London, United Kingdom) [30]. GenAlEx 6.5 software package (Peakall and Smouse [31]) was used for calculation of population differentiation by Wright's *F*-statistics [31]. Wright's F_{IS} is known as the inbreeding coefficient of individuals relative to the subpopulation. Wright's F_{ST} is a measure of population substructure and is most useful for examining the overall genetic divergence among subpopulations. The efficacy of the marker set was tested for use for individual identification. Values of match probability, defined as the probability of finding two individuals sharing, by chance, the same genotypic profile, were calculated according to published methods [32, 33]. Match probability values were computed for overall loci and for smaller marker sets to verify whether a satisfactory level of identification could be achieved with fewer than 13 STRs.

RESULTS AND DISCUSSION

Genetic variation

In Tab. 3, the number of detected alleles, the observed and expected heterozygosity, and the *PIC* for the 13 polymorphic loci are shown. A total of 165 alleles were detected in the dataset, and the observed number of alleles per locus varied between 8 (IOBT965) and 17 (BM888) with an average of 12.69 and a standard deviation of 2.49, the expected heterozygosity ranged between 0.702 (BM1818) and 0.893 (T156). The *PIC* over all loci was 0.80, revealing the satisfactory information content carried by the chosen markers.

A low variation was observed for expected heterozygosity (0.495–0.826), as in previous works analysing the beef cattle breeds (although using

Tab. 4. Results of Wright's *F*-statistics.

Locus	F_{IS}	F_{IT}	F_{ST}
OarFCB5	0.030	0.056	0.027
T156	0.448	0.462	0.025
BM888	0.176	0.200	0.029
RT1	0.055	0.060	0.006
RT13	0.397	0.408	0.018
T501	0.056	0.079	0.024
T26	0.050	0.060	0.010
RM188	0.131	0.150	0.021
IOBT965	0.271	0.308	0.051
BM1818	0.202	0.236	0.043
ETH225	0.286	0.295	0.013
CSSM19	0.639	0.646	0.019
Haut14	0.138	0.190	0.059
Mean ± *SD*	0.221 ± 0.051	0.242 ± 0.050	0.027 ± 0.004

Values were computed for 30 samples, 30 cured meat products and 30 randomly collected samples.
F_{IS} – index of fixation for an individual within the subpopulation, F_{IT} – index of fixation for an individual within the total population, F_{ST} – index of fixation for subpopulation within the total population, *SD* – standard deviation.

Tab. 5. Probability of identity considering different marker sets.

Group	Number of loci					
	2	4	5	8	10	13
RM	1.1×10^{-03}	7.8×10^{-06}	2.1×10^{-07}	1.6×10^{-11}	4.1×10^{-13}	2.5×10^{-17}
CM	1.1×10^{-03}	7.8×10^{-06}	2.1×10^{-07}	1.6×10^{-11}	4.1×10^{-13}	2.5×10^{-17}
RC	2.7×10^{-03}	1.1×10^{-05}	4.5×10^{-07}	1.2×10^{-10}	1.2×10^{-12}	3.7×10^{-16}

Values represent the probability of identity for increasing locus combinations.
RM – raw meat samples, CM – cured meat samples, RC – randomly collected samples.

12 STRs), which means results similar to the present study [8, 34].

Wright's *F*-statistics over all loci per dataset were calculated, and results are shown in Tab. 4. Estimates revealed an average value of homozygote excess of 24.2 % (in the total sample) due, in a large part, to the variation of gene frequencies among groups (F_{ST} = 2.7 %) and, to a lesser extent, to homozygote excess within the population (F_{IS} = 22.1 %). In Tab. 4, estimates of F_{IS} in each population and at each locus are shown.

Animal identification

Probabilities of identity calculated using different marker sets are shown in Tab. 5. In each studied group, the probability of identity values were first computed considering all 13 STRs. Following this, the probability of identity was calculated using smaller sets of markers until considering only two markers. These sets were built choosing different numbers of the most polymorphic STRs in each group.

Choosing only the five most polymorphic markers in the dataset, the probability of finding two animals sharing the same profile was, on average, two in ten million for raw meat and cured meat, and four in ten million for the entire dataset. Considering that only around 65 126 red deer were recorded in Slovakia in 2015 [35], using only five polymorphic markers would be sufficient to obtain a reliable individual genetic traceability system. It should be considered that ease-of-use must be achieved in order for such a genetic tracing system to be used as a routine procedure. For this reason, it would be beneficial to produce an STR set giving satisfactory results in all groups of samples, as discussed, which would simplify laboratory work and reduce analysis costs.

CONCLUSIONS

Based on the presented results, five loci are sufficient and critically necessary for identification of a deer meat sample of an unknown origin in Slovakia. Application of a unique marker set can avoid costs of setting up many different marker sets, and lead to easier laboratory analysis. The choice of an adequate marker set must consider knowledge of the population variability of red deer involved in the traceability system, which has been shown to affect marker discrimination, as well as marker genetic variation and polymorphism. A practical application of such a system should be complementary to the conventional traceability based on meat cut labelling.

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