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Tetraplex real-time PCR assay for the simultaneous identification and quantification of roe deer, red deer, fallow deer and sika deer for deer meat authentication

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ABSTRACT

Analytical methods are needed for the identification and quantification of meat species to detect food adulteration. Since game meat is more expensive than meat from domesticated animal species, it is a potential target for adulteration. We present a tetraplex real-time PCR assay that allows the simultaneous determination of the content of roe deer, red deer, fallow deer and sika deer. The tetraplex assay showed only moderate cross-reactivity with closely related species. After optimization the tetraplex assay had a limit of detection of 0.1% (w/w) and a limit of quantification of 0.5% (w/w) for each of the four deer species. The tetraplex assay was found to be robust, slight modifications of the experimental setup did not lower its performance. Recoveries obtained by analyzing DNA mixtures and DNA isolates from model game sausages were similar to those obtained with the singleplex assays.

1. Introduction

Food adulteration has become a global issue. Studies report that all over the world, consumers are confronted with food products that differ in their quality from the quality indicated by the producer (Asensio, González, García, & Martín, 2008; Ayaz, Ayaz, & Erol, 2006; Cubero-Leon, Peñalver, & Maquet, 2014; Premanandh, Sabbagh, & Maruthamuthu, 2013; Stamatis et al., 2015). The term food adulteration covers a variety of aspects (Abbas et al., 2018; Ballin, 2010). Meat products may be adulterated by substitution of more expensive meat species by cheaper ones. For economic reasons, game meat is a potential target for being adulterated with meat from domesticated animal species (Fajardo, González, Rojas, García, & Martín, 2010).

Since food fraud is illegal, analytical methods are needed that are applicable for food authentication. Meat species identification is most commonly based on the analysis of specific DNA sequences. Various PCR methods have already been published for the identification of frequently consumed game species such as roe deer (Druml, Mayer, Cichna-Markl, & Hochegger, 2015; Fajardo et al., 2006; Fajardo et al., 2008) and red deer (Fajardo et al., 2006; Fajardo et al., 2008; Kaltenbrunner, Hochegger, & Cichna-Markl, 2018b; Ramón-Laca, Gleeson, Yockney, Perry, Nugent, & Forsyth, 2014; Tobe & Linacre, 2008). In the last years, meat products declared to contain fallow deer or sika deer appeared at local food markets and in restaurants. Thus, we have recently developed real-time PCR assays for fallow deer (Kaltenbrunner, Hochegger, & Cichna-Markl, 2018a) and sika deer (Kaltenbrunner, Hochegger, & Cichna-Markl, 2018c). All our real-time PCR assays target fragments of single copy genes. Since the copy number of the mitochondrial genome depends on the cell and tissue type (Ballin, Vogensen, & Karlsson, 2009), methods targeting single copy genes are more suitable for quantification than methods amplifying fragments of mitochondrial genes. Accurate quantitative information is, however, a prerequisite to be able to verify if a product complies with food quality guidelines such as the Codex Alimentarius Austriacus. According to the Codex Alimentarius Austriacus, meat products must not be declared as “game products” if less than 38% of the meat content originates from game species (Codex Alimentarius Austriacus, 2005).

In this study, we aimed to combine the singleplex real-time PCR assays for roe deer, red deer, fallow deer and sika deer to a tetraplex real-time PCR assay. The tetraplex assay should fulfill several criteria. It should allow the simultaneous identification of the four deer species in one run to save time and costs. In combination with a reference real-time PCR assay published previously (Druml, Kaltenbrunner,

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Hochegger, & Cichna-Markl, 2016), it should enable the quantification of the contents of roe deer, red deer, fallow deer and sika deer. In addition, the tetraplex assay should be comparable to the singleplex assays with regard to the main analytical parameters, including limit of detection, limit of quantification, robustness, repeatability and accuracy. Therefore, we had to find experimental conditions leading to the amplification of the four target sequences as efficiently as the singleplex assays, even when one of the target sequences was present in surplus.

2. Materials and methods

2.1. Chemicals and food samples

RNase A (85.5 U/mg, 10 mg/mL) and phenol/chloroform/isoamyl alcohol (25:24:1) were purchased from AppliChem (Darmstadt, Germany), proteinase K (600 mAnsonU/mL) from Merck (Darmstadt, Germany). Ultrapure water (purity 18.2 MΩ cm at 25 °C) was produced in-house with a Synergy® water purification system (Merck Millipore, Molsheim, France).

Meat samples were collected between 2015 and 2016 and stored at −20 °C. All samples originated from lean muscle meat. Game meat was obtained from Wildpark Ernstbrunn (Ernstbrunn, Austria), the Research Institute of Wildlife Ecology (Vienna, Austria) and the University of Veterinary Medicine Vienna (Vienna, Austria). Meat from domesticated animals was bought at several supermarkets and meat markets in Vienna. Meat species identity was verified by sequencing.

Two model game sausages were prepared following the guidelines of the Codex Alimentarius Austriacus. One sausage, prepared by the Higher Technical College for Food Technology Hollabrunn (Hollabrunn, Austria), consisted of 21% (w/w) roe deer, 21% (w/w) red deer, 21% (w/w) fallow deer, 7% (w/w) wild boar and 30% (w/w) bacon (domestic pig). In addition, it contained sucrose (2 g/kg sausage), nitrite curing salt (28 g/kg sausage), dextrose (3 g/kg sausage) and common food allergens like celery; white, brown and black mustard; wheat; sesame; soy; milk powder and egg powder (27 g/15 kg sausage). The other sausage, produced at the Institute for Food Control (AGES), consisted of 28.6% (w/w) roe deer, 28.6% (w/w) red deer, 42.9% (w/w) pig, garlic, nitrite curing salt, onion, phosphate and sausage spices.

2.2. DNA isolation

The in-house CTAB protocol used for DNA isolation was described previously (Druml, Mayer et al., 2015). Concentration and purity of the DNA isolates were determined photometrically (QIAxpert spectrophotometer, Qiagen, Hilden, Germany). The isolated DNA was stored at −20 °C.

2.3. Primers and probes

The primers and probes for roe deer, red deer, fallow deer and sika deer had been designed in previous studies (Druml, Mayer et al., 2015; Kaltenbrunner et al., 2018a, 2018b, 2018c). All probes were labeled with a reporter dye at the 5′ end and a minor groove binding (MGB) quencher at the 3′ end. Primers were synthesized by Sigma Aldrich (Darmstadt, Germany), probes by Eurogentec (Seraing, Belgium). Primer and probe sequences, reporter and quencher dyes, amplicon lengths, target genes and accession numbers are given in Table 1.

2.4. Real-time PCR

Real-time PCR was done in an optical 96-well reaction plate (0.2 mL, Applied Biosystems, Foster City, CA, USA) sealed with an optical adhesive film (Applied Biosystems) using the ABI 7500 Real-time PCR System (Applied Biosystems). Reactions were performed in a total volume of 25 µL, comprising 12.5 µL QuantiTect Multiplex PCR NoROX Master Mix (Qiagen), 5 µL 5× primer/probe mix, 2.5 µL ultrapure

Table 1
Sequences and final concentrations of primers and probes, amplicon lengths, target genes and accession numbers.

Primer/Probe	Sequence (5′–3′)	Amplicon (bp)	Final concentration (nM)	Target gene	NCBI accession no.	Reference
Roe deer						
Roe deer Forward	TGGCTGCTGCGTGCAGAA	62	200	*Capreolus capreolus*	AY122040.1	Druml, Mayer et al. (2015)
Roe deer Reverse	TCTAAAATGCTTGGGAACCAGATAT		200	*lactoferrin* gene		
Roe deer Probe	**FAM** GAAGGGTCTCCGTCTGC **MGB EDQ**		100			
Red deer						
Red deer Forward	CAATAAGGCACAAACATTGACAAGT	87	300	*Cervus elaphus*	AY846793.1	Kaltenbrunner et al. (2018b)
Red deer Reverse	CACAATATGATGTTTTATTACCTCTAAACTATTACATT		300	*protein kinase C iota* gene		
Red deer Probe	**Cy5** TTCTGATATCATACACTTAAGC **MGB EDQ**		150			
Fallow deer						
Fallow deer Forward	GACACCATGGAGCCACAGATAA	64	300	*Cervus dama*	Y13963.1	Kaltenbrunner et al. (2018a)
Fallow deer Reverse	AGGCAGCTGTGGTGCTAC		300	*MC1-R* gene		
Fallow deer Probe	**Yakima Yellow** CGTCGATGACATTGTCCAG **MGB EDQ**		150			
Sika deer						
Sika deer Forward	GGTGCACTCTCAATAACTTCTGACAA	71	200	*Cervus nippon*	D14379.2	Kaltenbrunner et al. (2018c)
Sika deer Reverse	CCTACCACCGAAGCAATAGTGG		200	gene *for kappa-casein precursor*		
Sika deer Probe	**Dragonfly Orange** GCTTCTTGAGTAGCTACAGTG **MGB EDQ**		100			