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Application of next generation semiconductor based sequencing for species identification and analysis of within-species mitotypes useful for authentication of meat derived products

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ABSTRACT

In this study, we tested the Ion Torrent next generation semiconductor based sequencing technology for meat species identification in several highly processed and complex meat products and meat derived broths (a döner kebab, a beef/pork paté, a meat based filling of tortellini, one instantaneous granular preparation of broth stock made by meat and two ready to use meat broths from different producers). The detection protocol included the sequencing of targeted mitochondrial DNA (mtDNA) regions amplified with universal primer pairs and a bioinformatic pipeline designed to interpret sequencing results. Six libraries were sequenced producing a total of 1,363,351 filtered reads. Data mining detected expected and unexpected meat species in the analysed products. Pork was identified in the kebab and *Bubalus bubalis* DNA was identified in the beef/pork paté. For products for which the precise meat species ingredient information was not available, it was possible to obtain it. Human contamination based on human detected reads could be useful to evaluate the hygienic level of highly processed products. Mitochondrial haplotypes (mitotypes) were identified for several mtDNA-species combinations providing another level of information useful for the authentication of meat derived products. This work defined a methodological framework to establish assays using this sequencing platform for routine species identification in complex and highly processed food.

1. Introduction

Purposely complete or partial substitutions of species in meat products driven by economic advantages (i.e. frauds) and accidental and uncontrollable mislabelling or mixings of meat from different species (derived by errors occurring in the production plants or over different steps of the production chains), raise many concerns. Their implications are related to food safety and security, product marketing and consumers' acceptability, lifestyle, religious and ethical issues (Ballin, 2010; Fontanesi, 2009, 2017). Therefore, the identification of the species of origin of the products is becoming a very relevant aspect in meat science. Many efforts have been made to develop analytical methods and assays able to identify the species of origin in meat based and derived products, with applications also in other fields, including forensics (Fontanesi, 2017).

The identification of the species of origin in meat preparations is mainly based on the differences that can be detected at the level of biological components. Species-specific information useful for animal species discrimination can be retrieved from proteins or peptides, from some metabolites and from DNA (Fontanesi, 2017). DNA has been extensively used for species identification in different food products because i) it can be easily extracted from most biological matrices, even after cooking or other physical treatments due to its general high stability, and ii) it is possible to capture sequence differences using PCR based assays. These methods usually include an amplification step of targeted DNA containing species-specific sequences and the analysis of the amplified region with different approaches that have in common, as final step, the separation of DNA fragments by gel electrophoresis (i.e.: species-specific PCR, based on the presence or absence of amplification from targeted species; PCR-RFLP, based on the use of

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restriction enzymes that cut DNA palindromes; PCR-fingerprinting techniques, including PCR-RAPD, PCR-AFLP, PCR-SSCP, PCR-DGGE, high resolution melting, which produce species-specific DNA patterns using several methods; PCR-FINS, based on Sanger sequencing of amplified products; Mafra, Ferreira, & Oliveira, 2008; Fajardo, González, Rojas, García, & Martín, 2010; Rodríguez-Ramírez, González-Córdova, & Vallejo-Cordoba, 2011; Fontanesi, 2017). The targeted DNA assays usually amplify informative mitochondrial DNA (mtDNA) fragments [i.e. 12S, 16S, cytochrome b and cytochrome c oxidase I (COI) genes or the D-loop region] which are flanked by conserved sequences across many species that are used for the design of universal PCR primers, with intervening regions that contain species-specific sequences used for species identification. Species-specific PCR are designed as end-point PCR or quantitative real time PCR without the need of gel-electrophoresis separation of the amplicons (Fontanesi, 2017). All these methods can usually detect one or few species at the time. Therefore, the choice of the assays is quite difficult when more than one species is expected or when there is no at priori information on the presence of species in mixed and processed meat or in derived products like meat broth. To overcome the poor multiplexing detection potential of these methods, microarray detection systems have been designed to discriminate on the same chip, at the same time, mtDNA amplified fragments belonging to several species (Chisholm, Conyers, & Hird, 2008; Cottenet et al., 2016; Iwobi, Huber, Hauner, Miller, & Busch, 2011; Peter, Brünen-Nieweler, Cammann, & Börchers, 2004; Teletchea, Bernillon, Duffraisse, Laudet, & Hänni, 2008; Wang, Zhu, Chen, Xu, & Zhou, 2015). These systems are more informative than simple PCR based assays but are still limited by the construction bias of the tool that can detect only species for which probes are already included on the chip.

Next generation sequencing (NGS) technologies have changed the way in which DNA can be analysed. These technologies have increased of several orders of magnitude the sequencing throughput. Their advantage in the context of species identification relies on the possibility to combine in a single step the generation of species-specific information from the produced short sequence reads with limitless multiplexing potential and the possible relative quantification of the detected species by counting reads matching the same target specific sequence (even if, in many cases, this estimation might depend by the experimental design and technical procedures). The large potential and flexibility of NGS is obtained through bioinformatic analysis of the produced sequence data (Bertolini et al., 2015; Pabinger et al., 2014). Among the commercially available benchtop NGS platforms that can be used for species identification, it is possible to mention Roche 454, Illumina and Ion Torrent technologies (Bertolini et al., 2015; Goodwin, McPherson, & McCombie, 2016; Tillmar, Dell'Amico, Welander, & Holmlund, 2013). Ion Torrent technology is based on the detection of small pH modifications that occur during the sequencing phase and that are captured by semiconductor chips accommodating millions of reaction micro-wells that produce sequence reads (Rothberg et al., 2011). A few reports have tested the Ion Torrent Personal Genome Machine (PGM) sequencing platform for species identification in food products (i.e. Carvalho, Palhares, Drummond, & Gadanho, 2017; Giusti, Armani, & Sotelo, 2017; Muñoz-Colmenero, Martínez, Roca, & Garcia-Vazquez, 2017). We recently evaluated this technology for species detection in DNA mixtures of meat species and for species identification in dairy products (Bertolini et al., 2015; Ribani et al., 2018).

In this study, we tested the usefulness of this NGS technology for animal species identification in several highly processed and complex meat products and meat derived broths. The study defined a methodological framework to establish assays based on this NGS platform for species identification in complex meat derived matrices. The detection protocol included the sequencing of targeted mtDNA regions amplified with three different universal primer pairs and a bioinformatic pipeline designed to interpret sequencing results. In addition, this study described the possibility to identify within-species mitotypes, adding another level of information useful for the authentication of food products derived from meat species.

2. Materials and methods

2.1. Analysed products

Six different products were investigated in this study: one commercial döner kebab preparation; one beef/pork paté; one meat based filling of tortellini; one instantaneous granular preparation of broth stock made by meat; two ready to use meat-based broths from different producers. More details on these products, including their declared ingredients and origin, are reported in Table 1. These products represent highly processed meat preparations derived or potentially derived from different species.

2.2. DNA isolation

A total of 0.5 g or 0.5 ml for each of these products was used for DNA extraction. DNA was isolated using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions for DNA isolation from cell cultures and animal tissues. The quality of the extracted DNA was assessed using the Nanophotometer P-330 instrument (Implen GmbH, München, Germany) and by visual inspection on 1% agarose gel electrophoresis in TBE 1X buffer after staining with 1X GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA).

2.3. PCR

Amplicons from the selected products were obtained using three universal primer pairs that have been already shown to successfully amplify many different vertebrate species (Table 2). These primers were designed in conserved regions of the 12S and 16S mitochondrial rRNA genes (Bertolini et al., 2015; Karlsson & Holmlund, 2007; Kitano, Umetsu, Tian, & Osawa, 2007; Ribani et al., 2018). PCR amplifications were obtained using a 2720 thermal cycler (Life Technologies, Carlsbad, CA, USA) in a total reaction volume of 20 μL. PCR mix components were: 2X of the Kapa HiFi HotStart ReadyMix PCR kit (Kapa Biosystems, Boston, Massachusetts, USA), 10 pmol of each primer and 50 ng of template DNA. The amplification profile was the following: an initial denaturation step of 3 min at 98 °C; then 35 cycles of 20 s at 98 °C, 15 s at the primer pair specific annealing temperature (Table 2), 30 s at 72 °C; and a final extension step of 1 min at 72 °C. Results of the PCR amplifications were evaluated visualizing the obtained fragments by 2.5% agarose gel electrophoresis in TBE 1X buffer after staining with 1X GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA). Quantity and quality of the amplified fragments was estimated using a Nanophotometer P-330 instrument (Implen GmbH).

2.4. Ion Torrent sequencing

The amplified fragments were purified with ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA). Six libraries (one for each product) were prepared for sequencing with the Ion Torrent PGM (Thermo Fisher Scientific Inc.). Each library was based on amplicons of the three primer pairs obtained for the same product. The libraries included end-repaired and barcoded amplicons (200 ng of