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Review

DNA barcoding as a new tool for food traceability

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ABSTRACT

Food safety and quality are nowadays a major concern. Any case of food alteration, especially when reported by the media, has a great impact on public opinion. There is an increasing demand for the improvement of quality controls, hence addressing scientific research towards the development of reliable molecular tools for food analysis. DNA barcoding is a widely used molecular-based system, which can identify biological specimens, and is used for the identification of both raw materials and processed food. In this review the results of several researches are critically analyzed, in order to exploit the effectiveness of DNA barcoding in food traceability, and to delineate some best practices in the application of DNA barcoding throughout the industrial pipeline. The use of DNA barcoding for food safety and in the identification of commercial fraud is also discussed.

Contents

1. Introduction

High quality raw materials are fundamental to food production with adequate nutritional value and desirable taste (Konczak & Roulle, 2011; Pereira, Barros, Carvalho, & Ferreira, 2011). Food industry has developed several technological (e.g. microfiltration, ultra-heat treatment) and biotechnological (e.g. fermentation) processes to preserve and enhance the organoleptic properties of its products. Quality controls are made by various laboratory tests, which represent the mandatory starting point for a proper food traceability system. Governments have different national guidelines for the production and preservation of food (see, for instance, the recommendations of the World Health Organization—www.who.int/foodsafety/fs_management/infosan/en/ or regulations such as the European EC/178/2002), while the definition of which tests should be used in evaluating food quality and safety is the responsibility of several independent agencies, such as the American Food and Drug Administration,

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and the European Food Safety Authority. The demand for reliable food traceability systems has addressed the scientific research, hence producing different analytical approaches to the problem (Bottero & Dalmasso, 2011; Fajardo, Gonzàlez, Rojas, Garcìa, & Martìn, 2010; Hellberg & Morrisey, 2011; Mafra, Ferreira, & Oliveira, 2008). The validation of food authenticity relies mostly on the analysis of proteins and/or DNA sequences. Protein-based methods include immunological assays, electrophoretical and chromatographic techniques such as HPLC and TLC (Fügel, Carle, & Schieber, 2005; Kurtz, Leitenberger, Carle, & Schieber, 2010). While being effective in testing fresh products, protein-based approaches have a low effectiveness when applied to the analysis of heavily processed foods. In these cases, DNA-based methods are more effective, and can also be applied to different food matrices (Lockley & Bardsley, 2000; Mafra et al., 2008). Furthermore, DNA is more informative than proteins, and can be easily extracted also in the presence of small traces of organic material as well (Hellberg & Morrisey, 2011).

Thanks to the recent advancements in molecular biology, DNA markers have become the most effective instrument in the analysis of the DNA of plant cultivars and animal breeds, and are also used to track the raw materials in food industry processes (Kumar, Gupta, Misra, Modi, & Pandey, 2009; Mafra et al., 2008; Woolfe & Primrose, 2004). The aim of the present review is to summarize the state-of-the-art about the use of DNA barcoding as a universal tool for food traceability.

2. From molecular-based approaches to DNA barcoding

In general, DNA-based methods use specific DNA sequences as markers, and can be divided in *i*) hybridization-based markers, and *ii*) Polymerase Chain Reaction (PCR)-based markers. In hybridization-based methods, species-specific DNA profiles are discovered by hybridizing DNA digested by restriction enzymes, and comparing it with labeled probes (DNA fragments of known origin or sequence). PCR-based methods involve the amplification of target loci by using specific or arbitrary primers, and a DNA polymerase enzyme. Fragments are then separated electrophoretically, and banding patterns are detected by different staining methods, such as autoradiography.

PCR-based methods are extremely sensitive, often faster than other technologies, and are widely used in agriculture and zootechny (Doulaty Baneh et al., 2007; Grassi, Labra, & Minuto, 2006; Labra et al., 2004; Mane, Tanwar, Girish, & Dixit, 2006; Teletchea, Maudet, & Hänni, 2005). Discontinuous molecular markers such as RAPDs, AFLPs, as well as their variants (i.e. ISSR, SSAP, SAMPL) have been successfully used in the characterization of different kinds of raw material (Chuang, Lur, Hwu, & Chang, 2011; Fajardo et al., 2010; Mafra et al., 2008; Nijman et al., 2003). In recent years, the PCR-denaturing gradient gel electrophoresis (PCR-DGGE) has been largely used in the field of food traceability and safety in order to characterize bacteria and yeasts in fermented products (Dalmacio, Angeles, Larcia, Balolong, & Estacio, 2011; Muyzer, De Waal, & Uitterlinden, 1993; Peres, Barlet, Loiseau, & Montet, 2007; Zheng et al., 2012). By using this technique, microorganism composition is defined on the basis of the migration pattern of PCR-fragments belonging to specific genomic regions such as 16S and 26S rDNA (El Sheikha et al., 2009). PCR-DGGE was also used to monitor bacterial contamination in food products such as fermented drinks (Hosseini, Hippe, Denner, Kollegger, & Haslberger, 2012) and define the origin of raw material starting from the characteristics of its yeast or bacterial communities as in the case of fruit (El Sheikha, Bouvet, & Montet, 2011; El Sheikha, Durand, Sarter, Okullo, & Montet, 2012; El Sheikha, Métayer, & Montet, 2011) and fish (Le Nguyen, Ha, Dijoux, Loiseauet, & Montet, 2008; Montet, Le Nguyen, & El Sheikha, 2008).

The selection of the most suitable molecular approach depends on different aspects, including the amount of genetic variation of the analyzed species, the time needed for the analysis, the cost/effectiveness ratio, and the expertise of laboratories. Furthermore, genomic techniques require high-quality DNA to work successfully because their effectiveness can be negatively influenced by altered or fragmented DNA (Hellberg & Morrisey, 2011; Meusnier et al., 2008; Pafundo, Agrimonti, Maestri, & Marmiroli, 2007).

Regarding sequencing-based systems, Single Nucleotide Polymorphisms (SNPs) and Simple Sequence Repeats (SSRs), are largely used nowadays because of their high level of polymorphism and high reproducibility (Kumar et al., 2009). These approaches are used both in the identification of plant cultivars (Labra et al., 2003; Pasqualone, Lotti, & Blanco, 1999) and animal breeds (Nijman et al., 2003), and to prevent fraudulent commercial activities (Chuang et al., 2011). However, being highly species-specific, these approaches require access to the correct DNA sequence of the organisms (e.g. strains/varieties or ecotypes), and their application is often limited to a single taxon, or to closely related taxa.

Lack of both standardization and universality is the most relevant problem of DNA-based approaches. In 2003, a new identification system, DNA barcoding, was developed by researchers at the University of Guelph (Canada). This approach is based on the analysis of the variability within a standard region of the genome called "DNA barcode" (Hebert, Ratnasingham, & deWaard, 2003). This approach proved useful in solving taxonomic problems in several theoretical and practical applications (Hollingsworth, Graham, & Little, 2011; Rasmussen, Morrissey, & Hebert, 2009; Valentini, Pompanon, & Taberlet, 2009). In a strict sense, DNA barcoding is not completely innovative, because molecular identification approaches were already in use. However, it has the advantage of combining three important innovations: molecularization of identification processes (i.e. the investigation of DNA variability to discriminate among taxa), standardization of the procedure (from sample collection to the analysis of molecular outputs), and computerization (i.e. the not redundant transposition of the data using informatics) (Casiraghi, Labra, Ferri, Galimberti, & De Mattia, 2010).

The name DNA barcoding figuratively refers to the way an infrared scanner univocally identifies a product by using the black stripes of the Universal Product Code (UPC). An ideal DNA barcode requires two fundamental characteristics: high taxonomic coverage, and high resolution (Hebert et al., 2003). High taxonomic coverage (also called 'universality') refers to the correct amplification of the genomic region chosen as DNA barcode in the widest panel of taxa. On the other hand, a high resolution ensures the identification of different taxa, based on interspecific differences in DNA barcode sequences. As a general principle, DNA barcode regions should have a high interspecific, and low intraspecific variability.

The 5′-end portion of mitochondrial *cox1* gene was suggested by Hebert et al. (2003) as standard DNA barcode region for metazoans. This region does not assure a complete taxonomic resolution, but it does promise proximity (Hebert & Gregory, 2005). Based on preliminary results on *cox1* discriminatory power, specimens have been correctly identified at the species level with a success rate ranging from 98 to 100% in birds (Hebert, Stoeckle, Zemlak, & Francis, 2004), fish (Ward, Zemlak, Innes, Last, & Hebert, 2005), and in several other animal groups (Ferri et al., 2009; Galimberti, Martinoli, Russo, Mucedda, & Casiraghi, 2010; Galimberti et al., 2012; Hajibabaei et al., 2006). Nowadays, this region is considered the universal DNA barcode for metazoans, and is used to better distinguish even closely related taxa (see Uthicke, Byrne, & Conand, 2010; Wong et al., 2011), or to identify organisms from their parts, and also from traces of biological material (Dawnay, Ogden, McEwing, Carvalho, & Thorpe, 2007; Shokralla, Singer, & Hajibabaei, 2010; Vargas et al., 2009). In terrestrial plants, mitochondrial DNA has slower substitution rates than in metazoans, and shows intra-molecular recombination (Mower, Touzet, Gummow, Delph, & Palmer, 2007), therefore limiting its resolution in identification. The research for an analogous of *cox1* in terrestrial plants has focused on the plastid genome. Several plastidial genes, such as the most conserved *rpoB*, *rpoC1* and *rbcL* or a section of *matK*, which shows a fast evolution rate, have been proposed as barcode regions (Shaw, Lickey, Schilling, & Small, 2007). Intergenic spacers such as *trnH-psbA*,

atpF-atpH and *psbK-psbI* were also tested, because of their fast evolution rate (Fazekas et al., 2008, 2009). In 2009, the CBoL (Consortium for the Barcode of Life) Plant Working Group (Hollingsworth et al., 2009), suggested the use of 2-locus combination of *rbcL* and *matK* as core-barcode regions, because of the straightforward recovery rate of *rbcL*, and the high resolution of *matK*. Unfortunately, *matK* is difficult to amplify by using a single primer pair (Dunning & Savolainen, 2010). On the contrary, despite its limited resolution, *rbcL* is less problematic in terms of amplification, sequencing and alignment, and provides a useful backbone in the creation of plant DNA barcode datasets (De Mattia et al., 2012). Among other sequences, the *trnH-psbA* intergenic spacer is straightforward to amplify, and has a high genetic variability among closely related taxa (Bruni et al., 2010; Kress et al., 2010; Shaw et al., 2007). The nuclear ITS region was also indicated as supplementary DNA barcode region (Li et al., 2011). Although there is still debate on the effectiveness of these markers especially when users are dealing with closely related taxa, DNA barcoding showed consistent results when used to identify unknown specimens based on the comparison with reference sequences (Burgess et al., 2011; De Mattia et al., 2012).

Although the molecular approach at the basis of DNA barcoding is not new to science, the strength of this method relies on the availability of an international platform. BOLD (Barcode of life database), coordinated by the International Barcode of Life Project (iBOL), is a repository, which supports the collection of DNA barcodes, with the aim of creating a reference library for all living species (Ratnasingham & Hebert, 2007). BOLD is used to relate a given DNA barcode to both a vouchered specimen and other DNA barcode sequences belonging to the same or different taxa. This platform consists of several components, among which the Identification Engine tool (BOLD-IDS) is one of the most useful. BOLD-IDS provides a species identification tool that accepts DNA barcode sequences and returns a taxonomic assignment to the species level whenever possible. This engine assumes correct species identification for genetic distances up to 99%. Any researcher can use BOLD-IDS, and, if a reference record belonging to an unknown specimen is available in the database, the system provides identification at the species rank, or a list of the taxa related to that specimen. BOLD is a reliable resource both for research purposes and for practical applications, such as the traceability of food commodities.

3. DNA barcoding to identify and certify food raw material

The identification of organisms is fundamental to ensure high quality standards for the food industry and market (Myers, 2011; Novak, Gruber-Gréger, & Lukas, 2007). DNA barcoding is effective in certifying both origin and quality of raw materials, and to detect adulterations (e.g. by mixing products from different taxa) occurring in the industrial food chain. However, its performance is strongly influenced by the molecular variability of the organisms, and a high level of resolution is achieved when an organism has low intraspecific polymorphism, making it well distinguishable from closely related taxa (Casiraghi et al., 2010; Hebert et al., 2003).

Another critical element can be the availability of high quality repositories of reference sequences. For this reason, a high number of DNA barcode sequences from animals and plants (including farmed species) have been submitted during the last 10 years to both NCBI and BOLD databases (www.barcodeoflife.org), following the guidelines provided by the Database Working Group (http://barcoding.si.edu/PDF/DWG_data_standards-Final.pdf).

3.1. Seafood traceability and FISH BOL

DNA barcoding was proven to be particularly effective in the traceability of seafood (Becker, Hanner, & Steinke, 2011). The term "seafood" is normally used to indicate edible aquatic life forms, including fish, mollusks, crustaceans and echinoderms, which are available on the market as whole organisms, or as processed products. Seafood species are generally identified according to their area of origin and to several morphological descriptors. However, the increased demand of seafood, and the globalization of the market, has made the control of both the trade routes and the industrial processing systems (i.e. storage systems, freezing, drying) more difficult. In addition, several new species have been introduced in the market. Sometimes, these "new fish" have the same commercial name as organisms previously on the market, but do not correspond to the same species. They could also have different nutritional values, and/or be potentially antigenic (Barbuto et al., 2010).

DNA barcoding is successful when applied to seafood because: (1) in comparison to other animal sources (e.g. cattle, sheep, goat, horse) the number of species is higher, so that the effectiveness of this technique is enhanced; (2) classical identification approaches are not useful in many cases, in particular with processed food; (3) in seafood more than in other living groups, molecular identification can go further than the species level, allowing in several cases the identification of local varieties and hence identifying the origin of a certain product.

Several researchers have discussed the potential of DNA barcoding as a forensic tool for the traceability of edible fish (see for example Barbuto et al., 2010; Smith, McVeagh, & Steinke, 2008; Yancy et al., 2008). The *cox1* region showed a good discriminatory power in the identification of fish species (98% of probed marine species and 93% of freshwater species were successfully identified, Ward, Hanner, & Hebert, 2009). Successful results were also obtained starting from small portion of fresh or processed material by using few universal primer combinations (see Steinke & Hanner, 2011).

To date, more than 70,000 barcode sequences from 8300 species (26% of the total) have been stored in the framework of an international collaborative research: the Fish Barcode of Life Initiative (FISH-BOL—www.fishbol.org). FISH-BOL represents one of the most comprehensive resources for the analysis of fish and seafood products (Ward et al., 2009). Conceived in 2004, FISH-BOL involves hundreds of researchers, with the aim to obtain reference DNA barcode records for all fish species in the world. FISH-BOL data are available as a public resource in the form of an electronic database, which contains DNA barcode sequences (most of which freely available), images of reference specimens, and several sampling details. FISH-BOL data are also deposited and organized in the BOLD (Barcode Of Life Data) system (Ratnasingham & Hebert, 2007).

DNA barcoding was proposed by the US Food and Drug Administration for the authentication of fish-based commercial products (Yancy et al., 2008). In particular, the U.S. FDA planned to include DNA barcode data into the Regulatory Fish Encyclopedia, in order to help investigation of mislabeling and fish species substitution.

DNA barcoding was also proven effective in tracking seafood after industrial processing. Some species require only a primary processing, such as the freezing of the fresh fish for distribution to fresh fish retailers and catering outlets, hence preserving morphological characters useful for an accurate identification. However, when a complex manufacturing process is required (i.e. chilled, frozen and canned products for the retail and catering trades), or in the case of fish sold in parts (e.g. steaks, blocks, surimi, fish sticks and fins), classical identification processes are not effective, and DNA barcoding can be useful to obtain an identification.

Despite its proven effectiveness, few studies on the application of DNA barcoding on other categories of seafood have been made (e.g. crabs: Haye, Segovia, Vera, Gallardo, & Gallardo-Escàrate, 2012, holothurians: Uthicke et al., 2010, lobsters: Naro-Maciel et al., 2011). Furthermore, DNA barcoding approach based on *cox1* is not always suitable to identify some organisms, such as gastropods (Meyer & Paulay, 2005). More extensive studies are required to confirm the potential use of this technique on all kinds of seafood as a reliable "traceability tool".

3.2. DNA barcoding and meat traceability: the problem of the lack of data

Meat is normally subject to long production and distribution chains, which requires proper traceability systems. Pathologies related to meat as food (e.g. BSE, avian flu), and malpractices of some producers, have increased public awareness on the origin and quality of meat. Hence, the definition of accurate and reliable methods to identify the composition of food meat is necessary, besides the use of labels, which do not provide enough warranties about the actual content of a product. These new methods should protect both consumers and producers from frauds, and animal species from over-exploitation or illegal commerce (Manel, Berthier, & Luikart, 2002). A variety of DNA-based approaches for meat traceability, such as PCR-RFLP, species-specific PCR and PCR sequencing, have been developed (Mane et al., 2006; Teletchea et al., 2005). These approaches involve the use of mitochondrial other than nuclear markers. Recently, Teletchea, Bernillon, Duffraisse, Laudet, and Hänni (2008) proposed a microarray-based method, which make use of cytochrome b-derived probes, as a tool to identify commercial and endangered species of vertebrates in both food and forensic samples of meat. Cytochrome b region exhibits large interspecific and low intraspecific diversity, as well as conserved flanking regions, hence being a typical candidate as DNA barcode region. The choice of cyt b instead of *cox1* is due mainly to practical reasons. Several thousand *cyt b* sequences are deposited in public databases for a large range of edible mammal species, while only few *cox1* sequences are available in BOLD and GenBank. However, despite this lack of data, DNA barcoding technique based on *cox1* can be considered a reliable method for traceability of mammalian meat (see Cai et al., 2011; Francis et al., 2010; Luo et al., 2011). Similarly, as far as avian meat products are concerned, DNA barcoding based on *cox1 is* effective in identification (Hebert et al., 2004), but its use in the context of meat traceability is still limited.

As applied to the meat market, the relationships between DNA barcoding sequences and species names should be critically evaluated, because the commercial name of a meat product could refer to different molecular units (the so called Molecular Operational Taxonomic Units, or MOTUs, Casiraghi et al., 2010). As an example, Ludt, Schroeder, Rottmann, and Kuehn (2004) clearly showed consistent molecular differences within the species *Cervus elaphus*. As a consequence, deer meat should be identified with two different DNA sequences corresponding to *Cervus canadensis* (occurring in Asia and North America) and *C. elaphus* (inhabiting Europe). A similar situation occurred in bird species as in the case of the English and USA breeds of turkey (*Meleagris gallopavo*) that showed consistent genetic differences (Hird, Goodier, & Hill, 2003).

There are also several cases of species or breeds with the same DNA profile. In this case the DNA barcoding approach would not be able to return a correct identification, therefore making it impossible to track some meat products. This phenomenon, because of hybridization, which produces genetic introgression, is common in livestock. Cattle, where many breeds are derived from hybridization events (see Kikkawa et al., 2003; Nijman et al., 2003; Verkaar et al., 2003), is a typical example.

3.3. DNA barcoding of dairy products: a potential application

Dairy products are generally defined as foodstuffs made from mammalian milk. Due to the economic relevance, risk of allergies and religious practices related to this category of products, the development of techniques to assess authenticity and adulteration of milk-derived food is an issue of primary importance (Mafra et al., 2008). The use of molecular tools to characterize and trace dairy products is gaining large acceptance (Ponzoni, Mastromauro, Gianì, & Breviario, 2009) even if there are no studies based on a strict DNA barcoding approach. However, species-specific PCR has shown to be a reliable method to control the authenticity of this food category, because a specific target sequence (e.g. 12S rRNA, 16S rRNA, *cytb*) can be detected in matrices containing a pool of heterogeneous genomic DNA, such as milk (Mafra et al., 2008). Among the applications of these molecular tools, there is the possibility of detecting the adulteration of higher value milk by nondeclared cow's milk or the omission of a declared milk species. With regard to the characterization of milk origin and quality, an interesting application of DNA barcoding was recently described. The plastidial *rbcL*—the most universal marker for plant DNA barcoding—was found able to detect traces of feed-derived plant DNA fragments in raw cow milk and in its fractions (Nemeth et al., 2004; Ponzoni et al., 2009).This open new perspectives for the traceability of milk and dairy products in general.

On the whole, to obtain an accurate characterization of dairy products quality, a multilevel molecular approach is necessary. In particular, DNA barcoding-like techniques are useful in providing a reliable characterization of the composition of raw milk, while other approaches such as the PCR-DGGE can be useful to assess the microbial composition and provenance of processed milk products (Arcuri, El Sheikha, Rychlik, Piro-Métayer, & Montet, 2013; Borelli, Ferreira, Lacerda, Franco, & Rosa, 2006; Coppola, Blaiotta, Ercolini, & Moschetti, 2001; Dolci, Alessandria, Rantsiou, Bertolino, & Cocolin, 2010; Ercolini, 2004; Ercolini, Frisso, Mauriello, Salvatore, & Coppola, 2008).

3.4. DNA barcoding of edible plants

Plants are an essential element in human diet, both directly (cereals are the base of the food pyramid, followed by fruits and vegetables) and indirectly (plant products are used to feed cattle). Furthermore, several plants are used as food additives (e.g. soy). A reliable identification of crop species, as well as their origin and traceability, are key elements in the field of food safety. In the last 20 years several PCR-methods have been tested on several crop cultivars, such as rice, corn, sorghum, barley, rye (Pasqualone et al., 1999; Ren, Zhu, Warndorff, Bucheli, & Shu, 2006; Salem et al., 2007; Terzi et al., 2005). These methods are useful for both the producers, who are interested in protecting and certifying their crops (De Mattia, Imazio, Grassi, & Labra, 2008; Labra et al., 2003; Ren et al., 2006), and consumers, who are interested in the quality and origin of their food. The increasing diffusion of genetically modified (GM) crops has further increased the demand for molecular techniques to track transgenes (Auer, 2003). In recent years, a multiplex DNA microarray chip for simultaneous identification of GMOs, based on regulations of different countries, has been developed (Leimanis et al., 2006; Nikolic, Taski-Ajdukovic, Jevtic, & Marinkovic, 2009), as well as similar systems devoted to the identification of plant species and cultivars (Agrimonti, Vietina, Pafundo, & Marmiroli, 2011; Xie, McNally, Li, Leung, & Zhu, 2006). However, as previously discussed, these molecular methods have a common limitation in their high species-specificity. Due to globalization, an increasing number of plants originating from different areas of the world are now offered to consumers, but there are not reliable, universal tools for their identification. DNA barcoding could be a reliable alternative to DNA fingerprinting approaches in plants identification, with a higher effectiveness/cost ratio. In fact, DNA barcoding does not require an extensive knowledge of the genome of each organism, being based on the use of one or few universal markers (Hollingsworth et al., 2011).

Nowadays, the research on DNA barcoding in the field of botany is shifting from the analysis of the performance of different markers towards more practical applications. Among edible plants, this approach has been used to track spices (De Mattia et al., 2011). Species of the genus *Mentha*, *Ocimum*, *Origanum*, *Salvia*, *Thymus* and *Rosmarinus* were analyzed by using the core-barcode region (*matK* + *rbcL*), and the trnH-psbA intergenic spacer. With DNA barcoding, most common spices can be identified, with the exclusion of marjoram and oregano, which, belonging to the same genus

Oregano, have an intraspecific diversity which is higher than interspecific, because of several cases of hybridization.

DNA barcoding has shown high performances in discriminating basil species: *matK* and trnH-psbA were able to distinguish commercial basil (*Ocimum basilicum* L) from other *Ocimum* species, as well as different basil cultivars.

DNA barcoding was also used to investigate the genetic relationships between wild and cultivated plants, as well as their origin. Nicolè et al. (2011) used DNA barcoding on bean germplasm (*Phaseolus vulgaris* L.) observing distinct haplotypes for bean accessions corresponding to Mesoamerican or Andean areas. However, this study also highlighted the limits of approach in resolving genetic relationships between races and strictly related varieties.

Bruni et al. (2010) evaluated the effectiveness of DNA barcoding in separating toxic from edible species, evidencing a clear molecular distinction between cultivated species of the genera *Solanum* (*Solanum tuberosum* L., *Solanum lycopersicum* L. group) and *Prunus* (*Prunus armeniaca* L., *Prunus avium* L., *Prunus cerasus* L., *Prunus domestica* L.) and their toxic congenerics. This study suggested that DNA barcoding could be used to distinguish edible species from their non-edible or toxic congenerics (Jaakola, Suokas, & Häggman, 2010).

The limits of adopting universal barcode markers are evident at the cultivar level, where genetic variability is limited, and there are complications due to breeding events. To overcome these limits, Kane and Cronk (2008) proposed the ultra-barcoding methodology, which is based on the sequence of the whole plastidial genome, together with large portions of the nuclear genome. This combination provides enough information to evidence genetic diversity below the level of species, distinguishing hybrids from pure lines, hence it is far more sensitive than traditional DNA barcoding (Nock et al., 2011; Parks, Cronn, & Liston, 2009; Steele & Pires, 2011). Kane and Cronk (2008) evaluated the effectiveness of ultra-barcoding on cocoa (*Theobroma cacao* L.), and found several plastidial and nuclear SNPs, which were useful to identify different cultivars. This technique is promising, but it is difficult to apply on a large scale due to its high costs, and its excessive species-specificity.

Nowadays, there are no technical limitations to the application of DNA barcoding for the traceability of plant raw materials. However, the reduced genetic diversity at cultivar level often requires the analysis of large portions of the genome, which currently have a too high cost/effectiveness ratio to be widely used. Furthermore, this approach is contrary to the basic DNA barcoding methodology, which requires the analysis of short and universal DNA regions only.

4. DNA barcoding as a traceability tool during food industrial processing

An “ideal” traceability system would follow the “history” of a product from its origin to the moment it is used, taking into account all transformation and commercialization steps. Molecular identification and traceability systems were developed to work on raw materials. However, seeds, fruit, and different plant and animal parts are transformed in food with a definite shape, taste and smell through physical (i.e. heating, boiling, UV radiation) or chemical (i.e. addition of food preservatives, artificial sweeteners) treatments, which could alter DNA structure. For this reason, the application of DNA-based identification techniques (among which DNA barcoding) on transformed commodities can be ineffective because of the level of DNA degradation, and the simultaneous presence of several genomes belonging to different organisms.

4.1. Integrity of DNA during food industrial processes

DNA is normally more resistant to industrial processes than other molecules, such as proteins (Martinez et al., 2003), and DNA fingerprinting methods can be successfully used in identifying animal or plant materials, even when in small traces (Bottero & Dalmasso, 2011; Costa, Mafra, Amaral, & Oliveira, 2010; Kesmen, Sahin, & Yetim, 2007; Mane, Mendiratta, & Tiwari, 2009; Martin et al., 2009; Soares, Mafra, Amaral, & Oliveira, 2010). Nonetheless, food processing causes chemical and physical alterations, degradation and fragmentation being the most common effects (Bauer, Weller, Hammes, & Hertel, 2003). DNA integrity largely influences the effectiveness of molecular methodologies (Hellberg & Morrisey, 2011; Meusnier et al., 2008; Pafundo et al., 2007). DNA barcoding can have two advantages if compared to DNA fingerprinting approaches: *i*) it requires the amplification of a short DNA fragment (hence there is a lower risk of fragmentation), and *ii*) it is based on mitochondrial or plastidial genome (more preserved during processing).

Aslan, Hamill, Sweeney, Reardon, and Mullen (2009) showed that nuclear DNA is less preserved than the mitochondrial one in cooked meat. The analysis of different mtDNA regions can be effective in identifying bovine, sheep, and porcine meat even after boiling, pressure cooking or frying (Arslan, Ilhak, & Caliciogiu, 2006; Aslan et al., 2009; Mane et al., 2009). Complete *cox1* sequences were obtained from smoked fish products such as cod, groper, mackerel, salmon and tuna (Smith et al., 2008). Some difficulties were evidenced in obtaining full-length DNA barcodes from canned fish; in these cases, the use of shorter barcode sequences was considered a suitable choice, when limited to traceability purposes (Rasmussen et al., 2009). Similarly to mtDNA, plastid genome is conserved in most of processed food derived from plants. DNA barcoding was used to identify different aromatic species after industrial drying and shredding (De Mattia et al., 2011). DNA barcode markers were also efficiently used to identify commercial tea (Stoeckle et al., 2011), fruit species in yogurt (Knight, Ortola-Vidal, Schnerr, Rojmyr, & Lysholm, 2007), and fruit residues (e.g. banana) in juices, purees, chocolates, cookies, etc. (Sakai et al., 2010). Hence, DNA barcoding approach could be used for the analysis of different food matrices, its main constraints being: *i*) the level of degradation of DNA; *ii*) the development of reliable methods for DNA extraction, and *iii*) the effectiveness of different barcode methodologies (Hellberg & Morrisey, 2011).

4.2. Characterization of mixed food products

The DNA barcode region(s) and the primers used from DNA amplification are universal (Hebert et al., 2003). Given these assumptions, PCR amplifications performed on DNA samples deriving from mixed food matrices produce several DNA barcode fragments, which correspond to different species. Hence, Sanger-based DNA sequencing, although being effective when used for DNA barcode, is not a feasible approach for mixed food, unless preceded by a cloning approach, which could introduce biases, because of the co-amplification of DNA fragments from different individuals or taxa. Several techniques, such as digestion with specific restriction enzymes (i.e. RFLP), or electrophoretic analysis (Colombo, Chess, Cattaneo, & Bernardi, 2011; Mane et al., 2009; Teletchea, 2009) were used to separate different DNA fragments before the sequencing process. However, these methods are effective only when the food matrix is made of few species, and when they have relatively relevant differences in their DNA barcodes (i.e. different target regions for restriction enzymes, and sequences of different length). In other cases, amplicons should be cloned into plasmid vectors and introduced into bacterial competent cells (Zeale, Butlin, Barker, Lees, & Jones, 2011), in order to obtain single fragments. To date, this procedure has been used in dietary studies devoted to some animals, such as mammals and birds, or to identify plants present in the intestinal samples of mammoths (Van Geel et al., 2011).

A possible effective approach in applying DNA barcoding to complex food matrices could be the 454 pyrosequencing methodology, which produces several hundreds of thousands sequences per run, corresponding to the whole mix of DNA molecules extracted from the matrix. This approach allows to identify all raw materials,

including contaminants, or elements occurring in traces only. Pyrosequencing was used for several DNA barcoding analyses (see Hajibabaei, Shokralla, Zhou, Singer, & Baird, 2011; Valentini et al., 2009), including the identification of raw material of the diet of several animals (Raye et al., 2011; Soininen et al., 2009), as well as for analysing ancient DNA extracted from museum specimens (Shokralla et al., 2011). The restriction of this approach is the reduced length of barcode sequences, which range from 250 to 400 bp (Valentini et al., 2009). This limit has been partially resolved using minibarcodes: shorter fragments of *cox1* of about 150 bp (Hellberg & Morrisey, 2011; Meusnier et al., 2008; Shokralla et al., 2011), which can be also obtained through 454 pyrosequencing. The minibarcode approach provides enough information to identify species from different matrices (Hajibabaei, Singer, Clare, & Hebert, 2007; Hajibabaei et al., 2006, 2011; Meusnier et al., 2008), as well as to identify the content of seafood products (Becker et al., 2011; Botti & Giuffra, 2010; Rasmussen et al., 2009). However, the reduced length of minibarcode sequences may be not informative enough to identify closely related species.

5. Food safety and commercial frauds

Food safety is strictly related to the chemical and microbiological quality of raw material. Other important aspects include the hygienic practices adopted during industrial processes, and the distribution of final products. Several regulations, such as the European EC/178/2002 include the principal assumptions and rules related to the safety of food in general and to the authorities involved in food control procedures. Other regulations focus on more detailed aspects of the food industry, such as fishery and aquaculture products (EC/2065/2001). Symptoms deriving from the use of adulterated food are normally immediate, as in the case of seafood, which causes illness in 76 million USA citizens every year (Food and Water Watch, 2007), in many in many cases because of poor conservation practices, with consequent microbial contamination (see Shikongo-Nambabi, Chimwamurombe, & Venter, 2010). Nowadays, there are several microbiological tests to detect bacteria on raw materials and food (Boehme, Fernandez-No, Gallardo, Canas, & Calo-Mata, 2011; Boehme et al., 2010). Negative effects on human health could also derive from accidental or deliberate substitution of seafood species with others, which are not included in national or international regulations. As an example, the Nile perch (*Lates niloticus*), which is subject to commercial restrictions, is often used as a substitute for other perches, or several other species. In these cases, beyond obvious economical consequences, the substitutions could cause health risks. Nile perch coming from African rivers is contaminated by methylmercury and other pollutants (Filonzi, Chiesa, Vaghi, & Nonnis Marzano, 2010; Guallar et al., 2002). Another example of illegal and dangerous to health substitution is the toxic pufferfish, which is mislabeled as monkfish (Cohen et al., 2009).

Recent studies in Europe and North America reported that commercial frauds range from 15% to 43% of total commercial seafood, with 75% of frauds in the case of red snapper (*Lutjanus campechanus*, Hellberg & Morrisey, 2011; Rasmussen & Morrisey, 2008). The DNA barcoding methodology could be used to discover species replacement, evidencing commercial frauds. The *cox1* mitochondrial region was largely adopted to identify seafood species (Ardura, Linde, Moreira, & Garcia-Vazquez, 2010; Barbuto et al., 2010; Holmes, Steinke, & Ward, 2009; Hubert et al., 2008; Rasmussen et al., 2009; Steinke, Zemlak, Gavin, & Hebert, 2009; Valdez-Moreno, Ivanova, Elıas-Gutierrez, Contreras-Balderas, & Hebert, 2009; Wong & Hanner, 2008; Yancy et al., 2008). The availability of a well-populated reference database such as BOLD suggests that nowadays, DNA barcoding, can be used as a standard test tool by regulatory institutions (e.g. the U.S. Food and drug administration; Yancy et al., 2008).

In many cases commercial frauds are related to taxonomic problems: species can be identified by a common vernacular name, which can correspond to different taxa. Barbuto et al. (2010) reported the case of two *Mustelus* species (*M. mustelus* and *M. asterias*) which are sold in Italy under the same commercial name 'Palombo'. In other cases, different vernacular names are associated with the same species in different regions (Burgess et al., 2005). To avoid frauds and mislabeling, the vernacular name of edible species should be written together with the correct scientific name, and the reference to the DNA barcode sequence.

DNA barcoding showed a high effectiveness in the evaluation of the presence of allergenic species, both in fresh and in processed food. Nuts are considered one of the main sources of allergens (Hubalkova & Rencova, 2011), and their presence in food (also in traces) is detectable by molecular analysis based on different markers, including DNA barcode regions (e.g. *matK*) (Yano et al., 2007). Almond (*Prunus dulcis*), commonly used in several food products (bakery, pastry, snacks) due to its pleasant flavor, is also a potential allergenic (Costa, Mafra, Carrapatoso, & Oliveira, 2012). In this case, the distinction of almond DNA traces from other congeneric edible species such as cherry (*P. cerasus*), plums (*P. domestica* L.) and peach (*Prunus persica*) could be a problem. However, analyses conducted on the plastidial genome of these congeneric species evidenced some differences, which can be used in identification (Badenes & Parfitt, 1995).

The identification of allergenic material is one of the more interesting applications of DNA barcoding. It can be used to satisfy the requirements of FAO and European Commission, which list allergenic species that must be declared on food labels (Directive 2003/89/EC.1).

Similar approaches could also be applied to food intolerance as a consequence of substances present in some genera or species, such as gluten for people with celiac disease. Recently, PCR methods to identify the presence of rye, wheat and barley in products labeled as 'gluten-free', based on the analysis of plastidial markers (e.g. *trnL*), have been developed (Maskova, Paulickova, Rysova, & Gabrovska, 2012).

Food products which are in contrast with individual lifestyles or religious rules can also be included in the category of food frauds. This is the case of the addition of meat or of its sub-products in food consumed by vegetarians, or the undeclared use of pork meat, which is prohibited by Jewish and Muslim religions (Ibrahim, 2008; Kesmen et al., 2007; Montiel-Sosa et al., 2000). DNA barcoding can be an effective tool to discover these frauds.

In all these examples, the effectiveness of DNA barcoding methodology is strictly related to the presence of reliable and accessible reference sequences, which can be found only in a reliable reference database, developed by a joint effort from scientists from all over the world. This is particularly true in the case of plants, for which reference databases are practically absent, or underpopulated.

6. Conclusions

DNA barcoding can be used as a universal tool for food traceability. Even if, from a merely technical point of view, it is not completely innovative, in just a few years it has become widely used. This was ensured by a combination of factors: *i*) the dropping cost of molecular analyses; *ii*) the increasing availability of equipped laboratories and skilled personnel; *iii*) the presence of freely available web-based resources; *iv*) the increasing amount of informed consumers which require high standards of quality in food products. This scenario generated the request for a technique built around molecularization, standardization and computerization. In this sense, DNA barcoding is not only up to date, but is the natural product of the 2000s.

These case studies and technical advancements clearly indicate that DNA barcoding is a sensitive, fast, cheap and reliable method for identifying and tracking a wide panel of raw materials and derived food commodities (even in the case of strongly processed food

products), and for detecting allergens or poisonous components potentially occurring in food matrices.

Due to its universality, DNA barcoding can be used in different contexts, and by different operators. International agencies or institutions, which are responsible for quality control of raw materials or food commodities, can cooperate by exchanging their data, hence creating population reference databases, the lack of which is the only real limit of the method. In fact, while some groups of organisms (e.g. fish) are well represented, a lot of work is required to provide a reliable source of reference DNA barcoding data for groups which have been poorly investigated. For this reason, in the near future DNA barcoding is likely to become a routine test in many fields, and in particular in food quality control and traceability.

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