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The Evolution of DNA Typing in Agri-Food Chain

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Abstract

***Background*:** DNA typing has revolutionized not only diagnostics and forensics but also how we can analyze food. A number of techniques have been successfully applied for DNA analysis of plant-derived food. However, unlike forensics, a universally employed method has not yet emerged.

***Methods*:** A keyword-based search was performed using the ISI-Web of Science database to look for research articles on DNA testing in agri-food chain. After screening and eligibility check, a Systematic Review was compiled focusing on the techniques used to detect DNA polymorphisms.

***Results*:** The collection and summarization of the eligible peer-reviewed empirical studies indicated that PCR-based methods are the predominant technical approach for DNA testing in the agri-food chain. Simple Sequences Repeats are the preferred DNA molecular markers. In recent years, approaches based on DNA-sequencing are expanding, with the DNA barcoding representing the most popular option for species identification. Hypothesis-free NGS approaches are limited.

***Conclusions*:** The choice of the method is mainly dictated by the aim of the genetic analysis (e.g., to distinguish plant species or varieties) and the need of quantitative information rather than the features of the food product or beverages. The implementation of new

technologies (e.g., NGS-based) is growing but their use remains narrow compared to diagnostics.

Keywords
DNA test; frauds; food; molecular markers; plants; fingerprinting; forensics.

1. Introduction

1.1 Authenticity and DNA Analysis

Concerns about the “authenticity” of food products are growing in the field of genetic testing applied to the agri-food chain [1]. "Authentic" food is the one that conforms to the description provided by the producer, the manufacturer, or the vendor, with reference to the origin of the ingredients, the history, and features of the transformation process, the geographical origin, and the taxonomic identity of the species or variety used.

Although not a new concept, authenticity is increasingly being emphasized in the food sector because of its relevance to marketing and branding, and to reassure consumers. The analytical tools employed in food authentication are different [2, 3]. Those based on DNA testing are of considerable importance because, in principle, any claim related to the presence of a specific biological ingredient in food can be verified by DNA testing. DNA testing is also a powerful technique owing to the fact that processed foods often lose their morphological and diagnostic features. Moreover, food processing and storage make the analysis of other biochemical components less effective. In addition, DNA analysis can allow quantitative determinations also in mixed or complex food products. In theory, any DNA marker can be used to analyze food [4]. Among the available techniques, those based on the Polymerase Chain Reaction (PCR) offer significant advantages. The PCR is a simple, accurate, sensitive, inexpensive, and reliable technique that repeatedly replicates a DNA segment from virtually any material [5]. The classic procedure relies on thermal cycles but alternatives have also been developed. For example, isothermal amplification techniques (in brief, isothermal PCR) of nucleic acids are based on a constant temperature of DNA synthesis and thus they do not require a thermocycler [6]. DNA polymorphisms can be also revealed by DNA sequencing. DNA sequencing is a collective term for a series of techniques and/or processes that allow determining the order of the bases in a DNA molecule. Following its early days in the 1970s, the nucleic acid sequencing technology has been characterized by two breakthroughs. The first one was the automation of fluorescence-based capillary electrophoresis systems [7]. This advancement allowed a much more efficient and reproducible sequencing and significantly accelerated the production of the first human genome, ushering in the "genomics" era [8]. The development of automatic sequencing systems with remarkable spatial- (ability to distinguish fragments of different sizes) and spectral-resolution (ability to distinguish different fluorescent dyes) has also greatly favored the adoption of microsatellites as the marker of choice for forensic genetics [9]. A second milestone for DNA sequencing has been the evolution of the Next Generation Sequencing (NGS) technologies [10]. The strong demand for low-cost sequencing has driven the development of a number of technical

approaches. They share the ability to parallelize the sequencing process, thus producing from thousands to millions of sequences simultaneously. NGS sequencing technologies have greatly reduced the cost of DNA sequencing per base and currently, they are accessible by most research and diagnostic laboratories [11].

1.2 A Brief Overview of the Forensic Methods Based on DNA Analysis

Sir Alec Jeffreys is universally regarded as the creator of the first DNA analysis technique for forensics, popularized with the name of "DNA fingerprinting". His research group identified highly variable sequences of the human genome that can be revealed using a single probe [12]. This approach was found to be applicable in different biological areas but, when it was used in civil and criminal cases in England, the impact of the molecular analysis of hypervariable sequences went beyond the academia [13]. The original method was based on radio-labeled minisatellite probes, used for the Southern blotting technique. Despite its successful application for a wide range of legal cases, this approach had limitations, requiring a large quantity of high molecular weight (e.g., non-degraded) DNA. Since the mid-1990s, DNA fingerprinting methods based on hybridization have been progressively replaced by PCR-based methods [14]. Among the DNA markers based on PCR, microsatellites (aka STRs or SSRs) have quickly established themselves as the reference technique [15]. In human forensics, DNA profiling is currently performed with a panel of 12 (resp. 13) STR markers according to the EU standard (resp., the US CODIS standard) [13]. In recent years, ample progress has been made by exploiting DNA sequencing to detect sequence polymorphisms and high-throughput sequencing technologies are being increasingly used in clinical diagnostics [16]. In a nutshell, the NGS technology can be employed in two conceptual approaches. It can speed up the sequencing of specific, diagnostic PCR products or targeted libraries in a hypothesis-driven analysis and, it can be employed to sequence the whole DNA of the sample of interest for a hypothesis-free investigation.

1.3 Specificity of the DNA Testing in the Agri-Food Chain

Genetic traceability in the agri-food sector presents peculiar challenges compared to the genetic analysis in the human or animal sector. They include the biochemical and physical variability of the samples under examination, the very likely degradation of DNA (due to food transformation and storage), and the need to distinguish not only between different plant species but also varieties. For plant-derived food products, sometimes it is necessary to provide a systematic classification at the species level (e.g. detection of allergenic, poisonous, contaminant or toxic species). In other cases, it is necessary to identify the variety, because the price of a number of agricultural commodities is related to the plant variety (e.g., fruits, grains, vegetables, herbs, spices, etc.). Moreover, for plant breeding and more generally, for protection of Intellectual Property Rights (IPRs), often it is necessary to distinguish hybrids, which strongly favors the use of codominant markers. Finally, another specific feature is that plant species subject to intense breeding (e.g., many annual vegetables) have a limited genetic variability. Considering the specificity of the DNA testing of plant-derived food and the variety of the available methods, the aim of this paper was to analyze the adoption and evolution of the different DNA typing systems in the agri-food sector. In addition, we also scrutinized the diffusion of different methods in order to establish their relative importance and to allow determining future trends. To these goals, we

carried out a systematic review of the literature to identify, retrieve and manage scientific articles published in different indexed journals.

2. Materials and Methods

To collect and critically analyze peer-reviewed research articles, a systematic evaluation of the literature was conducted referring to the "Preferred Reporting Items for Systematic Reviews and Meta-Analyses" (PRISMA) [17]. PRISMA represents the evidence-based minimum standard of items for performing systematic reviews and meta-analyses in the scientific literature.

2.1 Identification

The literature search was performed on the Web of Science Science Citation Index Expanded database. The Science Citation Index (SCI) is an index of citations originally produced by the Institute for Scientific Information (ISI). We consulted the Science Citation Index Expanded section, the most extensive one, covering more than 8,500 journals in 150 disciplines. The indexed journals of this database are considered to be authoritative in the research area due to their rigorous selection process. The SCI ismade available online through various platforms, and we queried the Thomson Reuters Web of Science (https://clarivate.com/products/web-of-science/). The search was performed within the 2000-2018 time interval. The search statements (i.e., the queries that identify the information to be searched in the bibliographic database) were obtained by combining one term from each the following three fields: 1) Agro; Plant; 2) AFLP, Barcoding, CAPS, DNA, EST, Fingerprinting, ISSR, ISTR, molecular markers, NGS, PCR, RAPD, RFLP, Sequence, SNP, SSR, STR, VNTR; 3) Food, Mislabeling, Traceability. Specifically, the three query keywords were combined (ordered combinations without repetitions) with the Boolean operator "AND". The search field in the database was "Title / Keywords / Abstract". Additional records were identified through other sources (e.g., cross citations).

2.2 Screening and Eligibility

After removing duplicate entries, the bibliography was manually curated to exclude articles that did not fall within the area of interest. Essentially, we retained only articles that employed a nucleic acid detection technique to verify the genetic identity of plant species or variety in a food product (including beverages and herbal products used as a food supplement). For example, articles on the traceability of GMOs, pathogen or pest detection in food, identification of plant species in non-edible material (e.g., ingested by herbivores or insects, raw biomass, etc.) or honey, the recognition of wood species or of ancient food remains, or papers specifically dealing with procedures to isolate DNA from foodstuff were not considered eligible. Reviews were also excluded. The articles were classified according to the molecular method used. Considering the variety of techniques, names and synonyms (e.g., PCR-RFLP and CAPS) in the literature as a guiding criterion we took into account the technique used for the polymorphism detection, rather than the method of resolution of nucleic acids (e.g., the electrophoretic methods employed to reveal polymorphism). Each article was then assigned to one category, with the exception of papers based on the comparison or the combination of two or more analytical techniques.

3. Results and Discussion

The database search provided 91108 articles in the 2001–2018 period after removing duplicate entries. Many of these articles also referred to different areas of research that do not necessarily concern genetic traceability in the agri-food sector. After eligibility check, we included in the quantitative synthesis 243 studies.

3.1 Popularity of the Techniques

Being very heterogeneous, the articles were classified based on the type of DNA polymorphism in analysis. The main criterion to summarize the literature was to distinguish between methods based on polymorphisms due to the presence/absence of a diagnostic fragment (including techniques that reveal length polymorphisms of target sequences) and methods that reveal polymorphisms in the DNA sequence (e.g., the order of the bases). The articles were categorized under the following methodological approaches: amplification, arrays, sequencing (including NGS), and others. Under the term "amplification" we classified articles based on the direct DNA amplification to reveal, directly or indirectly, amplicon length polymorphisms. With the term "array", we categorized the articles that were based on the hybridization of nucleic acids (amplified or not) to DNA target molecules placed on a solid support. The classification "sequencing" was applied to the articles employing a technique that reveals polymorphism among samples based on the determination of the order and/or the presence of nucleotides in the DNA molecule, even if the sequenced products derives from amplification. In the category “others”, we included the remaining techniques, such as "sensors" (analytical devices that combine a biological component – typically a nucleic acid aka genosensor – with a physical-chemical transducer) and fluorescence transfer of resonance energy (FRET) approaches. Around 4% of the articles employed more than one technique of DNA analysis. In most cases, these were variations or modifications of the same basic technique (for example, standard PCR in comparison with real-time PCR, or two types of different DNA markers). More rarely, the comparison was made between more distant methodological approaches (e.g., sequencing techniques compared with PCR).

The data indicated that the techniques based on DNA amplification are by far the most widespread ones, followed by the sequencing-based techniques (Figure 1).

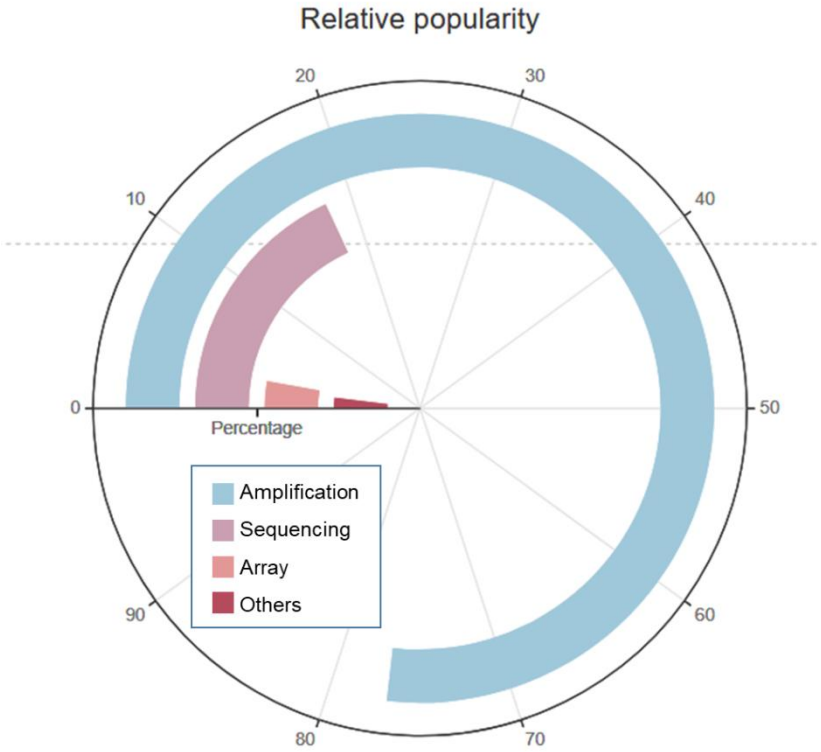

Figure 1 The radial chart illustrating the relative popularity of the technical approaches for DNA testing in the agri-food chain.

According to the literature, direct DNA amplification, such a PCR to reveal the presence of a diagnostic fragment, is taken often into account because it provides information even when a low amount of template DNA is available. Moreover, another frequently mentioned advantage is that the PCR gives the possibility of quantifying the template DNA, providing quantitative information about the presence of a specific plant species or variety. Moreover, the PCR allows the simultaneous amplification of different target molecules in the same reaction (multiplexing), allowing to increase the output-cost ratio. Finally, the use of highly selective primers to amplify a single and specific target sequence strongly limits the problem of working with contaminated samples and genetically complex mixtures. On the other hand, an obvious limitation of targeted PCR diagnostics is its ability to reveal only specific sequences depending on the primers used.

Compared to "amplification" methods, sequencing-based techniques are more limited. It is likely that their cost and laboriousness have limited their implementation. Among them, DNA barcoding has a predominant importance [18]. Although the usefulness and power of DNA barcoding for phylogeny is subject of debate, this technique is much less controversial as a method of taxonomic identification. In food testing, DNA barcoding is widely used for fish and meat products. For agri-food, the literature indicated that about half articles on the barcoding aimed at determining the authenticity of herbal products (typically infusions) or food. Another main area of interest is the identification of the plant species to reveal possible improper substitutions or contaminations. In relative terms, the barcoding represents the most widespread choice to reveal the presence of a plant species based on the analysis of sequence polymorphisms. Conversely, the standard DNA barcoding (e.g., Sanger's sequencing of PCR products) is not applicable to the mixtures of species [19].

3.2 Analysis of the PCR Based Techniques

Considering the prevalence of techniques based on "amplification", we carried out an investigation on the relative importance of the different methods. The techniques were classified into the following categories: "real-time", "standard", "isothermal", "SSR" (Simple Sequence Repeats) and "other markers" (i.e., SCAR (Sequence Characterized Amplified Region); STS (Sequence-Tagged Sites); ELISA-PCR (Enzyme-Linked Immunosorbent Assay; AFLP (Amplification Fragment Length Polymorphism); RAPD (Random Amplification of Polymorphic DNA)(Figure 2).

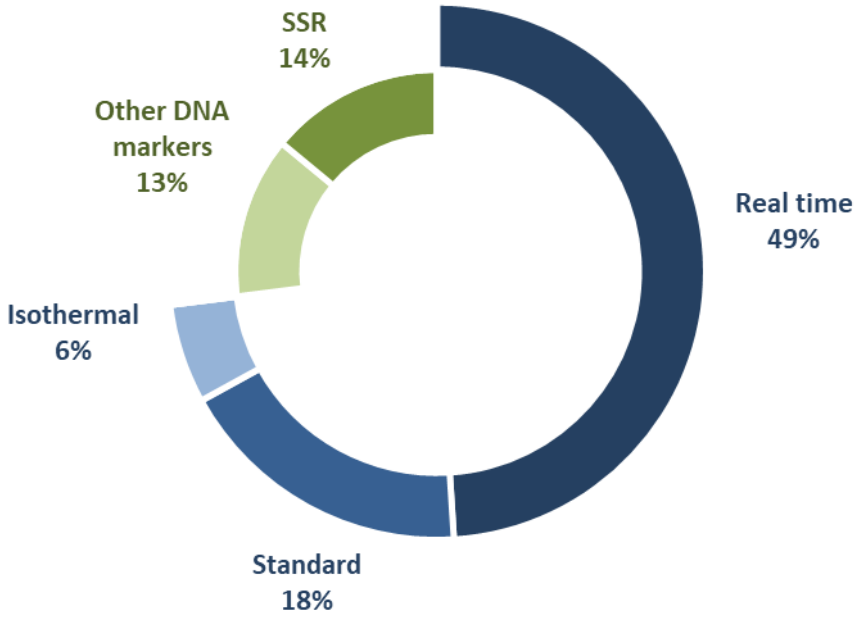

Figure 2 The relative importance of the amplification-based techniques for DNA testing in the agri-food chain. PCR-based DNA molecular markers are in green colors.

The most common technique is the real-time PCR (rt-PCR), which is a procedure that allows monitoring the accumulation of the amplicons during the whole PCR reaction in real-time. This technique offers the advantage of providing an easy quantitative evaluation of the template DNA present in a sample. Furthermore, since rt-PCR commonly relies on the detection of fluorescent molecules, it is more sensitive than traditional techniques. In addition, rt-PCR does not require the electrophoretic separation of the amplicons. However, as a disadvantage, it is more expensive than the traditional methods because of the high cost of reagents and equipment. The literature analysis indicated almost 80% of the research articles based on rt-PCR dealt with the identification of DNA molecules that code for an allergen.. Overall, the sensitivity and the ability to provide quantitative information are the most important assets of rt-PCR in the agri-food chain. Finally, the literature search indicated that in approximately 10% of the cases, the genotyping technique, High Melting Resolution, was performed in association with the rt-PCR [20].

The standard PCR, based on the analysis of specific DNA sequences, is the second most common technique based on direct amplification of a target, followed by isothermal amplification, which was mainly represented by the Loop-Mediated Isothermal Amplification (LAMP) method. The most important features of isothermal amplification are its speed and amenability to low-tech equipment, not requiring a termocycler. Further, in some methods, LAMP is coupled with

detection techniques that do not need an electrophoretic separation, which enhances the portability of the system [21].

In addition to the various methods for the direct amplification of diagnostic sequences (e.g., PCR, rt-PCR, isothermal, etc.), PCR-based DNA markers (i.e., to analyze amplicon length polymorphisms) represent the other important group of tools employed in genetic testing. Among them, SSRs have a predominant role. These are particularly useful for the traceability of organisms where limited genomic information is available, also because a small number of SSRs may be sufficient to discriminate a large group of samples. SSRs have become widely established in the field of plant traceability in the food chain and breeding also because they are codominant, thus making it possible to easily analyze hybrids. In the plant-food sector, most frauds involve the complete or partial replacement of a valuable variety with another of lesser value [22, 23]. These frauds are difficult to expose with other techniques, as it is not easy to find biochemical markers that allow identifying a specific plant variety in a complex food product. The literature search indicated that most of the works based on the SSRs were related to premium varieties such as those protected by European Union quality designations (PDO and PGI). Other PCR-based marker techniques (such as AFLP and RAPD) were rarely reported in the literature. There are various reasons that can account for their limited adoptability. A main reason could be that "anonymous" markers (i.e., those that are not based on the amplification of specific, already identified targets) do not have the “species-specificity” suitable to analyze mixed products, to taxonomically identify samples, or to detect contaminated food.

3.3 Trends in the Use of Molecular Techniques for Genetic Traceability in the Agri-Food Chain

The trend of the popularity of the different methods is illustrated in Figure 3.

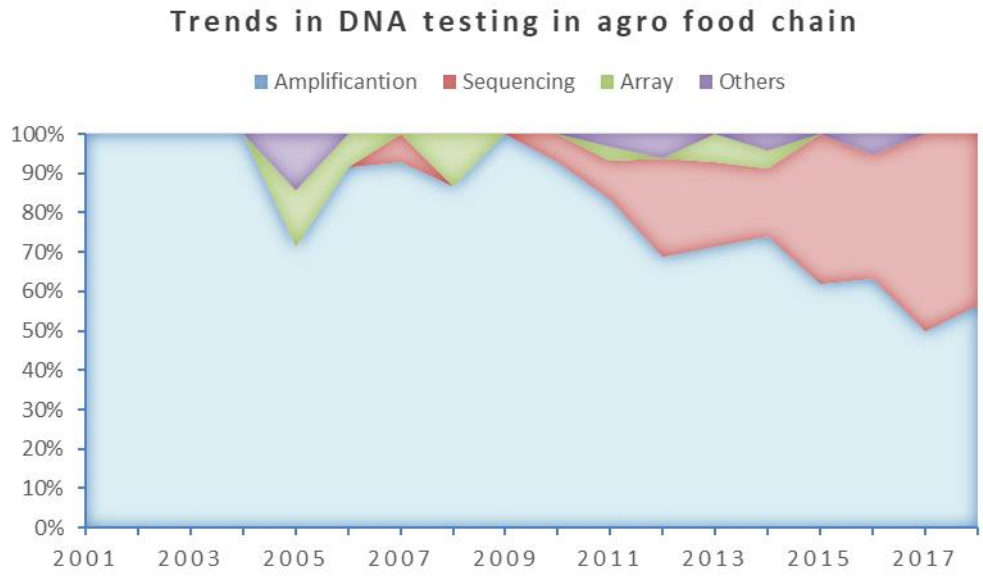

Figure 3 Trends in the relative popularity of the approaches employed for DNA testing in the agri-food chain.

The analysis indicated that the techniques based on the “amplification” were largely predominant in the past. Since the second half of the 2000s, techniques based on solid surface

hybridization (arrays) started to spread. The DNA array technology progressed rapidly mainly due to new fluorescent detection systems and automated production methods. After an initial success, however, the array technology did not rise in popularity, also in the field of genetic traceability in the agro-food chain. Advances in massively parallel sequencing in the late 2000s allowed a rapid decrease of the sequencing cost. The literature search indicated that the development of new sequencing techniques has been associated with the diffusion of barcoding. This was also because of the large increase of genomic information of non-model species. Although limited in number, metabarcoding approaches (e.g., based on the mass amplification and sequencing of samples that potentially contain more than one organism) [24] are also being recently used. The NGS technologies under a hypothesis-free approach have been rapidly adopted in human diagnostics and microbiology. Despite their advantages [25], their use in the field of genetic traceability in the agro-food chain remains very limited (less than five papers). The possible reasons are that NGS technologies are often expensive for large screenings, require a technically specialized workforce, extensive computational power, and higher amounts of DNA. In addition, the sensitivity and, more crucially, the performance of "omics" approach with the DNA isolated from products that have undergone an intense manufacturing process are to be evaluated experimentally. Unlike clinical diagnostics, genetic traceability refers to extremely different samples, for example, in terms of chemical and biochemical composition, conservation, and industrial transformation. Currently, NGS techniques for food analysis are much more widespread in the field of microbiology [26]. The plant genomes, from a computational and genetic perspective, are much larger and complex, mainly because of the abundance of repeated sequences, rendering their analysis challenging especially in complex mixtures. On the other hand, the power of NGS (e.g., higher data resolution and deeper coverage) provides greater statistical confidence for calling DNA polymorphisms [27], facilitating the analytical interpretation of the results.

4. Conclusions

Our analysis indicated that a large number of DNA typing systems have been successfully used in the field of genetic traceability in the agri-food sector [1, 22, 23]. The main goal of DNA testing is to reliably distinguish genotypes in food and to match food samples to specific profiles (source attribution). The term "identification" is cited in the title or abstract of more than half of articles reviewed. Different from forensics, the statistical evaluation on the rarity of DNA profiles to weight the strength of the evidence is seldom presented [28]. The choice of the technique is mainly dictated by the different purposes of the analyses (e.g., taxonomically distinguish species or varieties) and the genetic features of the plants under investigation, rather than the biochemical and physical variability of the food or beverages under examination. The majority of applications are based on the PCR technique, not only for its speed, sensitivity, and affordability but also for the need to work with degraded DNA (e.g., from food processing and/or storage) and to provide quantitative information, as indicated by the wide use of the rt-PCR approach. Amplification-based methods are still very popular because of the implementation of automated, robotic, high-throughput systems in diagnostics. Although applications are present in the literature, the popularity of portable methods for nucleic acid extraction or amplification is limited. Among the DNA markers, SSRs are largely predominant, while DNA barcoding represents the most diffused approach based on sequence polymorphisms. The advances in NGS technologies offer the

possibility to dramatically expand the amount of information accessible to DNA analysis in food-chains [29] For instance, implementation of (targeted) NGS technologies to microsatellite genotyping (the so-called SSRseq) will reduce the genotyping errors deriving from artifacts (e.g., stuttering) and also limitations (e.g., homoplasy) of the electrophoretic separation [30]. Moreover, the application of NGS to DNA barcoding is expected to overcome some limitations in the analysis of complex foods or contaminated samples. Currently, the read length of the commonly used NGS technologies is not yet adequate enough to cover the standard plant DNA barcoding loci, and thus consideration should be given to the "mini-barcodes" [31]. The recent marketing of NGS-platforms specifically designed to perform rapid and cost-effective genetic analysis will allow their applications in targeted (re)sequencing and the future development of analytically validated panels for food testing. Hypothesis-free NGS-studies are still limited and the evaluation and implementation of these new technologies and tools, together with the concerted efforts to increase information sharing and to establish standard operating protocols (e.g., in relation to sequence coverage, run quality, and variant interpretation), should be considered as priorities for the development of genetic typing in the agri-food chain.

Author Contributions

MF carried out the literature search and data analysis, GC conceived the work, analysed the data and wrote the paper.

Competing Interests

The authors have declared that no competing interests exist.

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