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DNA-based techniques to check quality and authenticity of food, feed and medicinal products of plant origin: A review

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ABSTRACT

Background: The increased attention in agri-food quality and safety has led to the progress of DNA-based tools aimed to fight adulteration issues. Among the various molecular approaches, those ones based on molecular markers and DNA barcoding have been adequately validated, whereas new tools such as droplet digital PCR (ddPCR), isothermal amplification and clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated (Cas) (CRISPR/Cas) system are starting to overtake the performances of the former ones and to be applied in the agri-food sector.
Scope and approach: Herein, an overview of the recent advances and exploitations of the DNA-based techniques for the authenticity and traceability of fresh and processed plant-derived food, feed and medicinal products is provided, including research about the monitoring of contaminant and allergen presence. Moreover, the potentials and flaws of these molecular tools are also discussed.
Key finding and conclusions: DNA-based technologies represent valuable tools for preventing agri-food frauds and adulteration of several vegetable products present on the market such as spices, extra virgin olive oil, wine, cocoa, and medicinal plants. The application of these methods can contribute to the protection of consumers and all stakeholders involved in the agri-food production and distribution.

1. Introduction

During the years, crisis in the food sector, including Bovine spongiform encephalopathy outbreaks (Wilesmith, Ryan, & Atkinson, 1991), dioxin poultries (Bernard et al., 2002), or the *Escherichia coli* occurrence associated with raw fenugreek sprouts (Foley et al., 2013), have strongly supported the need of a stringent food quality control. The necessity for robust, precise, and trustable analytical techniques for food authentication and traceability has continuously increased in the last decades also due to the strong competition to produce food products of high quality and price, which can lead to a higher chance of food frauds. In parallel, worries concerning origin and safety of food and an increased awareness related to the food characteristics have induced consumers to ask for more transparency from the food sector (Fanelli et al., 2021).

Traceability is defined as the capacity to track the source of a food product, from field to fork, at any point in the production chain, and represents a fundamental tool to find and prevent frauds and contaminant presence that can have, other than economic implications, heavy impact on consumer's health. In Europe, traceability became mandatory for the EU market with the EU Regulation 178/2002 implemented on January 2005. This European law establishes the traceability of produced, imported, or exported foodstuff within the Union. However, the traceability methods have never been clearly described by the regulation (Guyon et al., 2020). Other than authenticity, traceability deals also with the detection and labelling of authorized genetically modified organisms (GMOs) as regulated by the EU Regulation 1830/2003.

The need of a reliable food traceability system has been faced by scientific research hence producing different analytical approaches aimed to fight food authentication issues. Laboratory validation is based mainly on the study of the chemical composition, primary and secondary metabolites, and, lastly also on DNA analysis (Böhme et al., 2019; Fanelli et al., 2021). Studies involving the analysis of the DNA extracted from processed or fresh plant products started more than twenty years

ago, but it is particularly in the last decade that the sector has seen a growth of the number of published papers. A Scopus data search (https://www.scopus.com/search/form.uri?display = basic#basic; accessed on January 8, 2024) was carried out for English language papers for years between 2014 and 2023 using the following search terms: (PCR) AND (authentic*), (PCR) AND (traceability), (SSR) AND (authentic*), (SSR) AND (traceability), (SNP) AND (authentic*), (SNP) AND (traceability), (barcoding) AND (authentic*), (barcoding) AND (traceability), (isothermal amplification) AND (authentic*), and (isothermal amplification) AND (traceability), and selecting only publications concerning plant-based foods, feed and medicinal products and pertinent to traceability and authentication systems. Authors are aware that search carried out with the reported items can present some limitations in retrieving all the relevant articles. Results show that among the molecular analytical methods, the number of works regarding the application of DNA-barcoding was the highest (116 papers), followed by PCR-based methods (Fig. 1; Table S1). Despite Simple Sequence Repeats (SSRs) or microsatellite profiling presents the highest amount of research compared to Single Nucleotide Polymorphism (SNP) genotyping, however, the abundance of publications employing SNPs reached and even surpassed microsatellite in 2022 and 2023, respectively (Fig. 1; Table S1).

In the agrifood sector, frauds are frequently reported, and saffron, spices in general, extra virgin olive oil, wine, cocoa, and fruit juices are often considered as the most adulterated vegetable foods placed on the market. DNA analysis can be of extreme utility considering that the information required for the genetic characterization, often, goes beyond species identification, usually also requiring the identification of a particular variety. Indeed, for many plant species and products of plant origin the market price and the quality attributed by consumers highly depends on the cultivated variety (Galimberti et al., 2013; Zambianchi et al., 2021). Considering that DNA is unique among different
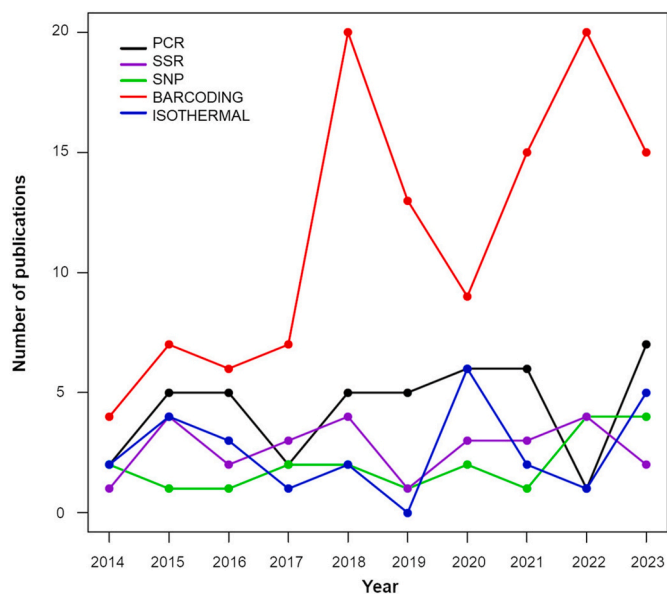

Fig. 1. Number of publications per year in traceability and authentication of foods of plant origin through PCR, SSR and SNP markers, barcoding, and isothermal amplification methods. Data search was carried out using Scopus document archive (https://www.scopus.com/search/form.uri?display = basic#basic; accessed on January 8, 2024) for English language papers for years between 2014 and 2023 using the following search terms: (PCR) AND (authentic*), (PCR) AND (traceability), (SSR) AND (authentic*), (SSR) AND (traceability), (SNP) AND (authentic*), (SNP) AND (traceability), (barcoding) AND (authentic*), (barcoding) AND (traceability), (isothermal amplification) AND (authentic*), and (isothermal amplification) AND (traceability), and selecting only publications concerning plant-based foods and pertinent to traceability and authentication systems.

individuals and is not influenced by environmental conditions or cultural practices, the species attribution and, mainly, the variety identification can be more accurate when DNA-based methodologies are used compared to other types of analysis (Catalano et al., 2016). Further challenges associated with food adulteration regard the addition of unknown components, the identification of GMOs, the proportion of ingredients, the allergen contamination as well as the verification of certified origin such as the quality label Protected Designation of Origin (PDO).

The present review aims to provide an extensive and updated (2014–2023) overview of the main applications of DNA-based methodologies in plant-derived products to prevent adulteration and mislabeling. The most established tools as those based on molecular markers and DNA barcoding along with the most recent ones such as the isothermal amplification, metabarcoding and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) (CRISPR/Cas) system, were explored (Fig. 2), also discussing their limitations and challenges.

2. PCR-based methodologies

So far, many PCR-based techniques have been used to identify commercially relevant species and varieties in food and feeds of plant origin. The advantage of these detection methods relies on the fact that sample identification is performed regardless its age, physiological status, condition and tissue type, and processing of the initial matrix (Kang, 2019; Lo & Shaw, 2018). In this regard, conventional and real-time quantitative PCR (qPCR) assays have been applied over the last twenty years along with more recent PCR techniques like the droplet digital PCR (ddPCR) (Kumar et al., 2022).

PCR is a routinely procedure of molecular biology and over the years it was proposed as a very useful approach for the detection of plant species in foodstuffs (Böhme et al., 2019; Kumar et al., 2022). During the PCR cycling, the exponential amplification of the target DNA allows the detection of desired sequences at very low concentrations even in presence of a complex starting matrix. When the quantification of different ingredients to ensure food authenticity is required, qPCR is the technique of choice for its sensitivity, rapidity, and multiplexing capacity (Böhme et al., 2019; Kumar et al., 2022). During a qPCR test, the DNA target is amplified, monitored cycle-by-cycle in real time and quantified by the measurement of the fluorescence released by a non-specific double strand DNA binding dye or by site-specific fluorescent probes. Real-time-qPCR performances are better compared to standard end-point PCR in terms of sensitivity, multiplexing capacity, speed and cost avoiding the post-PCR steps and reagents (i.e., gel electrophoresis); conversely, a specific fluorescence-detecting thermocycler and software are necessary (Böhme et al., 2019; Kumar et al., 2022). Recently, de Oliveira et al. (2022) proposed seven regions to be amplified by real-time PCR assays in processed cocoa-derived products and chocolate: ITS (ribosomal DNA), *rbcL* (*ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit* gene) and *lipids phosphate phosphatase gamma* (chloroplast genes) and *terpene synthase, vicilin, vicilin-like seed storage protein* and *albumin synthase* (nuclear genes) (Table 1). The specificity of amplification was tested *in silico* against NCBI database and showed that the cocoa *lipids phosphate phosphatase gamma* (chloroplast target) and cocoa *vicilin-like* (nuclear target) had specificity only against *Theobroma cacao*. These two targets were further evaluated for the amplification of DNA extracted from i) raw cocoa beans, ii) fermented cocoa beans, iii) dried cocoa beans, iv) roasted cocoa beans, v) six commercial chocolates with 55%, 70% and 93% of cocoa content (weight/weight), and vii) milk chocolates with almond and cocoa powder. Positive amplification signals were observed in all samples confirming their suitability and applicability on real-life products.

Single stranded conformational polymorphism (SSCP) analysis was described as an efficient tool for the investigation of PCR-amplified DNA fragments and the detection of polymorphisms between two alleles at
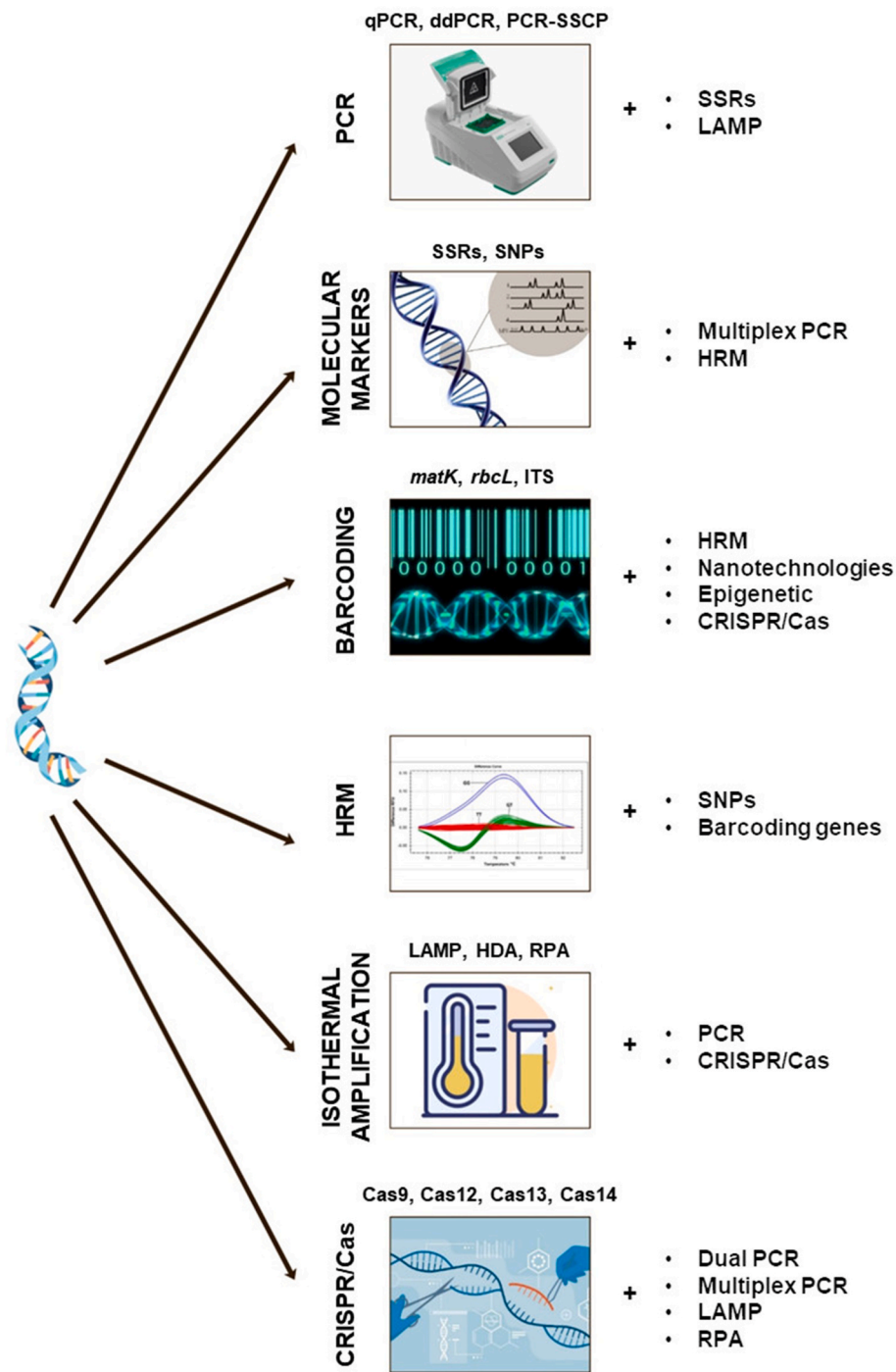
Fig. 2. Overview of the different DNA-based methodologies that can be applied alone or in combination with other molecular tools for the traceability and authentication of foods of plant origin. qPCR, real-time quantitative PCR; ddPCR, droplet digital PCR; PCR-SSCP, PCR single stranded conformation polymorphism; SSR, Simple Sequence Repeat; SNP, Single Nucleotide Polymorphism; HRM, High Resolution Melting; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR associated; LAMP, loop-mediated isothermal amplification; HAD, helicase-dependent amplification; RPA, recombinase polymerase amplification.

chromosomal loci (Kakavas, 2021). Coupled with PCR, it has been used for applications such as food traceability (Hirst et al., 2020) or genetic diversity discrimination between plant species (Sülü et al., 2020). Moreover, PCR-SSCP was applied as genotyping method to identify multiple indels in clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) (CRISPR/Cas)-induced mutants in rice (Zheng et al., 2016).

In more recent years, ddPCR has become a powerful technique for a simpler and more rapid detection of contaminants in food products. It

enables the absolute quantification of the DNA copy number based on a "divide and conquer" concept (Baker, 2012). The analyzed sample is partitioned in hundreds or millions of droplets of nanoliter-size and incapsulated in oil droplets, each of them miniaturized in a reaction tube containing up to few copies of the sequence of interest. The assay is carried out within the droplet and by counting positive versus negative reactions it is possible to calculate exactly the number of DNA molecules present in the original sample (Baker, 2012; Böhme et al., 2019; Kumar et al., 2022; Zambianchi et al., 2023). Compared to qPCR, ddPCR is

Table 1

Summary of recent PCR-based technique applications for food authentication and fraud detection.

Plant source	Purpose of analysis	Method	Target gene	Reference
Cocoa beans (*Theobroma cacao*) and chocolate	Cocoa authentication in processed cocoa-derived products	qPCR	ITS, *rbcL*, lipids phosphate phosphatase gamma, terpene synthase, vicilin and albumin synthase	de Oliveira et al. (2022)
Lemon, mandarin, citron, tangelo and tangor	Identification of genetic diversity among mutant lemon and mandarin varieties	PCR-SSCP	Expressed sequence tags	Sülü et al. (2020)
Rice (*Oryza sativa*)	Genotyping of CRISPR/Cas-induced mutants in rice	PCR-SSCP	*OsROC5* and *OsDEP1*	Zheng et al. (2016)
Various cereals	Quantification of cereal species	ddPCR, qPCR	*β-tubulin* and *γ-gliadin*	Schulze et al. (2021)
Common wheat (*Triticum aestivum* L.)	Identification of wheat varieties in flour mixtures	ddPCR	SSR markers	Ramos-Cabrer et al. (2022)
Medicinal plants: Mutong (Akebiae Caulis), Chuan Mutong (Clematidis Armandii Caulis) and Guan Mutong (Aristolochiae Manshuriensis Caulis)	Identification of plant adulterants in high processed samples	ddPCR	ITS2	Xu et al. (2022)
Various commercial products of both plant/ animal origin	Detection of celery (*Apium graveolens*) allergen	ddPCR	*Mannitol dehydrogenase*	Cau et al. (2021)
Soybean flour, roasted peanuts and other market samples	Detection of peanut and soybean allergens in foods	ddPCR, qPCR	*Arah1, Arah2, Glym30, Glym5* and *Lectin*	Pierboni et al. (2018)
Peanut	Detection of peanut allergen in foods	ddPCR, qPCR	*Arah2*	Temisak et al. (2019)
Ryegrass (*Lolium perenne*)	Detection of transgenes in pastures and pasture-derived products	ddPCR, qPCR	Target construct 1SST-6G-FFT	Giraldo et al. (2019)
Rice (*Oryza sativa*)	Detection of genome editing events	ddPCR	*OsMADS26*	Fraiture et al. (2022)
Rice (*Oryza sativa*) *Arabidopsis thaliana, Sorghum bicolor,* and *Zea mays*	Detection of genome editing events	qPCR	*Os06g0623700, AT5G05570, SORBI_010G072000,* and *Zm00001d038302.*	Peng et al. (2018)
Rice (*Oryza sativa*) Canola (*Brassica napus*)	Detection of genome editing events	ddPCR, qPCR	*Os06g0623700, LOC_Os2g42314, BnaA03g22900D/BnaC03g26960D,* and *BnaA06g36310D/BnaC07g48660D*	Peng et al. (2020)
Canola (*Brassica napus*)	Detection of genetically modified events	Ultrafast PCR	Several target events	Park et al. (2022)
Common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*F. tataricum*)	Differentiation between buckwheat species in food	Multiplex qPCR	*Fagopyrum esculentum major allergenic storage protein*	Kim et al. (2023)
Soybean (*Glycine max*)	Detection of genetically modified crops	Multiplex PCR, CRISPR/Cas12a and Cas13a	*CaMV35S* and T-*nos*	Cao et al. (2022)
Soybean (*Glycine max*), maize (*Zea mays*) and rice (*Oryza sativa*)	Detection of genetically modified crops	qPCR, CRISPR/Cas	*CaMV35S*	Peng et al. (2023)

qPCR, real-time quantitative PCR; ITS, internal transcribed spacer; PCR-SSCP, PCR single stranded conformation polymorphism; ddPCR, droplet digital PCR; SSR, simple sequence repeat.

more sensitive and does not need calibration or internal controls. Consequently, any variation among different matrices that may cause contrasting amplification efficiencies are limited. Despite ddPCR uses the same primers, probes, *Taq* polymerase, and reagents as conventional PCR, its specificity and repeatability are more significant due to the droplet method. Moreover, it shows an enhanced tolerance to PCR inhibitors and a higher resistance to primer mismatches. Additionally, this technique offers a greater flexibility of multiplexing and especially for the quantification of genetically modified events of the same species can outperform PCR in terms of cost-effectiveness (Dobnik et al., 2016).

Droplet digital PCR has been widely used in the authentication of plant species and food products (Table 1). Schulze et al. (2021) successfully developed probes for ddPCR and qPCR to discriminate four cereal species, while Ramos-Cabrer et al. (2022) applied SSR markers and ddPCR for the traceability of the local wheat cultivar 'Caaveiro' in flour mixtures. Xu et al. (2022) used ddPCR to distinguish between Mutong, a traditional Chinese herbal medicine derived from species of the genus *Akebia*, and Mutong adulterants. The detection of allergens, one of the main concerns in actual food industry, was also addressed through ddPCR by several authors (Cau et al., 2021; Pierboni et al., 2018; Temisak et al., 2019). A further exploitation of the method is the identification of genetically modified crops. In this regard, Giraldo et al. (2019) compared ddPCR and qPCR to identify and quantify transgenic events in a wide range of agricultural commodities including fresh

leaves, tillers, seeds, pollen, silage, and hay finding a higher sensitivity and repeatability for ddPCR. More recently, Fraiture et al. (2022) developed a duplex ddPCR method targeting specifically a gene-edited rice carrying a single nucleotide insertion. Similarly, Peng et al. (2018, 2020) used qPCR and ddPCR to discriminate several gene-edited plant species.

The possibility to develop or integrate different systems that can overcome traditional analysis and reduce the time to wait for results are very appreciated by food industries. In this frame, a portable qPCR system or ultrafast PCRs are of particular interest and thanks to their fastness and low expense they could be used for routine test and on field condition (Böhme et al., 2019). Park et al. (2022) developed an ultrafast PCR assay to detect eleven approved events in genetically modified canola. Kim et al. (2023) used the same system to discriminate between common buckwheat and tartary buckwheat (*Fagopyrum esculentum* Moench and *F. tataricum* (L.) Gaertn., respectively) in 22 different commercial products.

3. Molecular markers-based techniques

Molecular markers were widely used in biology to address questions related to population genetics and food traceability due to their ability to identify a particular sequence of DNA in a pool of unknown DNAs. Discontinuous molecular markers such as Random Amplified

Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLPs), Restriction Fragment Length Polymorphisms (RFLPs) as well as sequencing-based markers like SSRs and SNPs have been shown to be very useful for species identification and traceability purposes. However, the latter two markers are the most widespread for food authentication by reason of their polymorphism level, abundance, even distribution on the genome, automation, reliability and score ease (Böhme et al., 2019; Scarano & Rao, 2014).

3.1. Simple Sequence Repeats (SSRs)

Simple Sequence Repeats are repetitions of a short (2–6 bp) sequence, which is flanked by more conserved motives. By the repetitive nature of the sequence, it is likely that during DNA replication units of the core motive are erroneously added or removed creating polymorphism between individuals/varieties. SSRs are easy to detect by classical PCR approaches followed by agarose gel or capillary electrophoresis and being highly polymorphic at each locus they have been employed in several varietal identification and adulteration detection studies (Palumbo & Barcaccia, 2018, chap. 8; Scarano & Rao, 2014) (Table 2).

Recently, SSR markers have been successfully applied to distinguish durum wheat varieties in 25 samples of semolina and commercial pasta with an ability of foreign variety detection of 5% in hand-made mixtures (Fanelli et al., 2023). Microsatellite profiling was also employed to

Table 2
Summary of recent SSR and SNP marker applications for food authentication and fraud detection.

Plant source	Purpose of analysis	Method	Number of markers	Reference
Commercial semolina and pasta samples	Varietal identification of durum wheat cultivars	SSR profiling	Nine SSRs	Fanelli et al. (2023)
Cocoa (*Theobroma cacao*) beans and liquor	Identification of cocoa genotypes in beans and liquor	SSR profiling	Fifteen SSRs	Stagnati et al. (2020)
Grapes, musts, and wines	DNA traceability along the entire wine production chain	SSR profiling	Nine SSRs	Zambianchi et al. (2021)
Olive oil samples	Identification of olive genotypes	SSR profiling-HRM	Six SSRs and three plastid DNA loci	Gomes et al. (2018)
Commercial products containing zucchini	Identification of zucchini varieties	SSR profiling	Eight SSRs	Verdone et al. (2017)
Tea (*Camellia* spp.)	Identification of cultivar composition of the processed tea	SSR profiling-multiplex PCR	Eighty-three SSRs	Hu et al. (2023)
Apple juice	Identification of apple cultivars in apple juice	SSR profiling	Nine SSRs	Torello Marinoni et al. (2022)
Grapes, musts, and wines	DNA traceability in wine	SSR profiling	Nine SSRs	Zambianchi et al. (2022)
Rice (*Oryza sativa japonica*)	Varietal identification	SNP genotyping	Thirty-one SNPs	Sales et al. (2023)
Grapevine leaves, berries, and must	Varietal identification	SNP genotyping	Thirty-eight SNPs	Carrara et al. (2023)

SSR, simple sequence repeat; HRM, high resolution melting; SNP, single nucleotide polymorphism.

recognize different cocoa varieties from big lots of beans and liquors (Stagnati et al., 2020), to track monovarietal and polyvarietal wines (Zambianchi et al., 2021), and coupled with High Resolution Melting (HRM) to identify olive genotypes (Gomes et al., 2018). An important point in molecular traceability is the capacity to distinguish desired species/varieties in mixtures or in highly processed foods. Verdone, Rao, Coppola, and Corrado (2017) using SSRs identified and characterized several zucchini varieties in commercial processed products. Hu et al. (2023) developed a multiplex PCR assay consisting of 12 SSR markers to screen and identify Taiwanese tea varieties even in processed teas at different level of fermentation and roasting. Unfortunately, sometimes food production processes can negatively influence the detection outcomes obtained by SSR markers. In this regard, Torello Marinoni et al. (2022) used SSRs to distinguish apple varieties in apple juice but could detect alleles only at 3 or 4 loci out of 9 as consequence of procedures used during juice production. Zambianchi et al. (2022) evaluated the effect of storage time in bottled red and white wine finding that varietal identification was possible until 8 months from bottling, then DNA degradation became a serious issue especially for red wines.

3.2. Single Nucleotide Polymorphisms (SNPs)

Single Nucleotide Polymorphisms are point mutations causing a variation in DNA sequence involving a single nucleotide. SNPs are the most abundant type of mutations in living organisms, distributed along the entire genome, and usually having a biallelic state, which helps during allele calling (Fanelli et al., 2021; Scarano & Rao, 2014). They are widely used for studies of genetic characterization and are constantly generated by different Genotyping by Sequencing (GBS) experiments. SNP chips are now available for many species like maize, wheat, lentil, tomato, cocoa, grapevine, coffee, and others (Fanelli et al., 2021) allowing the identification of very similar cultivars or breeds for many plants of agri-food interest (Table 2). The possibility to generate SNP catalogs from GBS, the availability of publicly accessible large SNPs dataset and freely handy software allowed the development of simple and easy-to-use bioinformatics tools or methods like DNA biosensors and nanofluidic systems that are useful for the identification of varieties to place in the hands of breeders, seed certifiers, food companies and inspection agencies (Fanelli et al., 2021).

The use of SNPs for traceability is nowadays more common for animal-derived product compared to plant-derived product. The fact that SNPs are biallelic requires the use of a larger number of markers compared to multiallelic loci as SSRs, and around 50–100 SNPs are necessary to reach a certain level of discrimination or identification, whereas only 10–15 SSRs are required to get the same discrimination level. Moreover, food processing may induce chemical changes in the DNA and the presence of complex mixtures of different origin worsens the use of SNPs for traceability purposes (Corrado, 2016; Fanelli et al., 2021).

Despite the limitations that SNPs may have compared to other type of markers, progresses have been made in the recent past and more are likely to be expected in the near future. Sales et al. (2023) conceived a core set of SNPs able to distinguish among 210 japonica rice varieties cultivated in Spain. Carrara et al. (2023) employed a molecular toolbox to track grape varieties from the nursery to the must. Two complementary approaches were developed to exploit SNP markers: a high-throughput platform for varietal identification and a digital PCR system for varietal quantification. The toolbox was successfully applied along the Prosecco wine production chain to identify and quantify "Glera" variety with some limits in commercial aged wines (Carrara et al., 2023).

4. DNA barcoding, high resolution melting and next generation sequencing-based techniques

Over the last decade, DNA barcoding gained popularity due to its

precision, rapidity, and cost-efficacy in identifying plant species in food products (Antil et al., 2023). It provides taxonomic identification through the analysis of the variability in a short specific DNA region called "barcode", as developed by Hebert et al. (2003). The selection of the barcoding gene that displays low variability within a certain taxon, but also maximum inter-species divergence is crucial for this technique. The most targeted genes for plant species identification are the plastidial genes for *rbcL* and maturase K (*matK*), the mithocondrial cytochrome *c* oxidase subunit I (COI), the intergenic spacer region *psbA-trnH*, the genes encoding for RNA polymerase C1 (*rpoC1*), RNA polymerase B (*rpoB*), ATP synthase subunits CFO I and III (*atpF-atpH*), polypeptide K and L of photosystem II (*psbK-psbI*), and the nuclear internal transcribed spacer (ITS2) sequence (Ahmed et al., 2022; Soledispa et al., 2021). In this regard, the use of *rbcL* and *matK* as potential core DNA barcodes of plants is supported by the Consortium for the Barcode of life (CBOL,

http://ibol.org) within the global project Barcode of Life Data System (BOLD, http://www.boldsystems.org). This initiative aims to create public databases of short DNA fragments (barcodes) and primers useful in the generation of barcode sequences for every animal and plant species on the earth, representing a reliable resource in food traceability and safety (Ratnasingham & Hebert, 2007).

DNA barcoding has been widely used to identify and differentiate plant species (Table 3). Specific group of plants have been addressed by this technique for authenticity purposes, like medicinal plants and spices. Hence, DNA barcoding has been employed in discriminating herbal and medicinal plant-based products, as thyme, cardamom, anise, basil, turmeric, and ginger (Mosa et al., 2018), herbal infusions (Diaz-Silveira et al., 2021; Intharuksa et al., 2020; Negi et al., 2021; Zhang et al., 2020), and spices such as nutmeg (Swetha et al., 2017) and saffron (Khilare et al., 2019). DNA barcode assays were also applied in several

Table 3
Summary of recent DNA barcoding applications for food authentication and fraud detection.

Plant source	Purpose of analysis	Method	Barcode gene	Reference
Medicinal plants (Thyme, Turmeric, Basil, Ginger, Cardamom, Anise)	Authenticity investigation of commercially available herbal and medicinal plant-based products	DNA barcoding	*matK*, *rbcL*, and ITS regions	Mosa et al. (2018)
Herbal plants (*Harpagophytum procumbens*, *Harpagophytum zeyheri*)	Quality control in the manufacture of devil's claw supplements	DNA barcoding	*matK*, *rbcL*, *psbA-trnH*, and ITS2	Diaz-Silveira et al. (2021)
Herbal plants (*Terminalia* species)	Identification of species of genus *Terminalia* from commercial crude drugs	DNA barcoding	*matK*, *rbcL*, *psbA-trnH*, ITS1, and ITS2	Intharuksa et al. (2020)
Herbal plants (*Aconitum heterophyllum* and *Aconitum balfourii*)	Identification of Himalayan aconites	DNA barcoding	*rbcL*	Negi et al. (2021)
Herbal plants (*Pueraria montana* var. *lobata* and *P. montana* var. *thomsonii*)	Identification at the subspecies level and from raw material	DNA barcoding	ITS2	Zhang et al. (2020)
Spice (*Myristica fragrans* and *M. malabarica*)	Adulteration of traded *M. fragrans* mace	DNA barcoding	*matK*, *rbcL*, *psbA-trnH*, and ITS	Swetha et al. (2017)
Spice (*Crocus sativus*)	Identification of adulterants in saffron powder	DNA barcoding	*rbcL*	Khilare et al. (2019)
Oils (Olive, Hazelnut, Soybean, Sesame, Sunflower, Rapeseed, Corn, Cottonseed, Peanut, Safflower and Palm)	Detection of the botanical origin of olive oil	DNA barcoding	*trnL*	Uncu et al. (2017)
Rice (Twenty-one *Oryza* species)	Identification of rice species from seed banks	DNA barcoding	*psaJ-rpl33*, *trnC-rpoB*, *rps16-trnQ*, *rpl22-rps19*, *trnK-matK*, and *ndhC-trnV*	Zhang et al. (2021)
Saffron (*Crocus sativus*), *Calendula officinalis*, *Carthamus tinctorius*, *Gardenia jasminoides*, *Zea mays* and *Curcuma longa*	Authentication of Greek Protected Designation of Origin (PDO) saffron "Krokos Kozanis" from adulterants	DNA barcoding-HRM	*trnL*	Bosmali et al. (2017)
Coffee (*Coffea arabica*, Arabica, and *Coffea canephora*, Robusta)	Authentication of Arabica from Robusta coffee beans and the brewed beverage	DNA barcoding-HRM	ITS2	Bosmali et al. (2021)
Extra virgin olive oil, sesame oil, corn oil, sunflower oil, canola oil and soya oil	Detection of adulteration of olive oil	DNA barcoding-HRM	*rbcL*	Ganopoulos et al. (2013)
Rocket (17 *Diplotaxis* species)	Discrimination and taxonomy definition of rocket salad species	DNA barcoding-HRM	*trnL*, *trnF*, *rbcl*, *matk*, and ITS	Tripodi (2023)
Centella asiatica, *Hydrocotyle umbellata*, *Bacopa monnieri*, and *Bacopa caroliniana*	Species authentication in *Centella asiatica*-derived commercial products	DNA barcoding-HRM	ITS, *matK*, and *rbcL*	Nukool et al. (2023)
Amaranthus retroflexus and *Datura stramonium*	Discrimination between toxic and edible species	DNA barcoding-HRM	ITS2	Anthoons et al. (2022)
Plant products (teas, spices and herbal remedies)	Analysis of plant-containing products for species identification	DNA metabarcoding	ITS1	Omelchenko et al. (2019)
Twelve traditional medicinal herbs	Identification of endangered species in traditional medicinal herbs	DNA metabarcoding	ITS2	Arulandhu et al. (2019)
Fifteen samples of commercial herbal teas	Species authentication in herbal teas	DNA metabarcoding	*psbA-trnH* and ITS2	Frigerio et al. (2021)
Sixty-two herbs and spices containing oregano, paprika, and basil	Quality control for species identification and authentication in plant-containing products	DNA metabarcoding	ITS2	Raclariu-Manolică et al. (2021)
Saffron (*Crocus sativus*)	Authentication of food ingredients	DNA barcoding-nanotechnologies	ITS2	Valentini et al. (2017)
Liquid foods (Changyu white wine, Changyu red wine, Haitian soy sauce, Haitian vinegar, and Satine milk)	DNA identification in liquid foods	DNA barcoding-nanotechnologies	Exogenous short DNA marker	Ding et al. (2021)
Cocoa type Nacional (Arriba) and Colección Castro Naranjal 51 (*Theobroma cacao*)	Food authentication	DNA barcoding-CRISPR/Cas	Two regions located on the large single copy (LSC) region of the chloroplast genome	Scharf et al. (2020)
Cocoa type Nacional (Arriba) and Colección Castro Naranjal 51 (*Theobroma cacao*)	Food authentication	DNA barcoding-CRISPR/Cas12a	AT-rich target region in the chloroplast genome	La-Rostami et al. (2022)
Phyllanthus amarus	Plant species authentication	DNA barcoding-CRISPR/Cas	*trnL*	Buddhachat et al. (2021)

ITS, internal transcribed spacer; HRM, high resolution melting; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR associated.

investigations focused on identifying the varietal composition of olive oil. Uncu et al. (2017) using the plastid *trnL* intron barcode together with a PCR capillary electrophoresis approach detected small quantities of up to ten different plant adulterant oils in olive oil. The DNA from the reference tissue of 11 plant species (hazelnut, soybean, sesame, sunflower, rapeseed, corn, cottonseed, peanut, safflower, and palm) and their corresponding oils was amplified using the plastid barcode. All species displayed distinctive barcode fragments, whereas barcode size did not vary among the five olive cultivars, providing proof of the species specificity of *trnL* (UAA) intron length polymorphisms. Moreover, adulterant seed oils present at quantities as low as 5% in admixtures could be detected by the DNA-based assay (Uncu et al., 2017).

Although DNA barcoding is a well-proven routine molecular tool to evaluate food authenticity, there are still limitations to its employment, such as the disadvantage in designing species-specific universal primers and the low resolution to detect closely related species (Dawan & Ahn, 2022). Zhang et al. (2021) explored DNA barcoding to discriminate 21 species of *Oryza* comparing the performance of conventional plant DNA barcodes with rice-specific chloroplast and nuclear DNA barcodes, and a chloroplast genome super DNA barcode. The latter one consists in the whole chloroplast genome and was proven to be the most reliable marker allowing a rapid and accurate discrimination among rice genotypes (Zhang et al., 2021). Plastome sequencing represents a good choice for the identification of extremely closely related species when conventional DNA barcoding cannot make accurate identification. Chloroplasts are haploid and non-recombining and sequences are highly conserved, so they can act as a single locus. In addition, in contrast to a single gene, they have more variation presenting a high discrimination power. Moreover, with the advances in high-throughput sequencing, the cost of chloroplast genome sequencing lowered and the lack of close reference sequences for assembly has become less important than before. Despite the many advantages of the super barcode approach, DNA quality remains the main limiting factor. If DNA is degraded or not in insufficient amount it is challenging to obtain whole plastome sequence by assembly, and compared with a single-locus barcode, the cost of super-barcode is higher and data analysis complex. Overall, on account of all pros and cons highlighted above, super barcode represents a useful supplement to the current molecular identification and a feasible alternative when DNA barcodes do not work.

Recently, the DNA barcoding has been further improved through the combination with the HRM analyses, termed as Bar-HRM, that revealed great potential to differentiate cultivars and subspecies as well as to authenticate Protected Designation of Origin (PDO) of food products (Table 3; Bosmali et al., 2017, 2021). Bar-HRM uses the PCR-amplified universal plastid regions as an analytical target for the HRM curve assay to discriminate plant species. HRM analysis has higher resolving power than conventional melting curve analysis because the curves from HRM analysis can be distinguished on the basis of their shape, due to single nucleotide and/or the presence of insertions or deletions polymorphisms, even though amplicons present similar T_m values (Böhme et al., 2019; Ganopoulos et al., 2013). Recently, Tripodi (2023) applied HRM and DNA-barcoding to discriminate rocket salad species. The sequences of chloroplast DNA markers including the spacer between *trnL* and *trnF* and *tRNA-Phe* gene (*trnL-F*), *rbcL*, *matk*, ITS, along with a highly polymorphic marker (HRM500) were used to investigate allelic variance of 17 *Diplotaxis* species. The analysis of the five barcode regions were integrated with real-time PCR coupled with HRM to better identify taxonomic relationships, and three clusters were identified according to the common chromosomal set number (11, 9, and 8), with *D. siifolia* resulting the most distant species (Tripodi, 2023).

The association of HRM and DNA barcoding was also applied to differentiate between medicinal plants and possible adulterants (Nukool et al., 2023). By employing multiplex Bar-HRM with *matK1* and *rbcL1* loci *Centella asiatica* (L.) Urb. was successfully distinguished from other contaminant species that present common morphological traits and local names, like *Hydrocotyle umbellata* L., *Bacopa monnieri* L., and

B. caroliniana (Walter) B. L. Rob (Nukool et al., 2023). The same approach was also applied to distinguish poisonous species from their edible counterparts (Anthoons et al., 2022). ITS2-HRM was capable to amplify DNA from fragmented and/or artificially digested samples of the toxic thorn apple (*Datura stramonium* L.) and edible common amaranth (*Amaranthus retroflexus* L.) even 4 h after treatment (Anthoons et al., 2022).

With the growth of next generation sequencing technologies and the production of millions of sequences at reasonable low prices, DNA barcodes have been converted into metabarcoding. This technology was described as "designate high-throughput multispecies (or higher-level taxon) identification using total but degraded DNA extracted from an environmental sample (i.e., soil, water, feces, etc.)" (Toegl et al., 2012). Thus, the greatest advantage of metabarcoding is the capability to detect every species in a complex sample or processed mixtures simultaneously. Aside from its ability to release more information on species composition, a second major advantage of this technology is its ability to generate accurate, consistent identifications even in those species unidentifiable with morphological approaches, and data generation is provided at extremely low cost. This is due to the augmented sequencing output production that have determined a downfall of analytical costs. In addition, it analyses shorter DNA fragments alternatively to the standard barcodes longer than 500 bp used during traditional barcoding. A demanding task associated to the metabarcoding is the short length of markers (around 100 bp) required for facilitating sequencing, well conserved flanking primer binding sites to limit taxonomic bias during PCR amplification, and an enough varying target sequence for species discrimination (Antil et al., 2023). This technology has been largely employed in the field of medical herbs due to the complexity and the possible degradation frequently observed among the components of these plants. In this regard, Omelchenko et al. (2019) using optimized protocols of ITS1-based metabarcoding examined a broad set of plant products (teas, spices and herbal remedies), unmasking both the presence of extraneous components and the absence of those labeled. A similar strategy detected a wide range of declared and undeclared ingredients in traditional plant medicines belonging to different matrices, including endangered species (*Ursus arctos* and *Aloe* sp.) (Arulandhu et al., 2019). Moreover, a multi-locus DNA metabarcoding tool using two barcode regions, *psbA-trnH* and ITS2, studied not only the composition of herbal teas but also their relative quantities (Frigerio et al., 2021). DNA metabarcoding was also applied for the authentication of 62 products, containing basil, oregano, and paprika deriving from different retailers and importers in Norway, and diverse ranges of discrepancy between the constituent species and those declared on the product labels were reported (Raclariu-Manolică et al., 2021).

One approach trending in the last decade of research is nanotechnology and its association with DNA-barcoding is greatly promising in agri-food authentication and traceability (Munir et al., 2020). In this sense Valentini et al. (2017) developed a colorimetric test named "NanoTracer" able to identify DNA from specific species without sequencing and along the food supply chain outside the specialized laboratories. This tool relies on the PCR amplification of a short barcode polymorphic sequence and the further achievement of a single strand amplicon readily available for the subsequent hybridization–based colorimetric detection. Thus, NanoTracer can be exploited not only to detect the replacement of a fine ingredient, but also its dilution with cheaper adulterants (Valentini et al., 2017). More recently, Ding et al. (2021) combined DNA markers and gold nanoparticles for authentication in liquid foods. The analysis highlighted that gold nanoparticles allowed the colorimetric identification of DNA markers in liquors, condiments, and milk with a rapid readout based on the color solution. Moreover, markers showed an extended chemical stability and bioactivity in hybridization for months, enabling traceability of ingredients in long shelf-life liquid foods (Ding et al., 2021).

5. Isothermal nucleic acid amplification and epigenetic-based methods

To further simplify DNA amplification and identification, isothermal nucleic acid amplification technologies provide alternative strategies. These tools consist in the exponential amplification of a specific region of DNA maintaining a constant temperature and avoiding the lengthy steps of PCR. Moreover, they allow the on-site authentication as they do not require the use of a thermocycler for DNA amplification. An additional advantage of isothermal amplification methods is that some of them can also amplify non-DNA targets like messenger RNA or even thermally labile protein-nucleic acid conjugates. Further interesting features consist in an increased tolerance to biochemical inhibitors, the production of longer amplicons, a higher amplification efficiency and yield, and the easy availability of simple instruments that reduce costs.

Several isothermal amplification techniques have been conceived, among them the most popular are the loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), multiple displacement amplification (MDA), recombinase polymerase amplification (RPA), rolling circle amplification (RCA) and nucleic acid sequence based amplification (NASBA) (Xia et al., 2022). These methods were mostly applied for the identification of several microorganisms to control food-borne diseases; however, they also displayed high sensitivity and efficiency in agri-food authentication and traceability. Selected cases of isothermal amplification applications in plant-derived food products are shown in Table 4. For instance, Gonzalez Garcia et al. (2016) performed a helicase dependent amplification as an alternative to PCR for the detection of genetically modified maize.

Among the isothermal amplification technologies, LAMP assays are the most widely exploited. This method allows the identification of a target region in a single step using four different primers specifically designed to recognize six distinct regions of the template DNA (Xia et al., 2022). Moreover, LAMP shows a lower sensitivity to inhibitors, it requires DNA extracts that are not necessarily highly purified, and the incubation of visual LAMP reaction can be carried out using dry baths or conventional heating blocks, reducing equipment and running costs. One of the main applications for LAMP is the GMO detection (Singh et al., 2019). In this regard, LAMP assays targeting three construct regions between *Cauliflower Mosaic Virus* 35S promoter and *cry1Ac* gene (*p35S-cry1Ac*), *cry2Ab2* gene and *nos* terminator (*cry2Ab2-tnos*), and *cp4-epsps* gene and *nos* terminator (*cp4epsps-tnos*), were utilized for rapid detection of genetically modified crops (Singh et al., 2020). Additional

applications concerned adulteration issues. Sheu et al. (2021) developed a LAMP assay for the detection of *Curcuma longa* DNA for turmeric authentication. ITS2-26S rDNA was used for the LAMP primer designation and authenticated *C. longa* DNA within 30 min at 65 °C isothermally. Any cross-reaction with other adulterants was observed and the sensitivity of LAMP was 10-fold higher than that of PCR (Sheu et al., 2021). A LAMP protocol was also established to detect *Olea europaea* DNA for olive oil authentication (Sheu et al., 2023). Primers were designed based on the *oleosin* gene and rapidly amplified the target gene at 62 °C without cross-reaction with other DNA of plant oils.

At the molecular level, epigenetic changes as cytosine's methylation are important mechanisms that modulate gene expression and alter phenotypes without changing the DNA sequence (Lucibelli et al., 2022). Occasionally, some changes can be fixed in specific loci and be stably passed as epialleles through mitosis and meiosis. Epigenetic changes, among the others, can be associated to a different geographic origin or to different tissues of the same organism (Lucibelli et al., 2022). These aspects can support the application of epigenetic analyses to food traceability. One of the most frequent adulterations of saffron (*Crocus sativus* L.) is represented by the addition of the different parts of the crocus flower itself. DNA being the same in the different parts of the plant, it cannot be used to detect this kind of adulteration. On the contrary, the analysis of the cytosine's methylation can differentiate between the different tissues. Soffritti et al. (2016) used methyl sensitive AFLP markers to differentiate among tepals, stamens and stigmas showing the utility of epigenetic changes for traceability purposes (Table 4). Analysis clearly proved that, while at the genetic level the different parts of saffron flower were identical, from an epigenetic point of view they presented a high number of polymorphic signals enough to detect the presence of stamens or tepals in saffron stigmas (Soffritti et al., 2016).

6. CRISPR/Cas-based techniques

Recently, in addition to being gene editing tools, CRISPR and its associated Cas proteins have been widely applied for nucleic acid detection. In particular, Cas12, Cas13 and Cas14 present the collateral activity or *trans*-activity for cleavage of non-target single stranded DNA once forming a tertiary complex (Cas/RNA/target). In this way, the CRISPR/Cas system combined with multiple detection techniques can be used to identify different target nucleotide sequences simply by changing the crispr RNA and providing a new platform for rapid nucleic acid

Table 4
Summary of recent isothermal DNA amplification and epigenetic applications for food authentication and fraud detection.

Plant source	Purpose of analysis	Method	Target gene	Reference
Maize (*Zea mais*)	Detection of genetically modified maize	HDA	*ADH1* (alcohol dehydrogenase) and *cauliflower mosaic virus (CaMV) promoter P35S*	Gonzalez Garcia et al. (2016)
Genetically modified crops (cotton, maize, soybean, sugar beet)	Detection of genetically modified crops	RPA + CRISPR/Cas12a	T-*NOS* and P-*CaMV 35S*	Wang, Wang, Liu, et al. (2023)
Cotton, maize and soybean	Detection of genetically modified crops	LAMP	*p35S-cry1Ac, cry2Ab2-tnos*, and *cp4epsps-tnos*	Singh et al. (2020)
Turmeric samples (*Curcuma longa* and *C. aromatica*) and two adulterants (*Zingiber officinale* and *Alpinia galanga*)	Authentication of *Curcuma longa* turmeric powder in commercial food	LAMP	ITS2-26S ribosomal DNA	Sheu et al. (2021)
Olive (*Olea europaea*), camellia (*Camellia oleifera*), peanut (*Arachis hypogaea*), sesame (*Sesamum indicum*) and soybean (*Glycine max*)	Authentication of olive oil in commercial products	LAMP	*Oleosin*	Sheu et al. (2023)
Maize, soybean, peanut and rice	Detection of genetically modified crops	LAMP, PCR, CRISPR/Cas12a	*CaMV35S* promoter and *Lectin* gene	Wu et al. (2020)
Rice (*Oryza sativa*)	Detection of genetically modified crops	RPA + CRISPR/SpRY	*TGW* locus	Su et al. (2024)
Saffron (*Crocus sativus*)	Detection of adulteration and auto-adulteration with different part of saffron flower	DNA barcoding-Epigenetic	*matK* and *rbcL*	Soffritti et al. (2016)

HAD, helicase-dependent amplification; RPA, recombinase polymerase amplification; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR associated; LAMP, loop-mediated isothermal amplification; ITS, internal transcribed spacer.

detection (Wang, Wang, Li, et al., 2023). For instance, a portable biosensor for visual dual detection of the *CaMV35S* promoter and *Lectin* gene in soybean powders was conceived by Wu et al. (2020) (Table 4). Dual PCR and LAMP assays were employed to amplify the target DNA in the reaction tube, and after that the amplicons were separated into three different chambers, each of them contained CRISPR/Cas12a detection systems. Positive samples would produce green fluorescence while negative samples were black under the irradiation of 490 nm LED light. As low as 0.1% transgenic ingredients in soybean powders could be detected and the specificity of the system was confirmed with genetically modified maize and soybean powders, non-transgenic peanut and rice as targets. Similarly, Cao et al. (2022) developed a strategy that combined CRISPR/Cas12a and Cas13a for the simultaneous detection of *CaMV35S* and T-*nos* based on multiplex PCR and transcription (Table 1). Due to the different binding of crispr RNAs and targets by CRISPR/Cas12a and CRISPR/Cas13 systems, they were utilized to detect DNA-*CaMV35S* and RNA-T-*nos*, producing two different signals, yellow fluorescence at 556 nm and green fluorescence at 520 nm, respectively, with the limit of detection as low as 11 copies of T-*nos* and 13 copies of *CaMV35S*. Additionally, Peng et al. (2023) developed an amplification-free CRISPR-based short nucleic acid system consistent with qPCR to identify the *CaMV35S* promoter in genetically modified soybean, maize, and rice samples (Table 1). The reaction mixture was split into 20,000 evenly sized hexagonal wells within a silicon substrate on a microchip and the emission of green fluorescence resulting from the matching of genetically modified nucleic acid fragments and CRISPR-derived RNA was measured, enabling the detection of fragments at concentration as low as 0.1 % (Peng et al., 2023).

Beside fluorescence-based detection methods, CRISPR/Cas systems were also combined with gold nanoparticle based colorimetry assay. Wang, Wang, Liu, et al. (2023) used RPA combined with CRISPR/Cas12a system for the detection of *CaMV35S* and T-*nos* elements in 16 kinds of transgenic plant samples (Table 4). The strips were laid with gold nanoparticles (AuNP) labeled with fluorescein-5-isothiocyanate (FITC) antibodies, and the test line and the control line were labeled with goat anti-rabbit Immunoglobulin G and biotin ligand, respectively. When there was a target, the dual-labeled reporter (FITC, biotin) was degraded, and AuNP complex was formed in the T line for color development in case the result was positive. This approach highlighted a good specificity and an ultra-high sensitivity of 1–10 copies of standard plasmid and more than 0.01 ng/μL of genomic DNA. More recently, Su et al. (2024) designed a novel and efficient assay, named CRISPR/SpRY, for the rapid screening of gene-edited rice at the *TGW* locus (Table 4). The system could detect several types of mutations, including insertions, deletions, and nucleotide substitutions, with excellent sensitivity in less than 1 h with a limit of detection as low as 1% (Su et al., 2024).

The CRISPR/Cas system was also applied to improve the detection sensitivity of DNA barcoding for plant species and food authentication (Table 3). Scharf et al. (2020) used this technology to differentiate bulk and fine cocoa (*Theobroma cocoa*) selecting a SNP located within a PAM region attacked by the Cas9 and showing an alteration only in the bulk cocoa. In the same species, a CRISPR/Cas12a based system, which increased available detection sites on the AT-rich plastid genome in *T. cocoa*, was developed to distinguish two cocoa varieties, fine cocoa cultivar Arriba and bulk cocoa variety CCN-51 (La-Rostami et al., 2022). Admixtures of 5% CCN-51 (P < 0.01) and 10% Arriba (P < 0.05) along with processed cocoa products could be successfully detected with this approach (Table 3).

In recent research, Buddhachat et al. (2021) conceived a barcode-coupled Cas12a assay for plant species authentication using *Phyllanthus amarus* as a model. The guide RNAs (gRNAs) were designed from the barcode region *trnL* and proved to be highly specific to *P. amarus* even in contaminated condition with an accuracy degree of 90% (Buddhachat et al., 2021).

7. Limitations and challenges

The DNA-based methods for food authentication also present their challenges and limitations. Indeed, foodstuff manufacturing involves several physical and chemical treatments that can compromise DNA content, integrity, and quality making the DNA extraction a key step in food traceability (Bojang et al., 2021; Lo & Shaw, 2018; Stagnati et al., 2020; Torello Marinoni et al., 2022; Zambianchi et al., 2021). Many commercial kits are available on the market for DNA isolation from fresh tissues as well as from food or processed matrices of plant origin. However, the development and optimization of these kits are generally carried out for the most common matrices or fresh plant tissues collected from young seedlings without taking in account that in real case plant samples could be derived from adult tissues or preserved for a long time. Moreover, food ingredients are subject to multiple processing steps (freezing-thawing, cooking, mixing of different ingredients), thus, hindering the DNA extraction outcomes. Therefore, new sample-preparation protocols are required consisting in the accurate grinding of starting materials, adding a sample precipitation step for liquid food matrices, using polyvinylpyrrolidone to remove chemical inhibitors and able to deal with minute DNA concentration (Bojang et al., 2021; Lo & Shaw, 2018; Stagnati et al., 2020; Torello Marinoni et al., 2022; Zambianchi et al., 2021). The importance of the extraction protocol in complex matrices like olive oil has been investigated by Scollo et al. (2016) comparing four different DNA isolation methods, and the qPCR and ddPCR techniques. Výrostková et al. (2022) employed three different isolation methods to extract gluten DNA in gluten-free products of plant origin. The combination of glass and zirconium beads, proteinase K and a commercially produced isolation kit resulted in the most effective procedure allowing the further detection of gluten DNA by PCR in guaranteed and naturally gluten-free foods (Výrostková et al., 2022).

Extraction protocol adaptation and modification may require additional time before producing the final analytical report; for the future, the availability of commercial kits able to extract PCR-grade DNA from complex food matrices or recalcitrant plant tissues in a cost-time effective way would be of greater interest to researchers involved in molecular traceability. In this sense, nanoparticles and microfluidic tools are already enhancing the accuracy and efficiency of DNA isolation and purification in several plant species and food sources (Carvalho et al., 2018; Teixeira et al., 2023). A further option could be to replace nuclear DNA-based investigations with the employment of techniques involving the chloroplast genome that shows a higher copy number in plant cells (Wu et al., 2023).

DNA extraction is also sensitive to the presence of numerous additives, supplements and secondary metabolites present in the extract of foodstuffs. The coextraction of PCR inhibitors like polysaccharides and polyphenolics may subvert the amplification process by binding DNA or the magnesium cofactor ion. Moreover, tannic acids can inhibit *Taq* polymerase forming reactive free radicals that determine DNA strand damage and mutations (Lo & Shaw, 2018).

Once a suitable DNA extraction protocol has been established, a major point is the choice of proper molecular markers for the analysis. In the case of SSRs the availability of a reference primer set for plant species profiling is essential allowing the comparison between different studies and laboratories. Moreover, for both SNPs and SSRs markers the presence of public or private databases containing the correct DNA sequences of interest is required, and their application is often restricted to a single species (Fanelli et al., 2021).

DNA barcoding overcomes the limitation of knowing the whole genome of an organism, relying on the exploitation of one or few genomic regions, and frequently rests on the plastidial genome that is less endangered during industrial treatment. However, this method also has some drawbacks. In this regard, the most significant limitation is that no universal primers or genes exist in all living organisms having enough sequence divergence for species discrimination (Antil et al.,

2023). Any barcode system will be useful if there is a clear barcode gap necessary for cultivar distinction and if species are not polyphyletic and paraphyletic. Second, a limited number of reference DNA barcode libraries are available and fragmentary databases reduce the solidness of analysis (Antil et al., 2023). Third, the sole use of chloroplast barcodes is not suitable for discriminating hybrid species. In this sense, Besse et al. (2021) showed that using chloroplast loci, maximum species discrimination was around 70% and very variable among plant groups, whereas the integration with ITS region disclosed higher variations determining hybrid or closely related species resolution.

Recently, developed technologies such as DNA metabarcoding, DNA barcoding-HRM, LAMP, ddPCR and CRISPR/Cas are significantly improving the performances of DNA-based methods (Kumar et al., 2022). However, also for these approaches there are still some issues that need to be resolved.

Concerning metabarcoding, the main flaw regards the high-quality DNA required for sequencing, a more demanding sample preparation and the presence of trained personnel. Next generation sequencing methodologies are more complex and require the assembly of short sequence reads into a consensus sequence that could be harder in case of large genomes or de novo sequencing. In addition, despite their detection power, amplification bias caused by variable primer–template mismatches across species may constrain their quantitative potential and cause species drop. Moreover, while there are many bioinformatics pipelines available for the analysis of metabarcoding data, the resolution power of this method is directly related on the uniqueness of the barcode marker and the availability of a curated reference database. To overcome these limits, there is a need for screening of new barcodes and new variable regions within the same barcode as well as the presence of high-quality barcode sequence reference databases that allow good taxonomy and barcode coverage (Bruno et al., 2019). In the next years, massive research efforts should address not only technology progress of next generation sequencing but also cost decrease and more user-friendly options for analysis, in order to extent their adoption for agri-food traceability.

Also, HRM, in particular the LAMP assay, faces some challenges. The main constrain regards the complex primer design that limits the development of multiplexing approaches compared to conventional PCR. Non optimal primers and temperature cause unspecific amplification and primer-dimer products. Furthermore, the excellent sensitivity of this method makes it more susceptible to contamination. Several strategies were carried out to overcome this issue, such as the adding of uracil-N-glycosylase to the reaction mix, the use of DNA binding dyes or metal ion indicators that prevent the opening of the tube when the amplification reaction has ended, and the mix preparation performed on ice within very tight timeframe (Panno et al., 2020).

As regards ddPCR platform, from the perspective of technology progress, it relies on nucleic acid extraction procedures based on commercial kits and centrifugation equipment, which hampers the portability of its *in-situ* testing. The sample storage, delivery, and extraction are notably more error-prone than the ddPCR procedure itself, and these processes also need to be optimized. A further limit to consider is the prevention of droplet fusion and contamination during laboratory handling. Furthermore, the multiplexing capability of the current ddPCR technique is also strongly inhibited by the probe design of the detectors (Hou et al., 2023).

Regarding the CRISPR/Cas system, despite the very good detection accuracy and sensitivity, it still presents disadvantages in some aspect (Wang, Wang, Li, et al., 2023). For instance, the *trans*-cleavage efficiency of Cas effectors may be negatively influenced by the presence of protein, RNA or salt ions residues after the genomic DNA isolation. The off-target effect is a further issue to be figured out since it may lead to false-positive or -negative results. Constructing high-fidelity Cas9 effectors and optimizing guide RNA structure with a high GC content may minimize off-target effects. A protospacer adjacent motif (PAM) contained in the target sequences is required by most Cas effectors. This

aspect strongly limits the employment of CRISPR/Cas system in case there are mutations that make no suitable PAM sequences, requiring additional insertion. Last, the reaction process and sensitivity of Cas effectors may be limited by the concentration ratio of Cas themselves, RNA, Mg^{2+} and Mn^{2+} of the buffer, as well as the pH and reaction temperature, making challenging the achievement of standardization.

8. Conclusions

In recent years food safety, quality, and traceability acquired a great role and importance in the agro-food sector requiring sound and rigorous analytical tools assuring agri-food surveillance. DNA-based technologies with improved reliability, sensitivity, and high throughput capability provide better solutions for assessing plant-derived food, feed and medicinal product authenticity. In this sense, the combination of various approaches such as SSR profiling and HRM, DNA barcoding and CRISPR/Cas or nanotechnologies, as well as the recently developed technologies such as ddPCR, metabarcoding, and LAMP are surpassing the classical methods, allowing species and variety identification, geographic origin detection, and ingredient proportion verification. However, despite the technological progresses and instrumentation, the analysis of DNA from processed plant foods is still challenging and strongly depending on the DNA extraction step and the removal of metabolic inhibitors. Accordingly, in the present review along with the advances and the most recent applications of DNA-based technologies in the agri-food field, the main weaknesses and challenges of each tool are considered in order to orient towards the best detection method for the improvement of food safety and the protection of consumers.

Declaration of competing interest

All authors declare that there is no conflict of interest in publishing the content of this work.

Data availability

Data will be made available on request.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.tifs.2024.104568.

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