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Food Analysis Using Organelle DNA and the Effects of Processing on Assays

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Keywords

organelle DNA, mitochondrial DNA, chloroplast DNA, ingredient identification, food authentication, time-temperature indicator

Abstract

Extrachromosomal DNA such as organelle DNA are increasingly targeted in molecular detection assays where samples have been degraded by physical or chemical means. Owing to multiple organelles per cell and greater copy numbers than nuclear genes, organelle gene targets provide a more robust signal in polymerase chain reaction (PCR), quantitative PCR (qPCR), and other emerging molecular technologies. Because of these advantages, direct analysis of organelle DNA in food matrices is used for detection of contaminants and identification and authentication of food ingredients and allergens. Non-nuclear DNA is also used as an assay normalizer for detection of genetically modified organisms (GMOs) in foods. This review describes these protocols plus the effects of processing on efficacy, with special emphasis on thermally produced DNA fragmentation. Future research may incorporate molecular techniques beyond detection, used instead as time-temperature indicators in thermal food processing or quality indicators in food fermentation or acidification.

Mitochondrial DNA

(mtDNA): genes located in the eukaryotic, energy-producing organelle, the mitochondrion

Organelle DNA:

genes located outside the cell nucleus in the mitochondria or chloroplast (plastid)

Chloroplast DNA

(cpDNA): genes located in the photosynthetic organelle, the chloroplast, found in most plants and algae

INTRODUCTION

Consumers, processors, and government officials need to verify the identity of food ingredients for a variety of nutritional, personal, religious, safety, regulatory, and ethical reasons. Modern consumers demand transparency and accountability. They want wholesome foods with truthful labels. Questions of authenticity surround stated geographic origins, species identification or verification, Halal/Kosher requirements, and the identification of genetically modified organisms (GMOs) in the market. Safety considerations include traceability, the presence of allergens, and intentional or unintentional contamination by unintended ingredients. Unintended ingredients could include the presence of wheat in gluten-free foods, allergens such as nuts, beef, or pork in vegetarian or vegan foods, and taboo species hidden in ground meat products.

Originally, polymerase chain reaction (PCR), DNA sequencing, and other molecular assays were used as rapid methods to detect foodborne pathogens (Feng 2007) and for epidemiological source tracking of foodborne outbreaks (Barrett & Gerner-Smidt 2007). These rapid methods facilitated quick identification of bacteria of concern to human health. Recently, owing to concerns over food fraud and authentication, there has been a shift away from an exclusive emphasis on pathogens to include eukaryotic targets, namely the foodstuffs themselves. Molecular techniques using DNA technology to combat fraud, improve traceability and distinguish between closely related species are being increasingly utilized in food forensic analysis (Bosmali et al. 2012, Galimberti et al. 2013, Woolfe & Primrose 2004). Future use of molecular methods will employ DNA fragmentation as a way to validate thermal processes and evaluate shelf life (Caldwell et al. 2015a,b; 2016).

MITOCHONDRIAL AND CHLOROPLAST DNA AS TARGETS FOR FOOD ASSAYS

DNA is useful for the identification of many plant and animal products because of its relatively high heat stability compared with proteins and other cell components, which denature during mechanical and thermal processing (Doktycz 1997). All eukaryotes contain multiple copies of non-nuclear DNA in mitochondrial DNA (mtDNA) (**Figure 1**). Mitochondria are energy-producing organelles found in all eukaryotic cells. Each cell contains multiple mitochondria, and each of these possesses many copies (>1,000) of organelle DNA (**Figure 2**). These properties make mtDNA sequences excellent targets for amplification in terms of specificity, sensitivity, and robustness (Andreasson et al. 2002, Gerber et al. 2001). Because of these high copy numbers (**Table 1**), mtDNA offers an advantage over nuclear DNA for molecular detection methods and is used in disciplines such as archaeology and forensics, where highly degraded samples are typical. Chloroplast DNA (cpDNA) in photosynthetic plants and algae (**Figure 3**) is used for the same reasons as mtDNA (**Table 1**). The food industry uses organelle DNA (mt or cp) for detection because processing methods such as baking, boiling, frying, and microwaving degrade DNA and make it more difficult to detect (Gryson 2010). In plants, it is difficult to differentiate between species using either mitochondrial or chloroplast genes because these genomes are evolving slowly (Taberlet et al. 2007) and are conserved or very similar. Introns, or noncoding regions of DNA, evolve more rapidly because they do not have essential functions. Amplicons are genetic fragments targeted, then artificially created, by amplification protocols such as PCR. Introns, and therefore their amplicons, tend to have variable lengths between species and can be used to differentiate between them. As an example, the chloroplast *trnL* (UAA) intron, a noncoding region in the cpDNA, was described as a powerful genomic target because of size variations between plant species (254–767 bp) (Taberlet et al. 2007). Therefore, organelle genes are suitable for universal primers because of

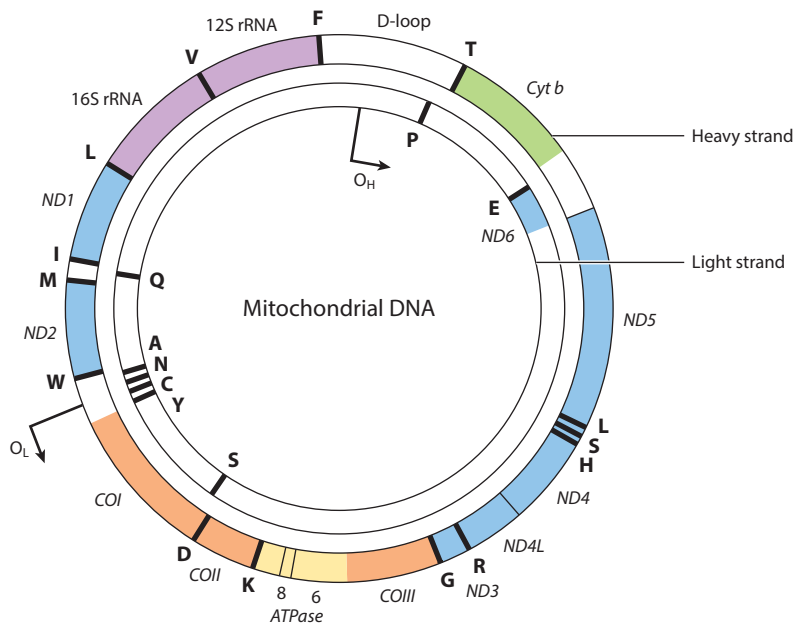


Figure 1

A map of the human mitochondrial genome. The genes that encode the subunits of complex I (*ND1-ND6* and *ND4L*) are shown in blue; cytochrome c oxidase (*COI-COIII*) is shown in orange; cytochrome b (*Cyt b*) of complex III is shown in green; and the subunits of the ATP synthase (*ATPase 6* and *8*) are shown in yellow. The 2 ribosomal RNAs (rRNAs) 12S and 16S (*shown in purple*) and the 22 transfer RNAs (tRNAs) (*indicated by bold black lines and denoted by their single letter code*) that are required for mitochondrial protein synthesis are also shown. The displacement loop (D-loop), or noncoding control region, contains sequences that are vital for the initiation of both mitochondrial DNA (mtDNA) replication and transcription, including the proposed origin of heavy-strand replication (shown as OH). The origin of light-strand replication is shown as OL. Adapted from Taylor & Trumbull (2005).

conserved sequences but can offer intronic variation between species based on amplicon length, creating an assay that can be useful for a wide variety of foods. Both mtDNA in animals and cpDNA in plants contain introns that can be used to find species-specific targets in these noncoding regions where variations in sequence length occur.

Molecular methods developed in the late twentieth century, such as conventional PCR, real-time or quantitative PCR (qPCR), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP) (Woolfe & Primrose 2004), DNA fingerprinting, DNA barcoding (Galimberti et al. 2013), and DNA sequencing, have begun to spread to commercial use in the food industry as they increase testing accuracy and decrease the time needed for ingredient validation. Conventional PCR and qPCR assays rely on the amplification of known DNA sequences. They utilize agarose gels or fluorescent signals to identify targets via fragment length or threshold cycle (Ct), respectively. RAPD, RFLP, and fingerprinting methods use small random primers to amplify unknown DNA sequences, which are then cut into smaller fragments using restriction enzymes. The DNA fragment patterns that result are diagnostic. Many of these molecular techniques require highly trained personnel, standardized protocols, and databases. Therefore, third-party validation is a growth industry in food testing. Protocols requiring gels, restriction digests, and sequencing are not rapid enough for inspection purposes. However, many

Quantitative polymerase chain reaction (qPCR):

molecular assay
utilizing fluorescent
dyes to record
amplification of target
genes in real time

Random amplified polymorphic DNA (RAPD): DNA segments are amplified at random, resulting in a unique profile; used where little or no sequence data are available

Restriction fragment length polymorphism (RFLP): a profiling technique using DNA cut by restriction enzymes; the visualized pattern detects variations in homologous sequence

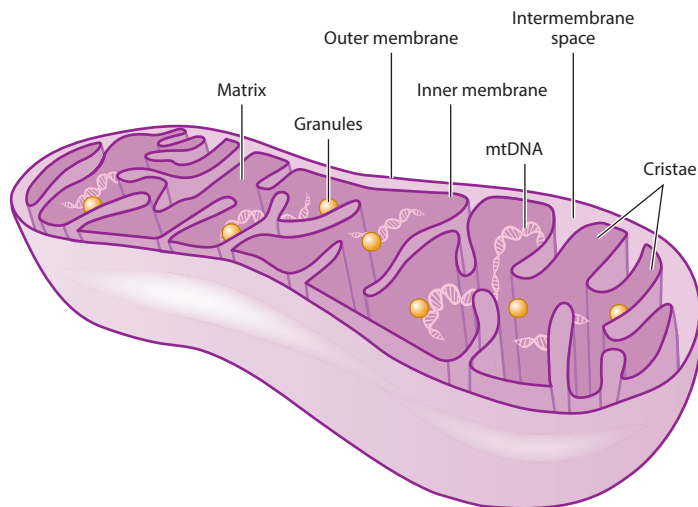


Figure 2

Cross-section of the mitochondrion showing multiple copies of mtDNA. mtDNA copies fluctuate according to the cell's energy requirements or phase in the cell cycle.

commercial kits relying on rapid qPCR methods are available for authentication, especially for identification of meat species.

FOOD APPLICATIONS FOR DNA TESTING

Verification of Meat and Milk

Meats are often ground for consumption. Opportunities for mislabeling or substitutions by other species can occur after meats are ground, as the resulting homogenate is hard to identify. Many consumers have taboos and religious laws concerning species allowed for consumption. Vegetarian diets, which exclude meats of all kinds, are the norm for many. In the United States, Europe, and other Western cultures, consumers view horses and dogs as pets and companions and do not want to eat them. Consumers want to know whether exotic meats such as kangaroo, rat, or snake have been added to ground meats, as these species are not traditionally consumed in certain

Table 1 Relative amounts of organelle DNA in some cells and tissues

Organism	Tissue or cell type	DNA molecules per organelle	Organelles per cell	Organelle DNA as percentage of total cellular DNA
Mitochondrial DNA				
Rat	Liver	5–10	1,000	1
Yeast ^a	Vegetative	2–50	1–50	15
Frog	Egg	5–10	10 ⁷	99
Chloroplast DNA				
<i>Chlamydomonas</i>	Vegetative	80	1	7
Maize	Leaves	20–40	20–40	15

^aThe large variation in the number and size of mitochondria per cell in yeasts is due to mitochondrial fusion and fission. Information from Alberts et al. (2002).

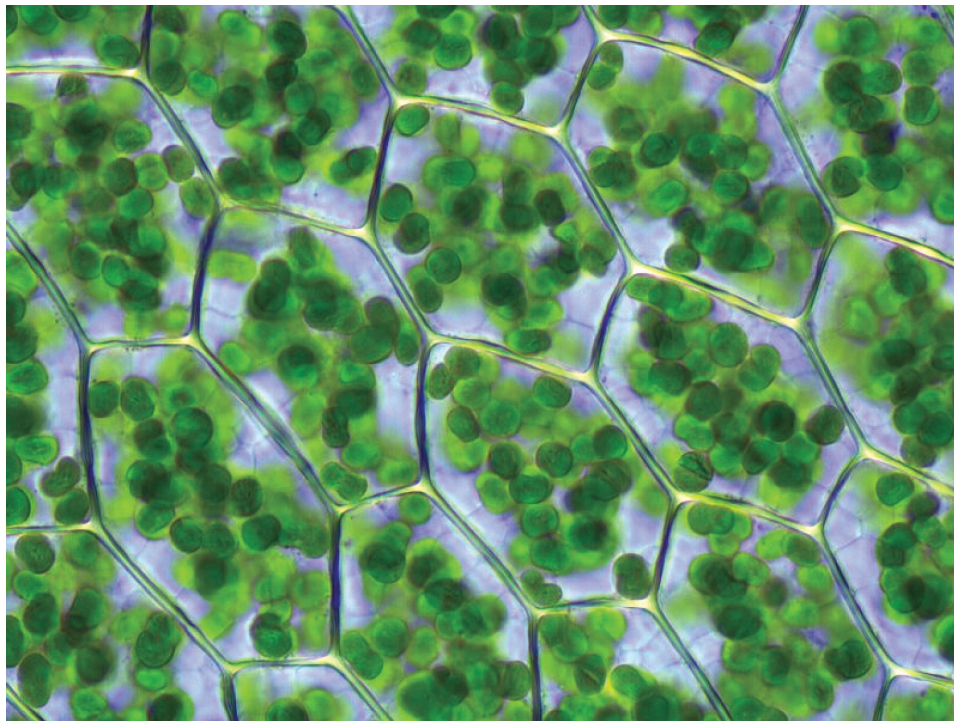


Figure 3

Chloroplasts visible in the cells of *Plagiomnium affine*, the many-fruited thyme moss “*Plagiomnium affine* laminazellen.” Photo courtesy of Kristian Peters. Licensed under CC BY-SA 3.0 via Commons - https://commons.wikimedia.org/wiki/File:Plagiomnium_affine_laminazellen.jpeg#/media/File:Plagiomnium_affine_laminazellen.jpeg.

cultures. Concerns over bovine spongiform encephalopathy and variant Creutzfeldt-Jacob disease from beef products spurred development of detection methods in both human food and cattle feeds containing meat and bone meals. Halal/Kosher laws forbid consumption of pork products. Speciation is also critical in nonmeat products created from animal derivatives. For these reasons, researchers have developed meat species detection assays based on mitochondrial genes (**Table 2**).

Although PCR detection methods using the bovine growth hormone gene in beef (Brodmann & Moor 2003) and the actin gene in chicken (Hopwood et al. 1999) were found in a survey of the literature, the majority of the meat species detection assays (>24) used mitochondrial genes (**Table 2**). Genes most commonly used were cytochrome b (*cytb*), 12S and 16S ribosomal RNA (rRNA), cytochrome oxidase I (*cox1*), NADH dehydrogenase subunits (*ND2*, *ND4*, *ND5*), ATP synthase subunits (*atp6*–*8*), the D-loop region, and transfer RNA (tRNA-valine). Two articles cited only the mitochondrial coding region (Arslan et al. 2006, Goncalves et al. 2012), and one laboratory tested the efficacy of a commercial kit that had proprietary primers and an unknown amplicon (Demirhan et al. 2012). Most PCR targets are chosen based on the amount of sequence available in public databases such as National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/pubmed>). For example, owing to thousands of available sequences in public databases, *cytb* is used more extensively than many other mitochondrial genes (Galimberti et al. 2013). In the literature cited, amplicons were larger in size for conventional

DNA fingerprinting:

a generic term describing molecular techniques used to identify eukaryotic individuals or species

DNA barcoding:

a taxonomic method to identify eukaryotic species using a short sequence of DNA, usually cytochrome oxidase I

Threshold cycle

(Ct): in qPCR, the cycle in which amplification of the target DNA becomes logarithmic. Ct is inversely proportional to gene copy number

Table 2 Detection of meat species using mitochondrial genes with PCR or qPCR

Target	Gene	Amplicon size (bp)	Other processing	Sensitivity	Reference
Beef	D-loop	513	None	<1%	Mane et al. 2012
Chicken	D-loop	442	None	<1%	Mane et al. 2009
Pork	12S rRNA	387	None	Not given	Che Man et al. 2007
Tuna	Cytochrome b (<i>Cyt b</i>)	59	Sequencing	Not given	Unsel et al. 1995
Turkey	<i>Cyt b</i>	81–565	None	Not given	Hird et al. 2006
Beef	Mitochondrial	271	None	Not given	Arslan et al. 2006
Tuna	<i>Cyt b</i>	126 and 146	RFLP	Not given	Lin & Hwang 2007
Fish species	<i>Cyt b</i>	464	RFLP	Not given	Wolf et al. 2000
Tuna	<i>Cyt b</i>	69 and 98	RFLP	10 copies	Terio et al. 2010
Tuna	<i>Cyt b</i>	276	RFLP, sequencing	Not given	Pardo & Perez-Villareal 2004
Muscovy duck	12S rRNA	64, 97, 400	None	0.1%	Martin et al. 2007
Dog	<i>Cyt b</i>	100	None	0.2%	Rahman et al. 2014
Cow, sheep, goat, water buffalo	Mitochondrial	118–197	None	<1%	Goncalves et al. 2012
Ruminants, poultry, fish, pork	12S rRNA, tRNA Val, 16S rRNA	104–290	Multiplex PCR	0.004–0.002%	Dalmaso et al. 2004
Beef, pork, buffalo, quail, chicken, goat, rabbit	<i>Cyt b</i>	359	RE	1%	Murugaiah et al. 2009
Donkey, pork, horse	<i>ND2</i> , <i>ND5</i> , <i>atp6</i> –8	74–183	None	0.0001 ng template	Kesman et al. 2009 Kesman et al. 2007
Pork	<i>Cyt b</i>	360	RE	Not given	Aida et al. 2005
Pork	<i>Cyt b</i> , 12S rRNA	149 and 183	None	0.1%	Soares et al. 2010
Poultry, game birds	<i>Cyt b</i> , 12S rRNA	470–500	None	Not given	Stamoulis et al. 2010
Pork	<i>Cyt b</i>	109	RFLP	0.01%	Ali et al. 2012
Pork	<i>Cyt b</i>	Commercial kit	None	1%	Demirhan et al. 2012
Pork lineage	Mitochondrial	75–114	Sequencing	Not determined	van Asch et al. 2011
Partridge	12S rRNA	89–140	RAPD	0.1%	Rojas et al. 2011
Universal animal	16S rRNA	<100	Sequencing	Not given	Horreo et al. 2013
Universal animal	16S rRNA, <i>ND4</i>	535 and 700	RAPD, sequencing	Not given	Rastogi et al. 2007
Meat meal	<i>Cyt b</i>	359	RFLP	Not given	Bellagamba et al. 2001
Crab meat	<i>COI</i>	610	Sequencing, DNA barcoding	34% sequencing success	Haye et al. 2012

Abbreviations: ATP, ATP synthase subunits; *COI*, cytochrome oxidase 1; ND, NADH dehydrogenase subunits; PCR, polymerase chain reaction; qPCR, quantitative PCR; RAPD, random amplification of polymorphic DNA; RE, restriction enzymes; RFLP, restriction fragment length polymorphism; rRNA, ribosomal RNA; tRNA, transfer RNA; Val, valine.

PCR, whereas qPCR assays utilized amplicons below the recommended maximum length of 250 base pairs because of the kinetics of the protocol. Most articles combined PCR with sequencing, DNA barcoding, RFLP, or RAPD and used single nucleotide polymorphisms (SNPs) or gel band sizes to distinguish between species (Table 2).

Molecular methods can utilize hypervariable regions of mtDNA sequence and SNPs to differentiate between animals, even closely related species (van Asch et al. 2011). Scientists have created conventional PCR assays for the identification of partridge meat sources, discriminating between red-legged, chukar, barbary, and gray partridge species using mitochondrial genes and genomic rhodopsin genes (Rojas et al. 2011). These assays are used in inspection programs to verify labeling of partridge meat products. Others have used mitochondrial genes and the actin gene to identify species in meat products to ensure reliable labeling (Rastogi et al. 2007). This research group was able to differentiate between buffalo, goat, cow, chicken, and pig samples using mtDNA-based PCR and RAPD. To commercialize this protocol, a library of reference fingerprint patterns for the meat species would be needed to quickly identify meats in foods. Horreo et al. (2013) created universal 16S rRNA primers for identification of different meats, including various types of fish, pork, beef, sheep, and poultry. This protocol is not rapid as it requires sequencing of PCR products. Researchers testing aquatic meats such as crab have used the cytochrome oxidase I gene to detect protected crab species and trace batches in the commercial chain (Haye et al. 2012). One article developed a multiplex PCR assay (Dalmasso et al. 2004) to differentiate between ruminants, poultry, fish, and pork. Some researchers have used mtDNA SNPs as molecular diagnostic tools to differentiate between Asian and European *Sus scrofa* (pig) species (van Asch et al. 2011).

Gelatin and gelatin-containing foods can be made from animal skin, ears, seaweed, or fish. It is important for adherents of Halal and Kosher practices to avoid gelatin made with pigskin. Demirhan et al. (2012) validated a commercially available real-time PCR detection assay using the mitochondrial cytochrome b gene to detect pig mtDNA. The limit of detection of the assay was cited as 1.0% adulteration on a weight-by-weight basis in two products: marshmallows and gum drops (Table 2).

In a recent study (Keim et al. 2015), researchers targeted the NADH subunit 5 gene to test human milk sold online. After anonymously purchasing 102 samples online, they found 11 samples to contain bovine DNA at quantities consistent with human milk mixed with 10% cow's milk.

Commercial kits are available for rapid detection of meat species by qPCR. One corporation specializing in food safety, Neogen (<http://www.neogen.com>), provides qPCR detection for beef, chicken, goat, horse, pork, rabbit, sheep, turkey, and seven fish species. Its closest competitor, Nimagen (<http://www.nimagen.com>), detects the usual suspects plus dog, donkey, buffalo, and warthog. The molecular targets used are proprietary.

Detection of Genetically Modified Organisms in Plant Ingredients

Authentication of plant foodstuffs has been recently driven by the inclusion of transgenic products in formulations. Public distrust of new plant breeding methods has caused concern over foods containing GMOs, organisms modified by nontraditional breeding methods such as transfection, gene insertion, or mutation. Despite acceptance of transgenic foods by the FDA, World Health Organization, the American Medical Association, and the United States National Academy of Sciences, molecular techniques have been developed for detection of GMOs in processed foods. mtDNA was included in some assays as the positive control, a test for amplification inhibitors in PCR and DNA extraction efficiency (Meyer 1999). Targeted products include grits, cornmeal, corn flour, and soybean flour as well as formulations containing tomatoes and potatoes. Researchers developed methods to detect and quantify approved transgenics such as genetically

modified potatoes, FlavrSavrTM tomatoes, Roundup ReadyTM soya, and Bt-maize (Debode et al. 2007, 2010; Lipp et al. 1999; Meyer 1999; Moreano et al. 2005). Detection relies on amplification by PCR of modified or inserted genes such as the 35S promoter and NOS terminator in genetically modified soybeans and maize (Lipp et al. 1999). Researchers were able to correctly identify samples containing 2% GMOs. False-positives were low (2 out of 105 samples) and were attributed to contamination of laboratory equipment, an ongoing problem with PCR detection assays.

Commercial kits for the quantification of Bt corn are available. Moreano and others used the GMOQuant Maximizer Bt176 Corn kit (GeneScan Europe; Bremen, Germany) to evaluate the effects of milling and heat on the quantification of GMOs in corn products such as grits, meal, and flour. They found that mechanical processes such as milling caused over- and underestimates of GMO products when comparing products with different particle size distributions, possibly due to physical damage of DNA by grinding. Heat-induced DNA degradation was blamed as the probable factor causing reduced detection in heat-treated corn products (Moreano et al. 2005). Gryson, in a 2010 review article, gave an excellent summation of the effects of food processing on plant DNA degradation and its effects on the efficacy of PCR-based GMO detection. Laboratories have also added new molecular technologies to PCR to increase its sensitivity. For example, Sforza et al. (2011) described the use of peptide nucleic acid (PNA) probes to increase the sensitivity of PCR by increasing the binding capacity of primers and using shorter primers for the same effect. Debode et al. (2010) designed plasmids for the purpose of multiplex calibration in GMO detection by qPCR.

Plant Food Allergens

Allergens are proteins that cause an immune response when ingested. Common plant allergens are found in peanut and various nuts, legumes, wheat, soybean, corn, potato, and rice. Because many proteins are denatured during cooking and processing, DNA is a more stable way to detect allergens in processed, complex mixtures. Many of the molecular protocol articles have been driven by allergenic food detection in mixtures containing plants (Table 3) and utilize the universal primers for chloroplast *trnL* intron to differentiate between plant species (Taberlet et al. 2007). The intron of the chloroplast gene *trnL* was used to detect rye, wheat, and barley in gluten-free products (Maskova et al. 2011). These researchers developed an alternative to the enzyme-linked immunosorbent assay (ELISA), an assay used to quantify proteinaceous substances, and instead used PCR to protect people with celiac disease or others who wish to avoid gluten. Wantanabe et al. (2006) created a qualitative detection method for peanut using two sets of primers: The first set is a universal primer pair from cpDNA for plant detection and assay normalization; the second set is used for detection of the peanut agglutinin gene. Simultaneous detection of 10 food allergens was achieved by using ligation-independent probe amplification using undisclosed DNA targets and noncoding regions of cpDNA for extraction verification (Ehlert et al. 2009). Sequencing produced electropherograms showing peaks of specific sizes for each of the ten allergen probes employed. This protocol allowed researchers to detect allergens in complex foods such as spread, sausages, dressings, yogurts, cookies, and baked goods. Universal primers for cpDNA using the *trnL* region were used to produce crop-specific amplicons containing introns of different sizes, which could then be separated and identified by gel electrophoresis (James & Schmidt 2004). Region IV of the gene had significant deletion and/or insertions that allowed them to characterize amplicon sizes of crops containing common allergens such as peanut, wheat, soybean, canola, corn, potato, and rice. Their future goals included adapting this protocol to microarray chips, which would hasten and semiautomate the process. Pea-specific primers using the intron between the cpDNA *trnL*

Table 3 Detection of plant species using chloroplast or mitochondrial genes with PCR or qPCR

Target	Gene	Amplicon size (bp)	Other processing	Sensitivity	Reference
Wheat, rye, barley	<i>trnL</i> intron	201, 196	None	0.02%	Maskova et al. 2011
Pea (<i>Pisum sativum</i>)	Intron between <i>trnL</i> and <i>trnF</i>	92	None	0.05%	Brezna et al. 2006
Peanut, soybean, canola, potato, wheat, corn, white rice, red rice	Region VI of <i>trnL</i> intron	642, 584, 387, 571, 662, 532, 615, 615, respectively	None	Not given	James & Schmidt 2004
Ten allergenic nuts: peanut, cashew, pecan, pistachio, hazelnut, sesame, macadamia, almond, walnut, brazil nut	Not given; cpDNA introns used for verification	88, 92, 96, 100, 104, 108, 112, 116, 120, 124, respectively	Ligation-dependent probe amplification, sequencing	5–1,000 mg/kg	Ehlert et al. 2009
Peanut	cpDNA intron, agglutinin gene	123, 95	None	0.001%	Watanabe et al. 2006
Olive oil	<i>trnL</i> intron, ribulose diphosphate carboxylase, NADH dehydrogenase	500, 1,172, 1,942	RAPD, AFLP	Not given	Busconi et al. 2003
Olive oil	<i>trnE</i> , <i>trnQ</i> , <i>ycf1</i> , <i>clpP</i> , <i>PetM/PsbM</i>	70–172	Melting analysis	0.1%	Ramos-Gomez et al. 2016
<i>Artemisia</i> species	<i>trnL</i> -F region	16–203	RFLP, RE	Not given	Lee et al. 2009
Grape varieties	VVMD5,6,7 and VVS2	Not given	SSR	8 of 26	Faria et al. 2000
Robusta/Arabica coffee	Intron between <i>trnL</i> and <i>trnF</i>	251	Gold nanoparticles	5%	Trantakis et al. 2012
Locust bean/guar gum	ITS (nuclear)	~200	RE	2–5%	Urdiain et al. 2005
Wine yeasts	10-mer mtDNA primers	250–3,000	RAPD	Not given	Martinez et al. 2007

Abbreviations: AFLP, amplified fragment length polymorphism; ITS, nuclear ribosomal internal transcribed spacer; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; qPCR, quantitative PCR; RAPD, random amplification of polymorphic DNA; RE, restriction enzymes; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat/microsatellite DNA analysis.

and *trnF* exons gave no false negative results when tested against 23 other plants, including corn, wheat, butter beans, and various seeds (Brezna et al. 2006).

Plant Food Authentication and Geographic Origin

Food authentication is concerned with the verification and validation of geographical, species, and ingredient claims. It proves that foods are consistent with ingredient labels and are wholesome and unadulterated with lower quality or less costly ingredients or fillers. PCR has also been applied to the authentication of plant ingredients. Because mitochondrial coding sequences in plants are highly conserved (Handa 2003), scientists use nuclear noncoding regions, microsatellite DNA, or chloroplast intron sequences to differentiate between plant species. Lee et al. (2009) used

Simple sequence repeats (SSRs):

repetitive DNA motifs noted for high mutation rate; used for molecular profiling and identification, also termed microsatellites

Amplified fragment length polymorphism (AFLP):

similar to RFLP, this assay amplifies selected DNA segments after restriction enzyme cuts to increase sensitivity

cpDNA to authenticate 21 *Artemisia* (wormwood herb) species. They combined PCR with RFLP to correctly identify different herb species. Faria et al. (2000) used microsatellite DNA analysis [simple sequence repeats (SSRs)] to differentiate between grape varieties. Discrimination between Robusta and Arabica coffee was facilitated by sequencing and discovering a unique SNP in the chloroplastic intraspacer region, resulting in different chlorotypes for each species (Trantakis et al. 2012).

Researchers have been able to extract DNA from difficult plant matrices such as gums and oils for use in forensic detection and identification. Two food additives, locust bean gum (LBG) and guar gum, are used as stabilizing agents in the food industry (Urdiain et al. 2005). Guar gum is less expensive than LBG and a suspected adulterant in commercial products. Molecular certification of commercial gums (Urdiain et al. 2005) was developed to differentiate between the two. Although both are polysaccharides, these gums contained enough contaminating plant DNA for gene detection and differentiation by PCR. Researchers had to find a DNA extraction method that eliminated the sticky and viscous polysaccharide coprecipitants. Using the nuclear ribosomal internal transcribed spacer (ITS) sequences as targets, Urdiain et al. (2005) created a rapid, simple molecular detection method to discriminate between the two gums via conventional PCR. DNA extracted from plant oils rather than leaves has been used to identify cultivars from various regions. Busconi et al. (2003) used three chloroplast genes (*trnL* intron, ribulose diphosphate carboxylase, NADH dehydrogenase) to determine the originating cultivars of extra virgin olive oils, combining fragment length analysis such as RAPD and amplified fragment length polymorphism (AFLP) with PCR because olive sequence data were not available at the time. Fragment analysis from olive oils corresponded to the profile of leaves from the same cultivars (Busconi et al. 2003). qPCR was used to identify olive oil from other oils such as sunflower, canola, soybean, maize, and sesame (Ramos-Gomez et al. 2016). It was concluded to be a reliable protocol, detecting 0.1% olive oil in commercial food products, including foods fried in olive oil.

Geographic origin is important for labeling of regional foods that have added value because of their origins. Researchers in Chile (Martinez et al. 2007) discovered a high correlation between molecular polymorphisms and geographic origins of wine yeast strains when using mtDNA restriction analysis followed by RAPD for construction of phylogenetic trees. They were able to confirm the geographic origin of 64 wine yeast strains using mtDNA and molecular methods.

DNA FRAGMENTATION

Basic Mechanisms

DNA destruction by heat occurs because of depurination or deamination (Gryson 2010). The most labile linkage in the DNA structure is the N-glycosyl bond, which is susceptible to hydrolysis (Lindahl 1993). Heat increases the rate of many reactions, including the production of reactive oxygen species that cleave the phosphodiester bond when scavenging electrons. Unlike the nucleus, which is sequestered from oxygen, the mitochondria participate in oxidative phosphorylation to provide energy to the cell; thus, mtDNA should be more prone to oxidative damage (Lindahl 1993). Above 100°C, helical strands are broken, causing an irreversible loss of secondary structure (Lindahl 1993). Acidic pH conditions also cause depurination of DNA, leading to strand cleavage (Sambrook & Russel 2001). However, DNA is relatively stable at alkaline pH values up to 9.5 (Gryson 2010).

The effects of temperature, pH, and the combination of the two parameters on DNA fragmentation were analyzed by Bauer et al. (2003) using plasmid DNA (pSG100, a derivative of pUC18). These researchers were able to monitor the first strand single break in the plasmid by HPLC,

expressing the results as a percentage of covalently closed circular plasmid. With un-nicked plasmid DNA expressed as 100%, they detected a decrease of closed plasmid to 85% after 90 min of incubation at 85°C, pH 8.4. Under acidic conditions at lower temperature (37°C, pH 4.0), the closed plasmid was reduced to 64% after 90 min. The greatest percentage of single-nicked plasmid occurred when temperature and acid were combined (65°C, pH 4.0), resulting in 1% of closed plasmid remaining after 90 min of incubation. This report looked at damage of the DNA phosphodiester bond (single-strand nicking) only. As mtDNA are also circular and relatively small compared with nuclear DNA, the analogy to plasmid nicking would be appropriate. Cleavage of the phosphodiester bond is the initiating step in DNA fragmentation and then oxidation and hydrolysis of nucleotides follow because of increased conformational availability to enzymes and reactive oxygen species.

Effects of Processing

Molecular methods are highly dependent on the quantity and quality of DNA available from the foodstuff. There are many articles that discuss the effects of processing on the sensitivity and reliability of the PCR assays. Heat, physical processes like milling, which reduce particle size, composting, pressure, drying, smoking, salting, and extrusion all have the potential to fragment cellular components including DNA (Gryson 2010). These processes can affect the primary and secondary structure of the molecule through hydrolysis and oxidation (Kharazmi et al. 2003, Meyer 1999). Fragmentation of DNA reduces the sensitivity of molecular methods, hinders quantification, and increases the limits of detection (Kharazmi et al. 2003). In qPCR for example, the Ct value is empirically determined as the cycle in which amplification of the target DNA becomes logarithmic. The assay is quantitative because Ct is proportional to number of amplicons in the test sample. The more target, the sooner the amplification achieves logarithmic growth. Amplicons can be fragmented by many processing methods, lowering the number of targets available for amplification and resulting in an increase in Ct value.

In a review article describing the effect of food processing on DNA degradation, Gryson (2010) provided a comprehensive list of processes that increase Ct value in qPCR: cooking, microwaving, fermentation, grinding, filtering, sterilization, acidification, salting, bleaching, degumming, centrifugation, drying, wet-milling, ensiling, and sonication. The article asserts the difficulty in obtaining good quality DNA from a highly processed food like corn flakes. Furthermore, of all the shear forces, grinding has the greatest effect on DNA fragmentation. This could impact the use of certain DNA extraction methods upstream from qPCR. The review concluded by saying that high temperature (>100°C) and low pH are the most important factors in DNA degradation or fragmentation.

DNA is also degraded at environmental temperatures, although at slower rates of destruction. Bovine mtDNA, as measured by qPCR, was reduced 93% as a result of composting of cattle carcasses under ambient environmental conditions for 147 days (Xu et al. 2009). The article did not speculate on the biochemical and physical mechanisms of DNA degradation. However, it is probable that the bovine mtDNA was degraded by the action of composting microorganisms and the release of cellular enzymes. Processes that result in the disruption of cell walls and membranes culminate in the release of endonucleases that fragment DNA (Kharazmi et al. 2003). However, under acidic pH conditions, the DNA-degrading enzymes are destroyed more rapidly than the DNA itself and their effect is minimized (Herman 1997). Endonucleases may also cleave DNA during storage of fresh foods (Chen et al. 2007). However, some chemical compounds were found to have a protective effect against DNA-degrading enzymes. Certain food components reduced DNA degradation by DNase I when measured by plasmid transformation frequency in *Escherichia*

coli (Bauer et al. 2004). Plasmids in the presence of maltol or putrescine had DNA degradation reduced by one order of magnitude. Degradation by DNase I was completely inhibited by octyl gallate and spermine (Bauer et al. 2004).

Effects of Heat

DNA can be brought up to temperatures approaching the boiling point of water (95°C), where it denatures and unravels; it then reforms into its original configuration when brought down to the annealing temperature of its base pairs. This describes the process of PCR, which works due to the stability and fidelity of nucleic acid chains at temperatures above physiological conditions. A temperature of 100°C does not degrade DNA very severely (Gryson 2010). However, above 100°C, DNA can become destabilized and permanently damaged. When the effect of thermal treatment on DNA degradation in legumes and maize was studied (Hrnčířová et al. 2008), researchers found that treatment at 80°C for 360 min had no effect on amplification of amplicons ranging in size from 874 to 1,371 bp as measured by conventional PCR. Treatments at 100°C and 200°C exhibited a drop in amplification after 60 and 30 minutes, respectively. When amplicons were longer than 1 kb, they were no longer detectable in conventional PCR after the higher heat treatments. Crab meat DNA obtained from canned samples was not successfully sequenced for DNA barcode analysis (Haye et al. 2012) because of mincing and extreme canning processes such as heat. However, researchers using PCR-RFLP were able to identify and differentiate between tuna species in cans because of the lower specificity and greater robustness of restriction site analysis of polymorphic fragments (Lin & Hwang 2007, Pardo & Perez-Villareal 2004, Terio et al. 2010, Unseld et al. 1995).

Despite high temperature (121°C) and pressure, it has been reported that autoclaving for 15 to 20 min did not completely fragment DNA samples placed on plastic and glassware (Suyama & Kawaharasaki 2013). Using a 2,682-bp DNA oligonucleotide sample and an 84-bp nested amplicon for detection, these researchers were able to amplify residual DNA from the bottom water of the autoclave pan after autoclaving at standard conditions for 20 min. Amplifiable DNA was destroyed only after autoclaving at 121°C for 80 min. Because molecular methods are extremely sensitive, they recommended using methods other than autoclaving to dispose of DNA and to prevent laboratory contamination.

Relationship Between Amplicon Length and Heat

Sakalar et al. (2012) studied the effects of several cooking regimes, including baking and boiling, on the identification and quantification of meat species. They concluded that the results of qPCR detection of mtDNA were influenced by time-temperature and the amplicon size. The baking of beef, pork, and chicken (amplicon sizes 374, 290, and 183 bp, respectively) caused an increase in Ct within 10 min at 200°C. They recommended employing small amplicons (<100 bp) to lessen the effect of heat on the sensitivity of the qPCR assay. Others studying the effects on heat and pressure on DNA fragmentation in turkey meat found baking and boiling had little effect when measured by qPCR using a TaqMan probe and small amplicon size (<200 bp) and targeting the mitochondrial cytochrome b gene (Hird et al. 2006). These researchers measured the effect of amplicon length by designing amplicons that had the same reverse primer and TaqMan probe but different forward primers. The forward primers were used to increase the length of the amplicons. The amplicons ranged in size from 81 to 116, 240, 317, 351, 427, 470, and 565 bp. They observed an increase in Ct value with increasing amplicon size. Amplicons up to 240 bp had similar Ct values, but over that value the increases in Ct value were more drastic. Canned and autoclaved turkey gave numerically

higher Ct values than raw, boiled, and baked product when using longer amplicons (<300 bp). They concluded that designing longer amplicons might improve specificity while still providing sufficient measurable signal after harsh thermal treatments of meats. However, this article did not provide standard curves for each amplicon size or specify PCR efficiency, linear range of detection, or lower detection limits, all of which are important parameters required in minimum information needed for quantitative experimentation (MIQE) standards for publication (Bustin et al. 2009). Therefore, the increases in Ct value may have been partly or fully due to lack of assay optimization. Other researchers contend that thermal processing results in not only increased DNA fragmentation but also changes in DNA extraction efficiencies that could affect downstream applications such as PCR (Murray et al. 2007).

Use of DNA Fragmentation as Time-Temperature Indicator

Many researchers recognize the effect of thermal processing of plant or animal food matrix as reducing amplification of the target in conventional and qPCR assays (Bauer et al. 2003, Gryson 2010, Hrnčířová et al. 2008, Kharazmi et al. 2003, Murray et al. 2007, Sakalar et al. 2012). Thermal processing can result in the elimination of detectable amplicon bands in electrophoretic gels and increase the Ct values for the respective methods. To reduce the effects of heat and other processing, researchers suggest the use of very small amplicons (<100 bp) (Aslan et al. 2009, Sakalar et al. 2012). However, the fragmentation of DNA has been shown to have a practical use as a time-temperature indicator (TTI), owing to its destruction over time-temperature while still providing a measurable outcome after treatment (Caldwell et al. 2015a,b). These researchers monitored the destruction of mtDNA over time-temperature. They documented linear relationships between Ct values of food matrices and reduction curves of indicator microorganisms such as *Geobacillus stearothermophilus* and *E. coli* O157:H7 for low-acid and high-acid foods, respectively. They also used mtDNA fragmentation to pinpoint the cold spot in a glassy solid such as peanut using noninvasive techniques (Caldwell et al. 2016).

Nonthermal processes have been examined by other researchers (e.g., Xu et al. 2009), who have looked at DNA as an indicator of biodegradation, albeit with animal carcasses and not food. Chen et al. (2007) observed that endonucleases may fragment DNA during storage of foods at lower temperatures. Caldwell et al. (2015a) found a linear relationship between fragmentation of mtDNA and ambient temperature processes such as fermentation and storage in high-acid products such as pickles.

Minimum information needed for quantitative experimentation (MIQE):

features a quality control checklist for publishing qPCR assays (Bustin et al. 2009)

Time-temperature indicator (TTI):

a device or constituent measuring variable temperature exposure by irreversibly mimicking the change of the target attribute

SUMMARY POINTS

1. Verification and identification of food ingredients are necessary for a variety of nutritional, personal, religious, safety, regulatory, and ethical reasons.
2. Molecular techniques are being increasingly utilized in food forensic analysis to combat fraud, verify and authenticate ingredients, improve traceability, and distinguish between closely related species.
3. Because of high copy numbers of genes in plant and animal cells, organelle DNA offers an advantage over nuclear DNA for molecular detection methods and is used in disciplines where highly degraded samples are typical, e.g., archaeology, forensics, and food processing.
4. Food processing can affect the sensitivity of molecular assays because of the fragmentation of target DNA by physical, chemical, and thermal means.

FUTURE ISSUES

1. Molecular assays need to be simplified and standardized to allow relatively untrained personnel to use this technology and eliminate false positives and false negatives.
2. For a molecular assay to gain widespread use and acceptance, it must be operator and laboratory independent. Conformity to MIQE standards for qPCR allows comparisons between labs and standardizes results.
3. For commercial use of organelle DNA in the food industry, a reference database will need to be created for some methods such as DNA barcoding (Galimberti et al. 2013) and any methods which use restriction enzymes for DNA fragment analysis such as SSR and AFLP (Busconi et al. 2003, Faria et al. 2000). Reference samples for comparison and internal amplification controls are also needed.
4. Collaborative validation studies by internationally recognized consortia such as the AOAC International or agencies with food safety prerogatives such as the US Food and Drug Administration or Department of Agriculture will have to be conducted to meet regulating body requirements in many countries or unions to qualify as official methods.
5. Instead of using organelle DNA for detection only, future use of molecular technologies can quantify degradation of DNA as a TTI in thermal, as well as nonthermal processes, such as storage, fermentation and acidification.
6. Because it is a rapid method, DNA fragmentation could be used as a presumptive test for process monitoring prior to shipping.
7. Fragmentation of intrinsic DNA could be used in accelerated shelf-life studies as a benchmark or normalizer and compared to sensory and chemical parameters.
8. Since DNA can be stored indefinitely at -80°C , food samples can be banked for future analysis if processing runs prove problematic.

DISCLOSURE STATEMENT

The author has a U.S. patent on protocols and data described in this review article.

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Errata

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