Advances in Food Authenticity Testing

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Introduction

1

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Food security is a primeval concern and people have developed systems of agricultural production, safe storage, processing, and transportation in order to satisfy their food requirement needs. Since the industrial revolution and the accelerated accumulation of scientific knowledge in the last two centuries, major inroads have been made in addressing the problems around providing sufficient food for earth's population, although about 793 million people globally were estimated to be undernourished in 2015 (FAO, 2015). For those living in the developed regions of the world, food insufficiency is not generally of relevance although societal problems concerning equitable food distribution are a current reality. However, issues around the safety and quality of the food supply are foremost in the minds of consumers and regulatory authorities in these countries. Food safety is of course paramount for the food industry and regulatory industries given the increased amount of dietary energy now consumed in the form of processed foods by the populations of developed countries (Monteiro et al., 2013) and the increasing complexity and length of food distribution and supply chains. However, consumer sensitivity to the possibility that their food may not be what it claims to be is generally assumed to be increasing.

A significant reason for this is sensitivity perhaps lies in the print and broadcast media's greater coverage of food scandals involving false labeling. Detailed descriptions of and investigations into recent scandals such as the presence of Sudan-1 dye in a wide range of foodstuffs in the United Kingdom in 2005, the detection of melamine in milk sourced in China in 2008, and the more recent horsemeat scandal uncovered in Ireland in 2013 have all been covered extensively in the public arena and have doubtlessly contributed to public concern. The melamine scandal in particular has been responsible for heightened awareness because this adulteration also had a significant food safety dimension due to the toxic effects of this compound, especially in babies and young children. Indeed, it is partly in response to this particularly egregious adulteration that consumers now increasingly treat food adulteration as a potential food safety issue with all the attention that this implies.

Regulatory agencies around the world are now devoting more resources to this problem not least because many of the adulterations, eg, those involving illicit alcohol, can involve revenue losses to the state. Operation OPSON is a multicountry (currently 47 states) joint cooperation between Europol and INTERPOL, which also includes public authorities and private companies. Operated through annual, targeted exercises, the most recent (OPSON IV) was conducted between December 2014 and January 2015 and, in this short period, OPSON seized more than 2500 tons of illicit and counterfeit food including mozzarella cheese, strawberries, eggs, cooking oil, and dried fruit (Europol, 2015). This type of cross-border organization is a recognition of the increasing globalization of trade in food and especially food ingredients and the extended nature of food supply chains.

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Within Europe, food fraud has been a long-standing subject of research and concern. The European Commission funded a number of research projects through its Framework Programmes 3–7 covering the period 1994 until now. These projects have ranged from collaborative research activities such as FAIM (1994–97) and TRACE (2005–10) to actions focused on specific analytical techniques (Food analysis using isotopic techniques, 1996–98) or authenticity issues (FATAUTHENTICATION; Authentication of fats and fat products used in food and feed, 2010–12). Recognizing the need for greater communication between states regarding intelligence around potential adulteration incidents, the Commission formed the Food Fraud Network (FFN) in 2015. This pan-European body is designed specifically to detect and prevent violations of food chain rules across borders and to collect information that is needed (in accordance with applicable national rules) to further refer a case to investigation or prosecution.

To support these regulatory and commercial initiatives, research scientists have devoted considerable resources to the development of analytical techniques to identify foods or food ingredients that are in breach of labeling requirements and may consequently be adulterated. A quick and rather crude search of the Web of Science using the search terms "food authentic*" and "food adulteration" revealed that 334 research articles with one or the other of these terms in the publication title had appeared in the decade between 2006 and 2016. Given that many publications will have more specific titles, it may not be unreasonable to conclude that the total number of such outputs may be 5 or even 10 times this number. Given this evidence of a flourishing research community focusing on analytical problems related to food adulteration, it was considered timely to gather together the most up-to-date information on the techniques and their use in specific application types in this book.

In Part 1, the focus is on advances in methods for food authenticity testing. This collection of 11 chapters contains contributions from experts in fields as diverse as differential scanning calorimetry to mass spectrometry and DNA fingerprinting. All of these authors have contributed information on the latest developments in these methods and the chapters will be invaluable to anyone practicing in the field or considering adding new techniques to their analytical repertoire.

Part 2 contains reports on advances in authenticity testing of a range of food types. These four chapters cover strategies for confirming the geographic origin of foods and testing for authenticity in meat, fish, and cereals.

Part 3 comprises 10 chapters, each of which deals with specific food products or analytical tools to support authenticity testing. Among the former are chapters dealing with adulteration testing methods for dairy products, cereals and cereal products, alcoholic beverages, and fruit and fruit juice products. Of increasing interest is the food supplement sector of the food industry, and one chapter focuses on the methods being currently applied to detect offenses in this industry segment. Last, but not least, is a chapter on the most appropriate chemometric tools suitable for and that are being used to tackle authenticity and adulteration practices.

I trust that you will find the book useful and profitable.

Gerard Downey
Dublin, February 2016

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Advances in DNA Fingerprinting for Food Authenticity Testing

2

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Chapter Outline

2.1 Introduction

For the last two decades, analysis of DNA in food has become a routine procedure to detect genetically modified organisms, allergens, pathogens, and adulterations in food. Many cases of food fraud have been uncovered by DNA testing, for example, undeclared horse meat in beef products (FSA, 2013), *Pinus armandii* in Chinese pine nuts (Nader et al., 2013), persipan declared as marzipan (Brüning et al., 2011), and overfished species like yellowfin (*Thunnus albacares*) or bigeye tuna (*Thunnus obesus*) in canned tuna declared as skipjack (*Katsuwonus pelamis*) (Chuang et al., 2012). The horse meat scandal stunned the food industry and trade in the European Union (EU) in 2013. Adulteration of pine nuts by the species *P. armandii* (Chinese

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white pine) affects food safety, since these latter cause significant and long-lasting taste disturbances (Pine mouth syndrome).

The authenticity testing mentioned herein is based on the detection of species-specific DNA sequences. Animal species are commonly differentiated based on sequences of mitochondrial genes, which code for the subunit I of cytochrome c oxidase. Plant species can be distinguished by differences of the intergenic spacer regions in the plastid chloroplast DNA or the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron (18S-5.8S-26S) (Nader et al., 2013).

Certain specialty foods like basmati rice and Angus beef require authenticity testing beyond the species level because crop varieties and breeds of livestock have to be identified and distinguished. Basmati and its adulterants, ordinary long-grain rice varieties, belong to the same species *Oryza sativa*. Angus cattle are a premium breed of *Bos taurus*, and meat from ordinary cattle can frequently be found declared as genuine Angus beef.

Traceability is a major requirement of EU food legislation (Regulation (EC) No 178/2002) and is particularly strict for meat products (Regulation (EC) No 1760/2000; Commission Implementing Regulation (EU) No 931/2011). Controlling this traceability requires the differentiation of individual animals, which can be achieved by DNA analysis as well (Vázquez et al., 2004).

Individual animals, breeds of livestock, and crop varieties cannot be identified based on the genetic elements commonly used for species differentiation (see previous discussion) because these DNA sequences are stable and well established within a species. For testing at this level, DNA sequences are required with a high degree of polymorphism, which changes frequently within generations. Genetic elements, which meet these requirements, are highly polymorphic mini- and microsatellites and single nucleotide mutations, the so-called single nucleotide polymorphisms (SNPs). These three groups of genetic elements provide the markers for DNA fingerprinting capable of analysis at the level of individuals, breeds, and varieties.

DNA fingerprinting used to identify human individuals was first applied by Sir Alec Jeffreys as a forensic tool to solve an immigration case in 1985 and later the Enderby murder in 1986 (Jobling and Gill, 2004). The original method applied by Jeffreys was based on polymorphisms of minisatellites and was later replaced by genotyping of microsatellites, also called simple sequence repeats (SSRs). SSR typing has revolutionized forensics since then and has now even become a major tool to solve decades-old cold cases.

Genotyping based on SNPs utilizes differences of single nucleotides and is widely used for breeding of plants and animals and for the identification and paternity testing of cattle (Heaton et al., 2002).

This chapter will describe in detail scientific and technological aspects of DNA fingerprinting and two applications of SSR genotyping in food, ie, authenticity testing of high-value rice varieties and traceability checks on pork meat. Legal and economic aspects will be discussed with regard to the DNA analysis of basmati and jasmine rice.

2.2 Scientific Background of DNA Fingerprinting and Its Applications

2.2.1 Mini- and Microsatellites

Minisatellites are often referred to as variable number of tandem repeats (VNTRs) and microsatellites as short tandem repeats (STRs) or SSRs. The names mini- or microsatellites are historically derived from the behavior of certain fractions of DNA in the cesium chloride density gradient during ultracentrifugation. These fractions form satellites, strong and localized bands above or below the main band.

VNTRs and SSRs are tandem repeats of short repeat units. The size of the repeat unit is the main feature to classify a tandem repeat as a mini- or microsatellite. It is generally accepted that repeat units of microsatellites contain less than 9 base pairs (Richard et al., 2008).

Tandem repeats occur in virtually all prokaryotic and eukaryotic genomes. The most simple repeat structures consist of mononucleotide repeats, homopolymers of poly(AT) and poly(GC) tracts, which were first observed in a very basic eukaryotic life form, the myxomycete *Physarum polycephalum* (Nader et al., 1985, 1986).

Mini- and microsatellites occur frequently in eukaryotes. About 253,000 (90 per megabase) microsatellites have been detected in the human genome (Richard et al., 2008), and according to Sonah et al. (2011) the rice genome contains over 135,000 SSRs with a frequency of about 360 per megabase.

Lengths of mini- and microsatellites can be highly variable, and mutations lead to the gain or loss of one or more repeats. Short microsatellites tend to expand while longer ones tend to contract. The variability of microsatellites depends on the length and structure of the tandem repeat and the location in the genome. The mutation rates are estimated at between 10^{-2} and 10^{-6} per locus and generation in the human genome (Eckert and Hile, 2009). In contrast, the average mutation rate for nucleotide conversions generating SNPs is much lower and estimated to be $\sim 2.3 \times 10^{-8}$ per nucleotide site. However, these might occur all over the genome and consequently are at least as frequent as changes in SSRs with 175 mutations at single nucleotide sites per diploid genome per generation (Nachman and Crowell, 2000).

Replication slippage is generally thought to be the main pathway causing the high mutation rates of tandem repeats (Ellegren, 2004). Slippage involves the transient dissociation of the replicating DNA strands and, during reassociation, loops might form on the template or nascent strands due to the tandem repeat structure. Loops on the nascent strand lead to an increase of the repeat length and on the template strand to a decrease. In most cases, the mismatch repair system corrects these changes, but in a small unrepaired fraction these mutations are manifested (Levinson and Gutman, 1987).

Mini- and microsatellites are often considered to be nonfunctional "junk" DNA. However, recent studies on variations of tandem repeats within functional genes of the yeast *Saccharomyces cerevisiae* reveal that these change the capability of the cells to adhere and form biofilms and thus contribute to the fast adaptation of these

microorganisms to changing environments (Verstrepen et al., 2005). Hypervariable repeats located within developmental genes of the dog genome supposedly permit the fast evolution of the limb and skull morphology of these animals (Fondon and Garner, 2004). Mini- and microsatellites might therefore significantly contribute to the genetic variability of organisms and may possibly be a driving force in evolution.

Mini- and microsatellites are highly polymorphic, and therefore these elements are used extensively in applied genomics. Their role in modern forensics has already been described herein but there are numerous other applications. Microsatellite typing was used for the identification of laboratory and industrial strains of the yeast *S. cerevisiae* and pathogenic *Candida* strains (Lunel et al., 1998). It was furthermore applied to investigate the origin of domestic horses (Vilà et al., 2001) and French wine grapes (Bower et al., 1999). Microsatellites play an important role in marker-assisted plant and animal breeding (Phuc et al., 2005; Gupta et al., 2001). In biological conservation, analysis of microsatellites became an important tool with which to determine the genetic diversity of populations and to prevent inbreeding of populations in captivity in reintroduction projects (Nader et al., 1999). Applications for the control of food authenticity and traceability will be discussed in detail later.

2.2.2 Single Nucleotide Polymorphism

DNA fingerprinting can be also performed on the basis of SNPs. This method has substituted SSR typing in many applications (Gupta et al., 2001). SNPs occur at single base pair positions in the genome, at which two different alleles exist in the individuals of a population or among different crop varieties. The abundance of the least frequent allele should be 1% or greater (Guerra and Yu, 2010). SSRs and SNPs are referred to as second- and third-generation markers, respectively (Gupta et al., 2001). Advantages of SNPs over SSRs include their higher abundance within genomes; in any genome, one SNP can be found in every 100 to 300 base pairs while SSRs occur less frequently by one to two orders of magnitude. The human genome reveals one microsatellite in every 10,000 base pairs and the rice genome reveals one in every 3000 base pairs (see previous mention). SNPs are also more homogenously distributed over the genome than are SSRs; they are also more stable due to their lower mutation rate (see previous mention). Due to these characteristics, SNPs are the markers of choice for genotyping of human and animal diseases, in personalized medicine (for example, in pharmacogenomics), genome-wide association studies and marker-assisted breeding of animals and plants (Gupta et al., 2001; Feltus et al., 2004).

One advantage of SSRs over SNPs is their polyallelic nature. For each SNP, four different alleles should occur, but in most cases only two alleles have been observed at a specific locus in the genome of a population. By analyzing only a small panel of SSR markers, crop varieties can be detected and differentiated in food. Similarly, meat can be traced back to individual animals or parents. The high abundance of SNPs largely offsets this disadvantage of only two alleles being present, and numerous SNPs can be analyzed simultaneously, for example, by applying multiarray DNA biochip technologies (see following discussion).

In contrast to SSRs, SNPs have not yet been established as routine markers for food analysis, and potential applications are still at the stage of fundamental research (Spaniolas et al., 2014).

Another alternative to SSR markers are insertions/deletions (InDels). In the rice genome, for example, 400,000 InDels have been mapped, many of these being polymorphic and therefore suitable for DNA fingerprinting (Steele et al., 2008). The cause of most of these insertions or deletions is unknown, but many of them have been created through the transposition of transposons and retroelements.

2.3 The Methodology of DNA Fingerprinting

2.3.1 First-Generation Markers: RFLP, ISSR, and RAPD

First applications of DNA fingerprinting were based on markers derived from restriction fragment length polymorphisms (RFLPs), inter simple sequence repeats (ISSRs), and randomly amplified polymorphic DNA (RAPD). These markers are considered as first generation.

RFLPs can be caused by all possible mutations including InDels, contractions and expansions of VNTRs and SSRs, and exchanges of single nucleotides. Therefore, RFLP analysis can also be used for SSR genotyping. Nader et al. (1999) applied RFLP typing to analyze the genetic diversity of the scarlet macaw (*Ara macao*) in Costa Rica. The method involves the isolation of DNA from the biological material and its digestion with restriction endonucleases to generate restriction fragments. The fragments are then separated by electrophoresis on agarose or polyacrylamide gels followed by Southern blotting. DNA bound to the membrane is hybridized with labeled probes consisting of repeats that are typical for microsatellites, eg, $(CAC)_5$ or $(GGAT)_4$. DNA hybrids are then visualized by labeling of the probes, for example, by the enzymatic cleavage of colorogenic substrates or radioactive isotopes. Band patterns are evaluated manually or by computer programs to differentiate individuals and determine parental relationships among them.

Inter simple sequence repeat−polymerase chain reaction (ISSR−PCR) has been used for testing genetic diversity (Kantety et al., 1995), cultivar identification (Charters et al., 1996), molecular mapping (Ratnaparkhe et al., 1998), seed authenticity testing (Kumar et al., 2001), and various other applications. In a study combining ISSR-PCR and fingerprinting with SSR markers, Nagaraju et al. (2002) evaluated the genetic relationships in traditional and evolved basmati and in semidwarf non-basmati rice varieties. ISSR analysis is based on the PCR amplification of the regions between adjacent and inversely orientated SSRs. These have to be close enough to allow efficient amplification. Inversions, insertions, deletions, and mutational events of microsatellites at multiple loci in the genome yield differences in the band pattern. It is not suited to differentiate between individuals in a population that show no or only few differences in their ISSR band patterns, whereas closely related subspecies and species reveal specific banding profiles or fingerprints. For the primer design, DNA sequences are selected that represent tandem repeats typically found in microsatellites.

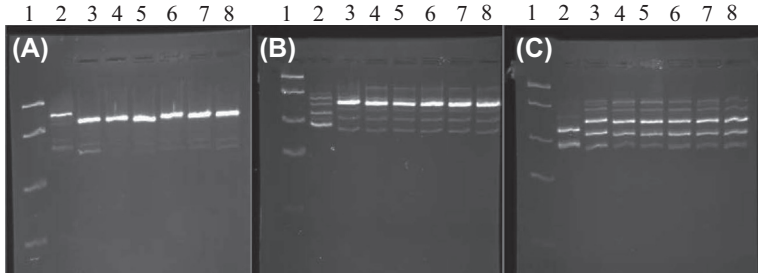

Figure 2.1 Analysis of basmati rice varieties by inter simple sequence repeat-polymerase chain reaction (ISSR-PCR). Amplification in the PCR reactions was performed with primers AGAGAGAGAGAGAGAGYT (agarose gel a), CACACACACACACACAG (gel b) and GAGAGAGAGAGAGAGAT (gel c). After amplification, amplicons were separated on agarose gels and visualized by staining with ethidium bromide. Lane 1 on the agarose gels contains the molecular weight marker FastRuler (*Life Technologies, Inc., Foster City, CA, USA*; fragment lengths: 1500, 850, 400, 200, and 50 base pairs). Amplicons of the following basmati varieties were separated on the other lanes of gels (A to C): Pusa Basmati-1 (lane 2), Taraori (HBC-19) (lane 3), Basmati 2000 (lanes 4 and 7), Basmati 515 (lanes 5 and 8) and Super Basmati (lane 6).

PCR amplicons are separated by gel electrophoresis and can then be visualized by staining with intercalating fluorescent dyes like ethidium bromide. Alternatively, one might use primers that are labeled by, eg, radioactivity or by conjugation with digoxigenin. Fig. 2.1A–C show results of an ISSR analysis of five different varieties of basmati rice and three different primers. These results will be discussed later in the context of basmati authenticity testing.

RAPD analysis is a special type of PCR involving short primers with arbitrary nucleotide sequences of generally 10 bases, which randomly bind to various regions in the genome. Amplified fragments are analyzed by agarose gel electrophoresis, and polymorphisms are identified on the basis of banding patterns resulting after staining with ethidium bromide.

Due to the rapid development of modern technologies for DNA analysis over the last two decades, RFLP, RAPD, and ISSR markers have lost their importance for DNA fingerprinting. They are still useful in certain cases in which there is no or only a little available information about DNA sequences of the targeted genome, for example, if genomes of rare or exotic organisms have to be analyzed. In food analysis, DNA fingerprinting based on ISSR markers has been applied to trace and differentiate wild coffee harvested in Ethiopian rain forests (Zeltz et al., 2005).

2.3.2 Second-Generation Markers: SSRs

Modern DNA fingerprinting methods based on SSR markers comprise the direct amplification of the microsatellite loci by the PCR with primers hybridizing to the flanking sequences of these multiple repeats. The amplified alleles of different repeat

lengths are then analyzed by gel or capillary electrophoresis. DNA sequence data for the design of primers fitting a large variety of different SSRs have become available due to various whole-genome sequencing projects and to the development of cost-effective high-throughput sequencing methods (Di Bella et al., 2013). Genomes of important crop and livestock such as rice (International Rice Genome Sequencing Project, 2005), wheat (Brenchley et al., 2012), cattle (Zimin et al., 2009), and swine (Archibald et al., 2010) have been sequenced and Websites like Gramene (www.gramene.org) hold SSR and SNP allelic data for these organisms.

In 2000 Bligh published her pioneering work on the differentiation of basmati from non-basmati rice varieties by SSR genotyping, and her method became a standard procedure for basmati authenticity testing (Nader et al., 2013). Like other self-pollinators, rice varieties are also particularly suited for DNA fingerprinting considering that they are, in most cases, homozygous pure lines that reveal only one allele per marker.

The method is capable of analyzing even complex mixtures in a reliable and quantitative manner, and the following example of the analysis of a rice sample obtained from a customer and declared as basmati describes the procedure in more detail.

DNA extracted from the sample and purified over silica membrane spin columns served as a template for the PCR amplification of 10 standard SSR markers, published by the UK Food Standards Agency (FSA) in 2004: RM1, RM44, RM55, RM171, RM201, RM202, RM223, RM229, RM241, and RM348. The markers belong to a panel of 50 SSR loci that are published on the Gramene Website together with primer sequences suited for their PCR amplification (Gramene, 2015). These SSRs were found to be highly polymorphic and are therefore used by the Generation Challenge Program for rice diversity analysis. Primers for amplification of the SSR markers RM1, RM201, RM202, and RM171 were labeled at the 5′-end with the fluorescent dye FAM (6-carboxylfluorescein), markers RM223, RM229, and RM241 were labeled with JOE (6-carboxy-4,5-dichloro-2,7-dimethoxy-fluorescein), and markers RM348, RM44, and RM55 with TAMRA (carboxy-tetramethyl-rhodamine).

After amplification, PCR products marked with different fluorescent dyes were combined into four mixtures: (1) RM202 and RM348; (2) RM1, RM223, and RM44; (3) RM201 and RM229; and (4) RM171, RM241, and RM55. Polynucleotides in each mixture were separated on an ABI 3130XL Genetic Analyzer with POP4 polymers on 36-cm capillary arrays (Life Technologies, Foster City, CA, USA) in the presence of ILS 600 internal size standards (Promega Inc, Madison, WI, USA). Fluorescent signals were quantified by laser-induced fluorescence and, due to the different labeling, alleles from three different SSR markers could be analyzed simultaneously during each chromatographic run. Chromatograms of the 10 markers are shown in Fig. 2.2.

Stutter peaks are observed for all markers with the exception of RM348. These are caused by replication slippage, which is also considered to be the main pathway causing the high mutation rates of tandem repeats in VNTRs and SSRs (see previous discussion). Stutter peaks represent minor amplification products, differing from the main amplicon by multiples of the length of the repeat unit (Ellegren, 2004). In contrast to SSR mutations in vivo, which lead to both extension and contraction of

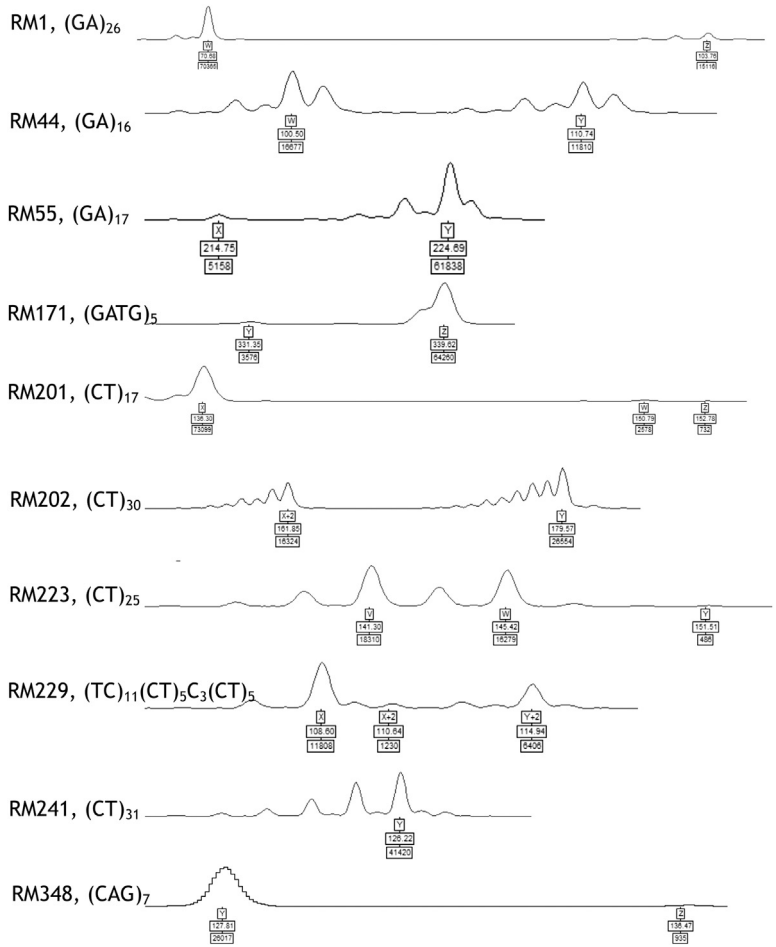

Figure 2.2 Separation of amplified alleles of simple sequence repeat markers in a rice sample by capillary electrophoresis. Allele type (W, X, Y, and Z), size of fragment (in base pairs), and intensity of fluorescent signals are indicated in the boxes under each allele peak. Details are described in the text.

the microsatellite locus, stutter peaks generally appear in PCR as products that are shorter than the size of the corresponding SSR allele. Strong stuttering is observed with markers RM1, RM44, RM55, RM201, RM223, and RM241, which are characterized by simple dinucleotide repeat structures $(GA)_n/(CT)_n$, whereas this effect is less pronounced in the markers with more complex repeat structures, RM171 $[(GATG)_5]$ and RM229 $[(TC)_{11}(CT)_5C_3(CT)_5]$, and absent in RM348 $[(CAG)_7]$.

In order to compensate for this stutter effect, only the peak of each allele with the highest fluorescence intensity is evaluated. Fragment sizes and peak areas are analyzed with the GeneScan/Genotyper or GeneMapper software and results are then transferred into an Excel sheet for further calculations (Table 2.1).

Table 2.1 Combinatorial Approach to Identify and Quantify Rice Varieties in a Mixture by Simple Sequence Repeat (SSR) Fingerprinting Evaluation of Data From Fig. 2.2

		RM1		RM44		RM55		RM171		RM201		RM202		RM223		RM229		RM241		RM348	
1	Pusa Basmati-1	W		Y		Y		Z		X		Y		W		X		Y		Y	
2	Pusa 1121	W		Y		X		Y		X		Y		W		X		Y		Y	
3	Pusa 1509	W		Y		X		Z		X		Y		W		Y+2		Y		Y	
4	Sugandha	Z		W		Y		Z		X		X+2		V		Y+2		Y		Y	
5	Analytical results	W	65%	Y	61%	Y	92%	Z	92%	X	95%	Y	67%	W	61%	X	61%	Y	100%	Y	96%
6		Z	35%	W	39%	X	8%	Y	8%	W	4%	X+2	33%	V	38%	Y+2	34%	–	0%	Z	4%
7		–	0%	–	0%	–	0%	–	0%	Z	1%	–	0%	Y	1%	X+2	5%	–	0%	–	0%
Hypothesis 1: Mixture of Pusa Basmati-1, Sugandha, and Pusa 1121																					
8	Step 1: 57% Pusa Basmati-1 subtracted	W	8%	Y	4%	Y	35%	Z	35%	X	38%	Y	10%	W	4%	X	4%	Y	43%	Y	39%
9		Z	35%	W	39%	X	8%	Y	8%	W	4%	X+2	33%	V	38%	Y+2	34%	–	0%	Z	4%
10		–	0%	–	0%	–	0%	–	0%	Z	1%	–	0%	Y	1%	X+2	5%	–	0%	–	0%
11	Step 2: 34% Sugandha subtracted	W	8%	Y	4%	Y	1%	Z	1%	X	4%	Y	10%	W	4%	X	4%	Y	9%	Y	5%
12		Z	1%	W	5%	X	8%	Y	8%	W	4%	X+2	0%	V	4%	Y+2	0%	–	0%	Z	4%
13		–	0%	–	0%	–	0%	–	0%	Z	1%	–	0%	Y	1%	X+2	5%	–	0%	–	0%
14	Step 3: 4% Pusa 1121 subtracted	W	4%	Y	0%	Y	1%	Z	1%	X	0%	Y	6%	W	0%	X	0%	Y	5%	Y	1%
15		Z	1%	W	5%	X	4%	Y	4%	W	4%	X+2	0%	V	4%	Y+2	0%	–	0%	Z	4%
16		–	0%	–	0%	–	0%	–	0%	Z	1%	–	0%	Y	1%	X+2	5%	–	0%	–	0%

Continued

Table 2.1 Combinatorial Approach to Identify and Quantify Rice Varieties in a Mixture by Simple Sequence Repeat (SSR) Fingerprinting Evaluation of Data From Fig. 2.2—cont'd

		RM1		RM44		RM55		RM171		RM201		RM202		RM223		RM229		RM241		RM348	
Hypothesis 2: Mixture of Pusa Basmati-1, Sugandha, and Pusa 1509																					
17	Step 1: 57%	W	8%	Y	4%	Y	35%	Z	35%	X	38%	Y	10%	W	4%	X	4%	Y	43%	Y	39%
18	Pusa Basmati-1	Z	35%	W	39%	X	8%	Y	8%	W	4%	X+2	33%	V	38%	Y+2	34%	–	0%	Z	4%
19	subtracted	–	0%	–	0%	–	0%	–	0%	Z	1%	–	0%	Y	1%	X+2	5%	–	0%	–	0%
20	Step 2: 30%	W	8%	Y	4%	Y	5%	Z	5%	X	8%	Y	10%	W	4%	X	4%	Y	13%	Y	9%
21	Sugandha subtracted	Z	5%	W	9%	X	8%	Y	8%	W	4%	X+2	3%	V	8%	Y+2	4%	–	0%	Z	4%
22		–	0%	–	0%	–	0%	–	0%	Z	1%	–	0%	Y	1%	X+2	5%	–	0%	–	0%
23	Step 3: 4%	W	4%	Y	0%	Y	5%	Z	1%	X	4%	Y	6%	W	0%	X	4%	Y	9%	Y	5%
24	Pusa 1509 subtracted	Z	5%	W	9%	X	4%	Y	8%	W	4%	X+2	3%	V	8%	Y+2	0%	–	0%	Z	4%
25		–	0%	–	0%	–	0%	–	0%	Z	1%	–	0%	Y	1%	X+2	5%	–	0%	–	0%

Fluorescence emission intensity directly correlates to the number of copies generated by PCR, permitting a quantitative analysis of each allele in relation to the whole allele mixture found for each SSR marker.

Shorter alleles of SSR markers are amplified more efficiently than the longer ones during PCR. Therefore, correction factors have to be applied on the fluorescent intensities to compensate for the allelic imbalance caused by the PCR reaction and to avoid longer SSR alleles being underestimated in relation to shorter ones.

Table 2.1 summarizes in rows 5−7 the quantitative results of the SSR typing illustrated by the chromatograms in Fig. 2.2 for each allele and after applying the corresponding correction factors. The letters V to Z characterize the alleles of each of the 10 microsatellite markers with V being the shortest and Z the longest tandem repeat (FSA, 2004). In a combinatorial approach, different hypotheses are generated to explain the allelic pattern determined for the sample by putative mixtures of rice varieties. For the present case, the allelic pattern can be explained by a mixture of the varieties Pusa Basmati-1, Pusa 1121, and the non-basmati variety Sugandha (hypothesis 1) or Pusa Basmati-1 with Sugandha and Pusa 1509 (hypothesis 2).

The allele patterns of these varieties have been determined from reference materials obtained from official sources and from trade (Nader et al., 2013, 2014) and are summarized in rows 1−4. These varieties are furthermore listed in Table 2.2. Initially, the dominant variety in the mixture is identified; this is Pusa Basmati-1 and would fit both hypotheses at a concentration of 57%. The percentage expresses the relative DNA content of the variety in the mixture in relation to the total rice DNA detected (see further comments following). Consequently, 57% are subtracted from all alleles that belong to Pusa Basmati-1. Residual alleles are listed in rows 8−10 for hypothesis 1 and 17−19 for hypothesis 2.

In a second step, the concentration of Sugandha is estimated at 34% to fit hypothesis 1 and 30% to fit hypothesis 2. These fractions are then subtracted from all alleles that belong to Sugandha and that are left over from the first evaluation step. Residual alleles are summarized in rows 11−13 for hypothesis 1 and in rows 17−19 for hypothesis 2. Finally, the concentrations of Pusa 1121 and 1509 are estimated at 4% for both hypotheses, and these fractions are subtracted from the alleles left over from step 2. Residual alleles are listed in rows 14−16 for hypothesis 1 and 23−25 for hypothesis 2. The residual allele patterns can be attributed to two different rice varieties, which cannot be identified due to the lack of reference materials. In addition to 57% Pusa Basmati-1, the sample therefore either contains 34% Sugandha, 4% Pusa 1121, and 4% of unidentified nonbasmati varieties or 30% Sugandha, 4% Pusa 1509, and 9% unidentified. For ISO 17025 accreditation, the test was fully validated by addressing precision, accuracy, linearity, and sensitivity (Nader et al., 2013). The analytical uncertainty U depends on the complexity of the mixture and might vary between $U = \pm 3\%$ (absolute) for samples with one major variety >95% and $U = \pm 6\%$ (absolute) for complex mixtures such as in the example described previously. In a quantitative proficiency test of the FSA (2006) involving 11 independent laboratories, an uncertainty of ±6% was determined, which corresponds to the result obtained by the authors (Nader et al., 2013).

Table 2.2 DNA Fingerprints of Various Rice Varieties Based on the Analysis of 10 Standard Simple Sequence Repeat (SSR) Markers

Variety	Origin	RM1	RM44	RM55	RM171	RM201	RM202	RM223	RM229	RM241	RM348	*fgr*
Traditional Basmati Varieties												
Taraori (HBC-19, Karnal local)	India	W	Y	X	Y	X	Y	W	Y	Y	Z	+/+
Basmati 386	India	W	Y	X	Y	X	Y	W	Y	Y	Z	+/+
Kernel (Basmati Pakistan)	Pakistan	W	Y	X	Y	X	Y	W	Y	Y	Z	+/+
Basmati 370	Pak./In.	W	X	Z	Y	X	Y	W	Y	Z	Z	+/+
Type 3 (Dehradun)	India	W	X	Z	Y	X	Y	W	Y	Z	Z	+/+
Ranbir Basmati (IET 11348)	India	W−1	X+1	Z	Y	X	Y	Y	Y	Z+2	Z	+/+
Evolved Basmati Varieties												
Super Basmati	Pakistan	W	X	X	Z	X	Y	W	Y	Z	Z	+/+
Basmati 385	Pakistan	Y	Y	Z	Y	X	X	W	Y	Z	Z	+/+
Basmati 198	Pakistan	Y	X	Z	Y	X	X	W	Y	Z	Z	+/+
D-98*	Pakistan	Y	X+2	Z	Y	X	X	W−2	Y	Y−2	Z	+/+
Pusa Basmati-1 (IET 10364)	India	W	Y	Y	Z	X	Y	W	X	Y	Y	+/+
Kasturi (IET 8580)	India	W	W	Y	Z	X	Y	V	Y	Z	Y	+/+

Punjab Basmati (IET 8580)	India	Y+2/Z+5	X+1	Z+3/Z	Y	X	X	Y	Y	X−4/Z	Z	+/+
Haryana Basmati IET 10367)	India	Z	W	Y	Z	X	X	Y	X	Y	Y/Z	+/+
Mahi Sugandha (IET 12601)	India	Y	W	Y	Z	Z	X	X	X	Z	Z	+/+
Basmati 2000	Pakistan	W	X	X	Z	X	Y	W	Y	Z	Z	y
Basmati 515	Pakistan	W	X	X	Z	X	Y	W	Y	Z	Z	+/+
Pusa 1121 (IET, 18004)	India	W	Y	X	Y	X	Y	W	X	Y	Y	+/+
Pusa 1509 (IET 21960)	India	W	Y	X	Z	X	Y	W	Y+2	Y	Y	+/+
Indian and Pakistani Nonbasmati Varieties Frequently Observed as Adulterants												
Superfine	Pakistan	Z	W	Y	Z	Z	Y−4	V	Y	Y	Y	+/+
Sugandha	India	Z	W	Y	Z	X	X+2/Y	V	Y+2	Y	Y	+/+
Sherbati	India	W	W	Y	W	Z	Y	Z	X	X	Y	−/−
Parmal	India	Z	W	Y	Z	Z	X	Y	X	Y	Y	−/−
Pak 386	Pakistan	Y	W	Y	Z	Z	Y	X	Y	Y	Y	−/−
IRRI-9	IRRI	Y+2	W	Y	Z	Z/W	X	Y	X+2	Y−4	Y	−/−
KS-282	Pakistan	W/Y+2/Z	W	Y	Z	Z	Y	Y	X	Y−4	Y	−/−
Supri	Pakistan	Z+2	W	Y	W	W	Y+4	Z	X	Y−2	Y	−/−
Other Rice Varieties of Importance												
Hom Mali (KMDL 105/ RD15)	Thailand	Z+5	W+2	Z+2	Z	W	Y	V	X	Y−2	Y	+/+
Phka Romeat, Rumdeng, Rumduol	Cambodia	Z+5	W+2	Z+2	Z	W	Y	V	X	Y−2	Y	+/+

Continued

Table 2.2 DNA Fingerprints of Various Rice Varieties Based on the Analysis of 10 Standard Simple Sequence Repeat (SSR) Markers—cont'd

Variety	Origin	RM1	RM44	RM55	RM171	RM201	RM202	RM223	RM229	RM241	RM348	*fgr*
Pathumtani 1	Thailand	Z+5	W	Y	Z+4	W	Y+4	V	X	Y	Y	+/+
Sen Kra Oub	Cambodia	W+6	W+2	Y	Z	W	X	W−12	Y+2	Y	Y	+/+
Vietnamese Jasmine rice	Vietnam	Y	W+2	Y	Z	W	Y	V	X	Y	Y	+/+
Paw San	Myanmar	W+10	Y−2	Z	X−3	W	Y+2	W−2	Y+2	Y	Y	−/−
Arborio Risotto rice	Italy	W+8	X	Z+2	X−3	X	Y−8	X	Z−2	Y−8	Z	−/−
Carnaroli Risotto rice	Italy	W+8	X	Z+2	X−3	X+2	Y−8	X	Z−2	Y−8	Z	−/−
Baldo Risotto rice	Italy	Y−8	Y	Z	X−3	X	Y−8	Z−4	Z−2	Y+6	Z	−/−
Vialone Nero Risotto rice	Italy	W+10	X	Z	X−3	X	Y−8	X	Z−2	Y−6	Z	−/−
Bomba Paella rice	Spain	W+12	X	Z+4	Z	X	Y−8	Y	Y+4	Y−2	Z	−/−

Note: DNA fingerprints were established by the authors based on reference materials obtained from authorities and traders. Each letter V to Z represents a DNA fragment of a distinct length (FSA, 2004). For several varieties more than one allele is observed for some markers and these are listed separated by a slash.

* According to a statement of the Pakistani Trade Development Authority, the variety D98 is the same as PK-198, which is an approved basmati variety. However, DNA fingerprints between the two varieties differ significantly.

The method determines the relative amount of DNA of each variety in relation to the total rice DNA in the mixture. Because DNA contents of rice kernels might vary depending on the processing stage and the variety, the results do not necessarily reflect the weight proportions of each type of rice. Brown rice with bran and germ contains more DNA than white milled rice, which primarily consists of the starch body. Furthermore, the DNA content might vary among rice varieties. In the validation study for the ISO 17025 accreditation process, which was mentioned before, DNA contents were determined for different rice samples (Brendel and Schubbert, unpublished results) and ranged between 17 ± 2 for white milled jasmine rice over 140 ± 67 for white milled to 296 ± 82 µg/g for brown basmati rice. Results are average values of six independent DNA extractions. However, the relative DNA amounts of rice varieties reflect quite well the weight proportions, because in practice mainly mixtures have to be analyzed, which consist of rice of one common processing stage (paddy, brown, milled, or parboiled rice) and of varieties that are related to each other and do not significantly differ in their DNA contents.

This was shown during routine preshipment inspections of basmati rice in Pakistan by analyzing basmati authenticity with the DNA fingerprinting method in comparison with the visual testing performed traditionally in this country. The latter method is based on morphological differences between kernels of different varieties, which are manually selected from the sample (Nader et al., 2014). Separated grains are then weighed to determine the relative content of each variety. Samples of 59 individual lots were analyzed with both methods in parallel. Contents of the variety Super Basmati determined by visual testing were in a range between 93.3% and 99% (w/w). The average difference of these results to the contents determined by DNA fingerprinting (DNA/DNA) was $1.39 \pm 1.18\%$ with a maximum deviation of 5.7%. These results indicate that both the relative DNA content and the relative weight do not differ significantly.

As an alternative to capillary electrophoresis, amplified SSR alleles can be separated by agarose or slab gel (polyacrylamide) electrophoresis. However, a study by Vemireddy et al. (2007) on authenticity testing of basmati rice revealed that capillary electrophoresis is essential for this kind of application due to its higher reproducibility and lower analytical uncertainty with regard to the quantification of SSR alleles in a mixture of various rice varieties.

InDel markers are longer than the relatively short SSRs and were introduced for basmati authenticity testing by Steele et al. (2008) as an alternative to microsatellite markers. Due to their length, InDels can be analyzed on agarose gels making this technology more cost effective and suitable for smaller laboratories without special equipment like DNA sequencers.

Another technology possibly suited for laboratories without special equipment such as DNA sequencers is a lab-on-a-chip method developed by Agilent Technologies, Inc., Santa Clara, CA, USA (Garrett and Clarke, 2007). PCR amplified SSRs or InDels are separated by capillary gel electrophoresis on a chip in the presence of intercalating fluorescent dye ethidium bromide.

2.3.3 Third-Generation Markers: SNPs

Mini- and microsatellite typing uses the analysis of length differences of the repeats by electrophoresis. In contrast, genotyping at SNP loci is based on the selective detection of the differences in one base pair and distinguishes the single base mismatch from the perfect match. New techniques have been developed for SNP analysis like oligonucleotide arrays on DNA chips and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) detection systems (for a review, see Gupta et al., 2001). For genotyping of individual humans, animals, and crop varieties only plus/minus assays are required and these can be automated. Many thousand SNP loci can be analyzed simultaneously on a DNA chip, making SNP typing a powerful tool for genome mapping and detecting even minor differences between individuals and populations.

For food, quantitative analytical systems are required since most often foodstuff contains materials from different individual animals or plant varieties. Quantitative SNP analysis is feasible with the MassARRAY iPLEX Platform, a MALDI-TOF MS technology (Gabriel et al., 2009). Real-time-PCR can be used for quantitative SNP analysis as well, eg, with two allele-specific TaqMan probes containing distinct fluorescent dyes. The KASP genotyping technology (Kompetitive Allele Specific PCR) is routinely used in plant breeding and is a cost-efficient alternative to chip-based SNP-typing systems in applications that require small to moderate numbers of markers (Semagn et al., 2013). The method is also suitable for quantitative SNP analysis by using two allele-specific primers conjugated with different fluorescent dyes, HEX (hexachloro-6-carboxy-fluorescein) and FAM (6-carboxy-fluorescein). Fluorescence emission intensities of each dye are measured by an end point fluorescent reading and are directly correlated to the number of templates of each SNP allele in the sample. The ratio of the fluorescence intensities of both d**yes** therefore corresponds to the ratio of both SNP alleles.

2.4 DNA Fingerprinting for Authenticity Testing of Rice Varieties

Rice is not only the most important staple food globally but it is also characterized by an extraordinary varietal diversity. The International Rice Information System (IRIS; www.iris.irri.org) recognizes around 5000 released varieties and the gene bank collection of the International Rice Research Institute, IRRI, holds more than 117,000 types of rice (Nader et al., 2014). Garris et al. (2005) analyzed 234 rice accessions representing the geographic range of *O. sativa* by SSR genotyping and sequence analysis of chloroplast DNA and classified the five subspecies *indica*, *aus*, *aromatic*, *temperate japonica*, and *tropical japonica*.

Rice varieties differ in many characteristics such as adaptation to different climates and soils, tolerance to salt and drought, resistance to pests, and shape. The chemical composition, morphology, and structure of the starch body are quite variable as

well, affecting the cooking characteristics, taste, and texture of rice. A large variety of traditional rice dishes are found worldwide, for example, risotto, paella, sushi, and biryani, and their diversity is mainly due to these aforementioned differences. Sushi, rice cakes, rice crackers, and rice paper for spring rolls are made from sticky *japonica* rice varieties. Biryani requires nonsticky, fluffy cooking rice varieties. Paella rice varieties are characterized by their high-absorption capacity for the associated broth, and cooked risotto rice by its creamy consistency. Special rice varieties best suited to prepare these dishes have been evolved by breeding and are traded as prime quality and at a higher price than varieties that mainly serve as staple food. Examples are the aromatic jasmine and basmati rice varieties, Arborio and Carnaroli risotto rice, Bomba paella rice, and Koshihikari sushi rice.

2.4.1 Case Study: Basmati Rice

Adulteration of high-value varieties with cheaper rice was first reported in literature for basmati rice. Basmati (the Hindi word for aroma) rice is indigenous to the Indian subcontinent and is characterized by its aroma and special cooking characteristics. The major aroma compound is 2-acetyl-1-pyrroline, which gives the rice its distinct popcorn flavor. Many other aromatic compounds have been found that contribute to the special flavor of basmati and that supposedly cause the differences in smell and taste observed between basmati and jasmine rice (Bryant and McClung, 2009). Basmati is furthermore characterized by its lengthwise excessive elongation during cooking and the soft and fluffy texture of the cooked rice.

Purity and quality of basmati rice has been traditionally tested in the countries of origin by chemical and visual methods. Experienced experts can visually distinguish basmati and nonbasmati rice varieties due to morphological differences of the milled kernels (Nader et al., 2014; see previous Section 2.3.2). Amylose content, alkaline spread, and gel length are chemical properties that characterize the typical cooking behavior of basmati and that can be tested by rather simple methods (Masoumias et al., 2013). Over recent decades, basmati has developed from a primarily ethnic food to one of the most important types of rice on the EU market. Due to its lower yields in the field, basmati rice is more expensive than other rice varieties that are mainly used as staple food. Depending on the harvests in Pakistan and India, prices can be twice as high as for normal long-grain indica rice. Consequently, basmati rice is frequently adulterated with cheaper nonbasmati varieties.

Based on the DNA fingerprinting method of Bligh (2000) which was described earlier, the Food Standards Agency (2004) examined 363 samples sold as basmati rice across the United Kingdom for authenticity and reported that 17% of samples contained more than 20% of nonbasmati and 9% were adulterated at levels in excess of 60%. As a consequence of these adulterations, the British Retail Consortium, Rice Association and the British Rice Millers Association released a Code of Practice on basmati rice in 2005, which became a legal standard in the United Kingdom and was also adopted as a general trade standard by the Grain and Feed Trade Association and the private sector in other EU member states.

The Code of Practice was developed in consultation with Indian and Pakistani rice exporters (All India Rice Exporters Association, AIREA; and the Rice Exporters Association of Pakistan, REAP) and defines 15 rice varieties as basmati. In the United Kingdom the code has been enforced as a legal standard by food control authorities and has since then led to a steady improvement of basmati authenticity (Nader et al., 2013).

Recently the French rice association, the Syndicat de la Rizerie Français (2015), published its own code of practice, which includes four new basmati varieties in addition to the 15 listed in the UK Code of Practice.

Basmati rice has been furthermore tested at the retail level by consumer organizations in France, Germany, and Switzerland (Union Fédérale des Consommateurs Que Choisir, 2009; Stiftung Warentest, 2010; Saldo, 2010) based on SSR analysis. The test by the German Stiftung Warentest revealed that 5 out of 31 samples did not comply with the UK Code of Practice and two of these did not even contain basmati rice but rather 100% of a nonaromatic rice without the fragrance gene *frg* (see later discussion).

Article 6 of the UK Code of Practice defines the DNA fingerprinting method published by the Food Standards Agency in 2004 as the standard procedure for authenticity testing. This method is based on the 10 standard SSR markers described in detail under Section 2.2 of this chapter. Table 2.2 reveals the allelic patterns of various rice varieties for these markers and the presence or absence of the fragrance gene *fgr*, a specific 8 base pair deletion in the gene *BADH2*, which codes for the enzyme betaine aldehyde dehydrogenase and supposedly causes the typical aroma of basmati and jasmine rice because of a lack of this enzyme (Bradbury et al., 2005). A quantitative screening test for basmati has been developed on the basis of this InDel marker, and the absence of the deletion indicates the presence of nonbasmati varieties (Bucher et al., 2014). However, as can be seen from Table 2.2, screening of the fragrant gene is not sufficient to test for basmati authenticity because numerous other rice varieties are also aromatic and homozygous for the *fgr* gene. These are not considered as basmati due to their different origin and cooking characteristics. Examples are the aromatic nonbasmati varieties Superfine from Pakistan and Sugandha from India, which would pass the screening as genuine basmati.

Of the 15 rice varieties listed as genuine basmati rice by the UK Code of Practice, seven are considered traditional varieties (Table 2.2). Taraori (HBC-19), Kernel (Basmati Pakistan) and Basmati 386 on one hand and Basmati 370 and Type 3 (Dehradun) on the other cannot be distinguished by genotyping with the 10 standard SSR markers because they are genetically closely related. They were collected from the same landraces by different institutions but were notified under different names. The Rice Research Station in Kala Shah Kaku in Pakistan notified Kernel Basmati and Basmati 370, the Haryana Agriculture University Taraori (HBC-19), the Punjab Agriculture University Basmati 217 and 386, and the Nagina Rice Research Station in Uttar Pradesh Type 3 (All Indian Rice Exporters Association, 2016).

The variety Ranbir was selected from the cultivar Basmati 370 and reported by the Sher-e-Kashmir University in Jammu (Siddiq et al., 2012). The DNA fingerprint obtained from a Ranbir reference material obtained by the Food Standards Agency

(2004) is different from Basmati 370 (Table 2.2) but all samples obtained so far as Ranbir by Eurofins revealed the same allelic pattern as Basmati 370 and Type 3. This indicates that the source of such reference materials is not always reliable even if these are official sources. The genotype of Ranbir as published by the FSA (2004) should be revised accordingly.

Due to the low yield of landrace basmati varieties, only Taraori, Type 3, and Ranbir are cultivated at significant quantities in India, whereas in Pakistan traditional varieties are no longer in use. By crossing basmati landraces with high-yield indica long-grain rice varieties, new varieties were developed of which the varieties Pusa 1121, Pusa 1509, Pusa Basmati-1, Super Basmati, and Basmati 515 play the most significant role in agriculture in India and Pakistan today. The most important basmati varieties cultivated in India are Pusa 1121 and 1509, which are exported on a large scale to the Middle East, Iran, and the United States. Pusa 1121 is also grown in Pakistan under the name Kainat 1121. Pusa 1121 and 1509 were reported in 2008 and 2013 as basmati and are characterized by their extraordinary kernel length and elongation during cooking. Because these varieties were reported after 2005, they are not listed as genuine basmati in the UK Code of Practice but are in the new French Code of Practice. Efforts to recognize Pusa 1121 in the UK as basmati failed due to the argument that an evolved variety only qualifies as basmati if at least one of the parents is a traditional basmati. Pusa 1121 is a sister line of Pusa Basmati-1, and evolved basmati varieties are the parents (Siddiq et al., 2012).

The French Code of Practice also includes the new Pakistani varieties Basmati 2000 and 515, which were evolved from Super Basmati and which cannot be distinguished from their ancestor by DNA fingerprinting using the 10 standard SSR markers (Table 2.2). The exact breeding history of these lines remains unclear. Based on ISSR- and RAPD-PCR analysis, no significant differences could be found between these lines and Super Basmati, whereas the genetic distance to Indian Taraori and Pusa Basmati-1 is quite obvious (Fig. 2.1). DNA fingerprinting with an extended panel of SSR markers revealed differences between Basmati 515, Basmati 2000, and Super Basmati but also a pronounced genetic diversity in the variety Super Basmati, which is probably due to the degeneration of the seed materials used in Pakistani agriculture and which complicates its differentiation from Basmati 515 and 2000. DNA fingerprinting of single kernels in samples of Super Basmati frequently reveals a diversity of alleles for the markers RM118, RM152, RM212, RM224, and RM252 (Gramene, 2015) with six different alleles observed for the marker RM252 (Nader et al., unpublished results). In contrast only one unique DNA fingerprint with one allele per SSR is observed with the 10 standard markers, defined by the FSA (2004; Table 2.2). This is probably due to differences in mutation rates of microsatellites (see previous mention under Section 2.2.1), which might be lower for the 10 standard markers than for the markers tested additionally.

DNA fingerprinting is furthermore applied to control basmati imports for compliance with EU customs legislation. According to Commission Implementing Regulation (EU) No 706/2014, husked brown cargo rice of nine basmati varieties qualifies for a zero rate of import duty. These include all landraces and the evolved varieties Pusa Basmati-1 and Super Basmati (Table 2.2). For tax exemption, only

one variety is allowed per consignment and admixtures of contrasting varieties (basmati and nonbasmati) are tolerated up to 5% (w/w) (Commission Regulation (EU) No 272/2010).

In contrast to EU customs legislation, both the UK and French Code of Practice tolerate mixtures of various basmati rice varieties and nonbasmati admixtures up to 7% (w/w). Analytical methods to control compliance with these codes therefore have to be able to identify and quantify various rice varieties even in complex mixtures; this can be achieved with the procedure described in detail in Section 2.2.

Currently 23 varieties are officially approved in India as basmati (All Indian Rice Exporters Association, 2016) and 9 in Pakistan. Of these 14 have been notified after the publication of the UK Code of Practice on Basmati Rice in 2005. Thus a revision of the current basmati definition in the EU and the adaptation of the DNA fingerprinting method to the rice breeding practice in the source countries seems necessary. Several of the new varieties cannot be distinguished from the older varieties based on DNA fingerprinting with the 10 standard SSR markers (see above). Examples are the varieties CSR 30 (Yamini) and Pusa Basmati-6 (Pusa 1401), which resemble Taraori Basmati and Pusa Basmati-1.

2.4.2 Case Study: Jasmine Rice

Theoretically, the DNA fingerprinting method can be applied to identify and quantify all rice varieties as long as reference materials are available to establish the corresponding genotype. Jasmine rice comprises aromatic varieties that are cultivated in Thailand, Cambodia, Laos, and South Vietnam and are imported into the EU in large quantities. In Thailand, jasmine rice is traded under the name Hom Mali (Thai words for fragrant jasmine) and comprises the two varieties Khao Dawk Mali 105 (KDML 105) and Kor Kho 15 (RD15), which are genetically identical with regard to the 10 standard SSR markers. Varieties Phka Rumduol, Phka Rumdeng, and Phka Romeat are traded in Cambodia as Phka Malis, which means "fragrant flower." They are genetically identical to Hom Mali with regard to the 10 standard SSR markers and the additional markers RM72, RM212, RM252, RM348, RM440, and RM525, but differences are reported for the markers RM152 in the case of Phka Rumdeng and RM263 for Phka Rumduol (CARDI, 2013). Varieties of Hom Mali and Phka Malis are photosensitive, can only be grown during the rainy season and harvested once per year. They are genetically closely related to each other and were probably derived from one single landrace.

The photo-insensitive varieties Pathumthani 1 and Sen Kra Oub have probably evolved from these landrace varieties by breeding and can be cultivated and harvested throughout the entire year. They can be readily distinguished from each other and from Hom Mali and Phka Malis by DNA fingerprinting based on the 10 standard SSR markers (Table 2.2).

In the EU, the term "jasmine rice" (Riz Jasmine) is defined in the French Code of Practice as fragrant rice from Thailand or Cambodia. "Riz Jasmine Thaï" must contain a minimum of 92% (w/w) Hom Mali or 80% (w/w) Pathumthani. In contrast only Hom Mali and Phka Malis varieties are called "jasmine rice" in Thailand and Cambodia.

2.4.3 Further Candidates for Rice DNA Authenticity Testing

DNA fingerprinting based on the 10 standard markers can be also applied for authenticity testing of risotto rice; distinct allele patterns were derived for the varieties Arborio, Carnaroli, Baldo, and Vialone Nano (Table 2.2).

Bomba paella rice is characterized by its high absorption capacity for the cooking broth and is grown traditionally in Southern Spain. Wankhade et al. reported in 2010 that Bomba cultivars from four different regions in Spain were highly polymorphic with regard to six out of 10 SSR markers. A different set of markers was used by these authors than in the study reported in Table 2.1 with only RM171 in common. The genotype for Bomba in Table 2.2 therefore represents only one cultivar and further studies are necessary to characterize this genetically diverse variety in more detail for authenticity testing. The genetic diversity of Bomba is supposedly caused by the high level of heterozygosity of this variety.

DNA fingerprinting is also the method of choice to give new or so far unknown rice varieties with specific characteristics an identity and to market these as specialties at a higher price. As an example, the aromatic variety Paw San from Myanmar is widely unknown outside this country. It significantly differs from basmati and jasmine rice with some unique features in particular related to cooking characteristics, which might create a new market and export opportunities for Myanmar. A reference material of this rice was obtained from the trade and its DNA fingerprint is listed in Table 2.2. In contrast to jasmine and basmati rice, Paw San does not contain the fragrance gene *frg*, which is characterized by an eight−base pair deletion in the *BADH2* gene (see previous mention). Instead, Myint et al. (2012) detected a three−base pair insertion in this gene in eight of 18 different accessions of Paw San, obtained from different sources and regions in Myanmar. Similar to the eight−base pair deletion of *frg*, this insertion might cause the inactivation of the betaine aldehyde dehydrogenase and therefore the typical aroma of the rice.

2.5 Meat Traceability

In the light of the bovine spongiform encephalopathy (BSE) crisis, the EU imposed a compulsory traceability and labeling system for fresh beef and veal (Regulation (EC) No. 1760/2000). The system imposed full traceability of the meat back to individual animals. Traceability can be verified by fingerprinting (Vázquez et al., 2004) of DNA extracted from meat and from samples obtained from individual animals prior to slaughtering. Practical systems, integrated into the ear tags every animal has to wear according to Regulation (EC) No. 1760/2000, have been developed to sample and conserve DNA from the animals.

In addition, regulation (EU) No. 1169/2011 imposes stricter labeling rules for unprocessed meats from swine, sheep, goat, and poultry while Commission Implementing Regulation (EU) No 1337/2013 requires food business operators to establish a traceability system that provides the link between the meat and the animal or group of animals from which it has been obtained. DNA fingerprinting would actually be the

method of choice for the verification of these legally imposed traceability systems. However, experience after the implementation of beef traceability in 2000 has shown that the industry is quite reluctant to establish such voluntary verification systems.

Notwithstanding this, some companies are setting up universal DNA-based traceability systems to support certain claims made on their products and to distinguish themselves from the competition. Claims can be on a certain geographic origin or a distinct quality due to a special breed. For swine, such a system is under development at the laboratory of the authors (Brendel and Schubbert, unpublished results) to verify that pork labeled as a Berkshire or Gloucester Old Spot sired breed contains only meat that is sired by particular Berkshire or Gloucester boars used on distinct farms.

In principle, such traceability systems can be based on two concepts. A direct link between animals and meat products can be made by matching the DNA fingerprints. For this approach, DNA of all animals in the herd would require analysis and fingerprints would be kept in a data base, but this is costly. Alternatively, DNA fingerprints may only be established for the breeding animals and meat samples can then be traced back to specific breeds by paternity testing.

The latter "proof of descent" concept is cost efficient and was tested by Eurofins in a feasibility study. DNA was extracted from bristles of breeding boars (Berkshire and Gloucester Old Spot) and fingerprints were established based on 11 tetrameric (repeat units of four base pairs) SSR markers with a commercially available kit (Animaltype Pig, Biotype Diagnostic GmbH, Dresden, Germany; Caratti et al., 2010). In a blind study, meats of different origins were then analyzed with the same marker panel, and DNA profiles were compared with the fingerprints of the breeding animals. Using the panel of tetrameric SSR markers, it was possible to unambiguously trace specific meat samples back to a sire.

2.6 Future Trends

Until now the most prominent example of the application of DNA fingerprinting in food authenticity testing is that of basmati rice due to a strong industry and retail interest to ensure the reputation of this specialty as a premium product. The method became the main pillar for the Code of Practice on basmati rice, which has developed to a trade standard far beyond the United Kingdom.

Similar success stories can therefore be expected for other premium foods, when industry and trade have a strong commercial interests to safeguard the reputation of such products. Such interests might concern other rice varieties like premium sushi, jasmine, risotto, or paella rice, but also special cocoas or coffees that are sold at premium prices. Arriba cocoa is a highly aromatic variety from Ecuador, and there are strong indications of adulterations with bulk cocoa. Methods to distinguish the premium Arriba varieties have been recently developed and are based on sequence differences in the chloroplast DNA (Hermann et al., 2014) or DNA fingerprinting with SSR markers (Hermann et al., 2015).

The legislators in the EU have obliged business operators handling meat to impose traceability and to provide a direct link between their product and the individual animal or animal herd; this was first done for cattle in 2000 and since 2013 also for goats, sheep, swine, and poultry. Under current legislation, this link only has to be provided by documentation and does not require a parallel analytical system that allows its verification. So far there are not many examples of industry and the retail sectors establishing such verification systems voluntarily. But consumer trust was severely affected by the horse meat scandal, and this might encourage business operators to establish such verification systems to protect their brands. DNA fingerprinting is the most elegant and straightforward approach to provide the direct link between the final product and the individual animal or animal herd.

Costs involved in the establishment of such systems are often overestimated. Not every animal has to be tested, and it is sufficient to keep backup samples of tissues, like hair, of each slaughtered animal and to control traceability by spot-checking meat. Alternatively, databases containing DNA fingerprints of the sires can be established and meat checked randomly by paternity testing, as discussed under Section 2.5.

The horse meat scandal concerned processed food for which exact labeling concerning the origin of the animals is not required by EU legislation and which may contain meat of different animals. As was shown for DNA fingerprinting of basmati rice, the quantitative analysis of several varieties is feasible in a mixture. Also, in processed meat, the differentiation and quantification of different animals should be possible in order to verify traceability claims made by business operators and to confirm the origin of these meats in a more efficient manner.

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