Chapter

Tracing the Domestic Pig Using the Omics Technologies

Ivona Djurkin Kušec and Kristina Gvozdanović

Abstract

Pork represents one of the most important sources of protein in the human diet. Consumers today expect their food to be safe and of expected quality. Therefore, traceability and originality of the product must be guaranteed. This chapter provides an overview of the different approaches used for traceability and authentication of pork and pork products. Different DNA-based methods for meat speciation and authentication are described and their potential for use in the pork industry is highlighted.

Keywords: traceability, authentication, genomics, pork, product

1. Introduction

Pork is today one of the most important sources of protein in the human diet. Modern consumers expect their food to be not only safe but also of the expected quality, especially if the product is labelled with one of the quality trademarks, such as PDO (Protected Designation of Origin), PGI (Protected Geographical Indication) or TSG (Traditional Specialty Guarantee). The originality of the product, but also its traceability, which ensures the claimed quality, must therefore be guaranteed at all stages of production, from the raw material to the final product. In this context, it is necessary to distinguish between authenticity and traceability. The term authenticity refers to the genuineness and integrity of the food product. It is considered the key to accuracy, helping consumers to choose a particular product based on its claims [1]. On the other hand, traceability is defined as the ability to trace a food, feed, food-producing animal or ingredients through all stages of production and distribution [2], which can protect consumers from fraud and producers from unfair competition. In meat adulteration, the meat of a declared animal species is replaced (in whole or in part) by cheaper meat of lower quality, vegetable proteins or undeclared ingredients such as components of animal origin (e.g. blood plasma). In addition, non-meat ingredients such as water and additives are added, the geographical origin of the meat and/or the feeding of the animals are falsely declared, and undeclared processing methods are used in meat products [3]. All of this constitutes not only consumer fraud but can also have a negative impact on the safety of the final product. Furthermore, they restrict the freedom of choice of consumers, especially those who opt for a strict diet for religious reasons, as the consumption of certain types of animals is not allowed in some religions.

There are several methods available for the authentication and traceability of pork products, but the most accurate results are obtained using omics technologies, and more specifically DNA-based technologies. Although proteomics and metabolomics are also considered as "omics" technologies, these two approaches are not in broader use because of their shortcomings, such as the low number of species that can be determined simultaneously, the low sensitivity of some methods and the high cost of sample examinations, the instability of biomolecules of interest and the lack of species-specific biomarkers that could enable quantitative evaluation in order to detect mixed samples. The reasons why DNA-based technologies are preferred over other, is the stability of DNA, the ability to simultaneously detect more than one species or an unknown species in a product, the simplicity of the methods and their cost-effectiveness. On the other hand, one should bear in mind that DNA can degrade in highly processed or thermally treated products. In these cases, another method of analysis should be considered.

Traceability in terms of detection of the species within a product is usually achieved by analysis of the mitochondrial genome (mtDNA) due to its highly polymorphic nature, and the fact that it is almost exclusively maternally inherited and without genetic recombination [4]. Within the mtDNA cytochrome b (cytb) gene is almost always targeted for meat speciation purposes.

The cytb gene is one of 37 genes within the circular mitochondrial genome [5] and is ideal for species identification because it has limited variability interspecies within and much greater variation between species [6]. Other target genes and DNA fragments used as markers for species identification include cytochrome oxidase subunit I (COI), the mitochondrial D-loop region, 16 s rRNA and 12 s rRNA.

The aim of this chapter was to give an up-to-date overview of the available omics technologies for pig traceability and pork product authentication, to point out their advantages and possible disadvantages, and to present their cost-effectiveness taking into account the robustness of the method, simplicity, human effort and price.

2. DNA methods for meat speciation

2.1 Species-specific polymerase chain reaction

Most DNA-based methods used for speciation of meat are based on the polymerase chain reaction (PCR), which can be used alone for this purpose or supplemented by further analyses such as sequencing or RFLP (restriction fragment length polymorphism). The specificity of PCR assays depends on the target sequences, which depend on the choice of appropriate primers for the detection of the species of interest. In this way, efficient detection of the target species can be performed with high sensitivity (even as low as 10 picograms; 0.1%; [7]) and in different meat mixtures (0.01% pork/meat: w/w; [8]) with relatively low cost and labour. The disadvantage of the method is that the meat species of interest have to be targeted, so there is no possibility to detect additional species that might be present in the meat product (**Table 1**).

2.2 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP presents a method of detecting species within a mixture by digesting the resulting fragments and then separating them according to size by gel

electrophoresis. It should be noted that in the event that one needs to detect several species simultaneously in one reaction, more than one digestion enzyme usually needs to be used. Nevertheless, the method itself is qualitative, very sensitive, quite cost-effective and has been successfully applied to various mixed products, which is why it is still used as the preferred method in various laboratories (**Table 2**) [22].

Species	Product	Gene	Reference
Pig	Sausages	12S rRNA	[9]
Pig, sheep, cattle, buffalo, goat	Meat mixtures	Mitochondrial cytochrome b	[10]
Pig, cattle, goat, horse, donkey, chicken	Raw meat	Mitochondrial cytochrome b, 12S rRNA, 16S rRNA	[11]
Pig, cattle, duck, chicken, turkey, goose	Sausages	Cytochrome Oxidase Subunit I (COI)	[12]
Pig, chicken, beef, sheep	Sausages	Mitochondrial cytochrome b	[13]
Pig, cattle, chicken, bata fish, sheep	Raw meat	Mitochondrial 12S rRNA	[14]
Pig, camel, pigeon, chicken, duck, horse, beef	Processed meat products (sausages, meatballs, kebab)	Mitochondrial cytochromes b	[15]

Table 1.
Overview of studies using species-specific PCR for the detection of pork in different products.

Species	Product	Gene	Reference
Pig, beef, chicken	Processed meat products (meatballs, streaky bacon, frankfurter/hot dog, and burger)	Mitochondrial Cytochrome B	[16]
Pig, wild boar	Raw meat	Mitochondrial Cytochrome B	[17]
Pig	Meatballs	Mitochondrial Cytochrome B	[18]
Pig	Sausages	Mitochondrial D-loop, Cyt-b, and eukaryotic 18S rRNA	[8]
Pig, dog, cat, chicken, goat, dromedary, rat, rabbit, donkey, turkey	Meat mixtures	Mitochondrial cytochrome b	[19]
Pig, beef, buffalo, chicken, duck, goat, sheep	Raw meat	Mitochondrial cytochrome b (cytb) and *NADH* dehydrogenase subunit 5 (*ND5*)	[20]
Pork, beef, poultry	Meat mixtures	12S rRNA, 16S rRNA, Cyt-b	[21]

Table 2.
Overview of studies using PCR-RFLP for the detection of pork in different products.

2.3 Quantitative PCR (qPCR)

Quantitative (often called real-time PCR) is perhaps the most widely used method for species detection. Although quantitative in nature, it is also qualitative and therefore can be more widely interpreted than other methods that are used for detecting pork. The main principle of the method relies on exponential quantification of the starting amount of specific DNA by using different fluorescent reporters for monitoring the amplification of the DNA in real-time. Fluorescence is measured after each cycle and the intensity of the signal reflects the amount of DNA amplicons in the sample at a given time [23]. The point at which the fluorescence intensity rises above the detectable level is proportional to the initial number of template DNA molecules in the sample. This point is called the quantification cycle (Cq) and allows the determination of the absolute amount of target DNA in the sample. This is achieved from a calibration curve generated from serially diluted standard samples with known concentrations or copy numbers [24]. Two strategies are available for visualising DNA amplicons: non-specific fluorescent DNA dyes (e.g. Sybre Green I and EvaGreen) and fluorescently labelled oligonucleotide probes (e.g. TaqMan Probes; [25, 26]). Although both approaches are used for the speciation of meat, probe-based chemistry predominates due to its higher specificity mediated by an additional oligonucleotide (i.e. probe) and also due to its ability to simultaneously detect more than one species in a single reaction (multiplex PCR). Multiplex PCR can be designed either as a single-template reaction, in which multiple primer sets are used to amplify specific regions, or as a multi-template reaction, in which multiple templates and multiple primer sets are used in a single reaction. It is less expensive but more complicated to design and can be less sensitive than PCR with one primer set. The PCR products obtained can be further analysed by massively parallel sequencing, gel/capillary electrophoresis or by real-time PCR (**Table 3**) [31].

2.4 Loop-mediated isothermal amplification (LAMP)

By loop-mediated isothermal amplification (LAMP) the DNA is amplified with high specificity, efficiency and speed under isothermal conditions. The method is performed using a DNA polymerase and a set of four designed primers that recognise four to six distinct target gene sequences. The following primer pairs are used in the

Species	Product	Gene	Reference
Pig, cow, chicken, goat	Processed meat (meatballs)	Mitochondrial cytochrome b	[27]
Pig, chicken, fish, cuttlefish, shrimp, beef	Commercially processed foods	Mitochondrial cytochrome b	[28]
Pig, horse, beef	Processed meat	Beta (β)-actin (*ACTB*) gene, growth hormone receptor gene (GHR)	[29]
Pig, chicken, beef, camel, rabbit, sheep, goat	Processed meat, sausages	Mitochondrial 12S rRNA	[30]
Pig, cow, sheep, goat, chicken	Processed meat (burger)	Mitochondrial cytochrome b	[16]

Table 3.
Overview of studies using quantitative PCR for the detection of pork in different products.

Species	Product	Gene	Reference
Pork, chicken, cattle	Raw meat	Mitochondrial 12S rRNA	[33]
Pig, cattle, sheep, chicken, duck	Meat mixtures	Mitochondrial cytochrome b	[34]
Pig, cattle, buffalo, sheep, goat, fox, rabbit, rat, dog, chicken, duck, fish	Heat-processed meat, meat mixtures	Mitochondrial DN1	[35]
Pig, lamb, chicken, beef	Raw meat	Mitochondrial D-loop, 18S rRNA	[36]
Pig, beef, buffalo, mutton, chevon	Raw meat, sausage, salami	Mitochondrial D loop	[37]
Pig, chicken, duck, sheep	Sausage	Beta-actin	[38]

Table 4.
Overview of studies using LAMP technology for the detection of pork in products.

method: the internal primers (forward internal and internal backward); the external primers (forward and backward); the optional loop primers (loop primer forward and loop reverse primer). The internal primers are 45–49 bp in length and complementary to two widely separated sites on the template; the external primers are 21–24 bp in length and are added to the reaction mixture at lower concentrations so that they bind to the template more slowly than the internal primers. The internal and external primers, both forward and backward, in combination with Bst DNA polymerase (from Bacillus stearothermophilus DNA Polymerase I), which has high strand displacement activity at 60–65°C, produce a dumbbell-shaped DNA structure [32]. In this way, up to 109 copies of the amplified DNA can be produced in less than one hour (**Table 4**).

2.5 Digital PCR

Digital PCR (ddPCR) improves the sensitivity of qPCR by allowing precise absolute quantification of nucleic acids. The method is based upon sample partitioning, i.e. the division of samples into discrete units before amplification. The sample is prepared in a similar way qPCR, but then divided into approximately 20,000 partitions (droplets), each of which (ideally) contains zero or one (or at most a few) template molecules. Each partition is treated as a single PCR reaction. After amplification, each partition can be analysed to determine whether it contains a template molecule. Samples containing an amplified product are treated as positive (1, fluorescent) and those without (or with very little) fluorescence as negative (0). The method is not based on the number of amplification cycles (as in qPCR), but on Poisson statistics by which the exact copy number of the template can be estimated. Compared to qPCR, ddPCR is more accurate, but is also less sensitive to factors that can affect PCR reaction (such as the quality of the extracted DNA due to thermal processing of the meat, primer-dimers etc.). Furthermore, ddPCR is easier to perform compared to real-time PCR, although the costs for consumables and equipment are somewhat higher [39]. In meat speciation studies, the method proved to be very sensitive for different types of meat (e.g. beef, pork, horse, sheep, turkey) with a limit of quantification of 0.01% and a limit of detection of 0.001% [40]. Furthermore, the study of Basanisi et al. [41] showed that the analytical sensitivity, defined as the lowest DNA concentration from each species for which at least 95% of the replicates were positive, was 0.1 pg. μL^{-1} for

#ID	Sample type	Number of DNA samples per µL					
		Horse	Beef	Pork	Sheep	Chicken	Turkey
1	Turkey/chicken sausage	—	—	54	—	156.5	674
2	Pork sausage pesto	—	—	27.90	—	1.5	2.8
3	Chicken burger	—	—	—	—	17.40	235
4	Pork/beef burger	—	437	5.3	—	—	—
5	Minced beef/pork meat	—	970	36.9	—	—	4.5
6	Minced pork meat	—	3.3	979.5	—	—	—
7	Pork sausage	—	12.5	941	—	—	2.6
8	Beef burger	—	4575	—	—	—	—
9	Beef meatball	—	5155	—	—	—	—
10	Sheep meat	—	—	—	7320	—	—
11	Horse fillet	2820	—	—	—	—	—
12	Minced horse meat	584	18.3	—	—	—	—
13	Pork sausage	—	—	4765	—	—	—
14	Turkey burger	—	—	5.5	—	5	1681
15	Beef burger	—	1393.5	—	—	—	—
16	Pork burger	—	—	918	—	1.6	—
17	Turkey/chicken/pork burger	—	—	572	—	685	2627
18	Pork sausage	—	—	742	—	18.1	—
19	Horse/pork sausage	467.5	49.5	227.5	—	1.2	1
20	Horse sausage	1702	—	849.5	—	—	—

Table 5.
Species identification in commercial products after digestion by ddPCR [41].

pig and chicken and 0.01 pg. μL^{-1} for bovine, horse, sheep and turkey. A typical result of ddPCR in different products is shown in **Table 5**.

Table 6 gives an overview of studies using ddPCR for the speciation of meat (with emphasis on pork) in different products.

2.6 DNA-barcoding

DNA barcoding is a fairly new molecular and computational system that combines DNA sequencing with bioinformatics analysis to accurately identify all species of animals, plants, fungi and bacteria. The method was developed in 2003 by Herbert et al. [46] who proposed the usage of the mitochondrial gene of cytochrome c oxidase subunit 1 (cox1 or COI) for the creation of a "DNA barcoding system". The method is based on sequencing the full-length DNA barcode with an approximate length of 650 base pairs (bp) in the mitochondrial gene for cytochrome c subunit I and then comparing it with the reference sequence organised in the barcode database (The

Species	Product	Gene	Reference
Pig, beef, horsemeat	Meat mixtures	Mitochondrial cytochrome b	[40]
Pig, beef, horse, rabbit, donkey, sheep, goat, dog, chicken, duck, pigeon, goose, turkey	Raw meat	Replication protein A1 (*RPA1*)	[39]
Pig, chicken	Meat mixtures	Beta (β)-actin (*ACTB*)	[42]
Pig, beef, horse, sheep, chicken, turkey	Raw meat, sausages	Mitochondrial cytochrome b	[41]
Pig, beef, chicken, mutton	Meat mixtures	Beta (β)-actin (*ACTB*)	[43]
Pig, cattle, buffalo, chicken, sheep, duck, horse	Sausages	Mitochondrial cytochrome b	[44]
Pig, chicken, beef	Raw meat	Beta (β)-actin (*ACTB*)	[45]

Table 6.
Overview of studies using ddPCR technology for the detection of pork in different products.

Barcode of Life Data System (BOLD); http://www.barcodinglife.org). The method proved effective for detecting fish adulteration [47], but also for distinguishing different types of meat in a product [12], and was employed for detecting adulteration of deer meat with pork in canned food in China [48]. It should be however emphasised that the barcoding method works better in individual meat types than for mixtures. In addition, for moderately or highly processed foods, where DNA may be highly degraded, PCR amplification of full-length barcodes can be challenging [49]. In these cases, a mini-barcoding method that focuses on the analysis of short-length DNA markers is the preferred method [50]. Mini barcodes are 100–300 bp long DNA fragments in COI barcode region that can provide information for single species identification with a resolution of ≥90%. In addition to COI, other mini-barcodes such as cytochrome b or 16 s ribosomal RNA genes can be employed for phylogenetic analyses or for COI barcode complementary analyses. For example, the study by Xing et al. [50] showed that in highly processed meat products complete COI barcoding failed in 44% of cases, however, the authors successfully obtained the 16S rRNA mini-barcodes from nearly 90% of these cases.

2.7 Microsatellites (SSRs)

Microsatellites are molecular markers that represent short sequences of up to 6 nucleotide repeats. They are often referred to as Simple Sequence Repeats (SSRs), Variable Number Tandem Reapats (VNTR) or Short Tandem Repeats (STRs). The basic characteristics of microsatellites are a codominant mode of inheritance, a high representation in the genome and a high degree of polymorphism. One of their characteristics is the high mutation rate, which is up to 1000 times higher than for intron DNA [51, 52]. The method is based on a robust selection of fluorescently labelled markers that can be combined into multiplexes depending on size and annealing temperature. This is followed by a PCR reaction in a thermocycler and then sequencing of the products obtained. The sequencing results are interpreted with different programmes depending on the goal of the profiling. For example, in breed/product authentication studies, the number of microsatellite markers is optimised based on several parameters such as allele frequency and the number of private alleles in the population, polymorphism information content (PIC) and match probability value.

For meat speciation studies these steps are not necessary, only the match probability is calculated. The match probability method is based on the probability of finding two individuals in the population who share the same genotype, taking into account the likelihood ratio (LR).

Due to their high polymorphism, the amount of information that we can obtain, and also the low cost of analysis compared to other available DNA methods, microsatellites are widely used as molecular markers in genetic diversity studies, parentage analysis and authentication of meat from different livestock species [53–57]. However, the method is not so commonly used for meat speciation. Nevertheless, there are successful implementations of meat speciation using STRs, such as a panel of 16 microsatellites used by Rębała et al. [58] to distinguish between wild and domestic pigs.

2.8 Single nucleotide polymorphisms (SNPs) and SNP chips

Single Nucleotide Polymorphism (SNP) is the most common variation that occurs in a DNA fragment. A nucleotide is considered polymorphic if it exists in at least two versions, with the frequency of the more common version being less than 99%. The SNPs discovered so far are stored in a publicly accessible database at http://www.ncbi.nlm.nih.gov/SNP; http://snp.cshl.org. Given the large number of SNPs discovered, it is important to distinguish the important from the unimportant SNPs, i.e. those that affect gene function and expression [59]. Negrini et al. [60] noted that the frequency of SNPs can be as high as every 500 base pairs and that they often occur outside the coding regions. Given their high frequency in the genome, their genetic stability and simplicity together with the cost-effectiveness of the method, makes SNPs preferred markers in many situations.

The number of SNPs used for traceability purposes varies: there may be one within a gene or many, as in SNP microarrays (SNP chips). For example, the study by Fontanesi et al. [61] identified a g.299084751C > T SNP in the NR6A1 gene that determines the number of vertebrae in pigs to distinguish the meat of wild boar from that of domestic pigs. This was later confirmed by Koseniuk et al. [62].

SNP chips (microarrays) are a high-throughput laboratory tool used for the detection of a large number of SNPs within a species/breed/individual. The technology is based on known nucleotide sequences which are then used as probes to hybridise with the DNA sequences tested. The method is very robust and nowadays quite inexpensive, and is therefore used in many genetic analyses. Its major drawback in meat speciation studies is that SNPs used for the creation of the microarray have to be known, and thus cannot detect an unknown species in the product. The other disadvantage is that they do not provide quantitative information. Currently, the method is used for authentication of breed/individuals rather than for specification of meat. However, there are examples of microarray-based technologies used for meat speciation, such as the Chipron Meat 5.0 LCD kit (Pacific Image Electronics Co., Ltd), which enables the simultaneous detection of 17 mammalian and seven avian species. The kit is based on LCD chip technology, where each LCD chip contains eight identical microarrays separated in small reaction chambers.

2.9 Next-generation sequencing (NGS)

In recent years, tremendous progress has been made towards using next-generation sequencing technology in many different areas of genetics, including meat speciation. Preparation steps have been simplified, commercial kits have been produced

and software for bioinformatics analyses has evolved, allowing for a simplified and friendlier environment for end users. Unlike other DNA technologies, there are no major restrictions on the amplification of targeted/non-targeted PCR fragments with NGS analysis, allowing great flexibility in performing meat speciation analyses. A major advantage of this technology over others is the ability to identify unknown species (if present in a product) and the ability to perform the analysis on mixed and highly processed products in which DNA can be heavily degraded. The targeted approach is performed with amplicons generated by a set of universal primers that target some of the mitochondrial DNA genes, such as 16 s rRNA, while in the untargeted approach, only a set of universal primers is used instead of taxa-specific primers. This process is also called metabarcoding [63]. The DNA metabarcoding method combines DNA barcoding with NGS thus offering an ideal approach for detecting multiple species present in the product, including the unexpected species [64]. It should be noted that the database for species identification must be comprehensive in order to allow accurate identification of the species present in the product (**Table** 7).

After the development of NGS sequencing, new methods for determining different species in mixed samples have emerged. These include already described metabarcoding, metagenomics [71, 72] and mitochondrial metagenomics [72]. Currently, two bioinformatics pipelines are available: the All-Food-Seq (AFS) method, proposed by Ripp et al. [71] based on non-targeted deep sequencing of whole genomic DNA from different mixtures followed by comprehensive bioinformatics analysis to accurately identify species from all kingdoms; and FASER (Food Authentication from SEquencing Reads)

Approach	Identified species	Meat type	Gene	Reference
Targeted	Pig, cattle, horse, wild yak, greylag goose, human, tuna	Mixed meat samples (laboratory), beef stuffing, mutton roll, roasted camel meat, smoked horsemeat, lamb kebab, sausage, beef sausage, dog food	16 s rRNA	[65]
Targeted	Pig, cattle, chicken, turkey, horse, donkey, sheep, goat, alpine ibex, sika deer, red deer, roedeer, reindeer, elk, hare, rabbit, chamois, Muscovy duck, goose, ostrich, pheasant	Meat samples, model sausages (cattle/pig/chicken/turkey/horse)	16 S rRNA	[66]
Targeted	Pig, cattle, sheep, chicken, duck	Mixed meat samples (laboratory)	GHR	[67]
Targeted	Pork, beef, mutton, chevon, chicken, turkey, ostrich, duck, kangaroo	Raw meat, sausages, minced meat, biltong	16 S rRNA	[68]
Untargeted	Pork, horse, chicken, sheep, turkey, beef	Raw meat; processed samples (powder, cooked, canned)	N/A	[69]
Untargeted	Chicken, pork, ostrich, Australian parrot, quail, shrimp, cattle, duck, cat	Complex admixed samples prepared by mixing a variety of meat sources	N/A	[70]

Table 7.
Overview of the studies that employed NGS technology for meat speciation (including pork) in different products.

proposed by Haiminen et al. [72]. The latter pipeline is used to determine the relative mixture composition of eukaryotic species using RNA or DNA sequencing. The authors developed a comprehensive database that includes >6000 plants and animals that may be present in food. Comparison of FASER with All-Food-Seq on the same input data of the mixed raw sausage showed that FASER correctly identified the main ingredients and their relative proportions in the observed matrix and found an additional 1% horse meat. Of the unexpected matrix components, 1.83% of goat meat was observed, which was not included in the database used for All-Food-Seq and therefore was not present in their results and was a false positive in the FASER analysis.

Although the AFS and FASER methods are ideal in theory, they have some limitations: first, the methods are likely to be extremely complex for bioassessment and biomonitoring, as a whole genome has a high degree of complexity. Second, although whole-genome databases have grown rapidly, it takes many years to obtain high-quality whole-genome sequences for a species. And third, the studies use simulated and not experimental data [73]. These limitations can be overcome by mitochondrial metagenomics. This is a metagenomic method that uses mitochondrial genomes (mitogenomes) instead of nuclear genomes as a reference. The main advantages of mitochondrial metagenomics are: the conserved structure of metagenomes, their easy reconstruction and large public databases. To date, only one study by Jiang et al. [74] has used the mitochondrial metagenomic method (3MG) for meat speciation. The authors successfully developed a 3MG method that identified 12 of the 15 animal species tested. In addition, 12 of the 15 (80%) animal species tested could be identified in model mixed samples of pork and chicken meat. Analysis of the composite samples of the two animal species yielded correlation coefficients of 0.98 for pork and 0.98 for chicken between the number of uniquely assigned reads and the mass fraction.

3. Authentication of pork products

Authentication as a concept refers to the originality and integrity of a food product so that the consumer can rely on the claimed quality of a specific product. This is particularly important for high-quality products, which are usually labelled with certain quality marks (such as PGI, PDO or TSG) and achieve high prices on the market.

The authentication process in such cases often refers to the identification of a particular breed within a product. The methods used for this purpose usually rely on DNA-based techniques that can directly detect differences between breeds using specific markers. Farmers' breeding decisions have always favoured certain traits within a given breed. In most cases, this selection pressure has led to the fixation of a few phenotypes (coat colour, stature or similar) and left selection signatures in animal genomes that influence these traits. Thus, if DNA markers affecting specific traits of a breed can be found, they can be used to authenticate the breed. Markers that are usually used for this purpose include PCR-RFLP markers, microsatellites and SNPs. Margeta et al. [75], for example, found a simple PCR-RFLP method based on the MC1R gene related to coat colour, to distinguish the Black Slavonian pig (Crna slavonska) from its crosses with other breeds and wild pigs. In the study conducted by Fontanesi et al. [76], a SNP g.43597545C4T) was identified in different KIT gene haplotypes in different pig breeds. The T allele was found to be fixed in Cinta Sense (95.9%), but its presence was also not detected in other pig breeds. The authors concluded that the SNP found can be considered as a DNA marker with which it is possible to distinguish the breed from other non-belted breeds. Gvozdanović et al.

[77] employed microsatellite markers combined into three sets for the genetic characterisation of the seven breeds included in the study; subsequently, a set of eight microsatellite markers was created for traceability of Black Slavonian pig based on the match probability value. The results showed that when eight highly polymorphic loci were combined, the chance of finding an identical genotype in two random individuals was about three in ten million (10^{-7}). This formed the basis for establishing a reliable genetic traceability system for meat from Black Slavonian pigs. Moretti et al. [78] used a commercially available 60 k SNP chip to identify a small number of SNPs for traceability of the Nero Siciliano pig and its products. A panel of 12 SNPs was sufficient to distinguish the Nero Siciliano pig from cosmopolitan breeds and wild boars, while the final panel of 20 SNPs allowed the discrimination of all breeds involved in the study, but also correctly assigned each individual to its breed and distinguished the Nero Siciliano from first generation hybrids.

It should be noted that in cases where information on breeds and their contribution to the final product is required, as is the case for some products derived from Iberian pigs and their crosses, a multilocus approach can be used [79]. The main disadvantage of this method is that it cannot be used to determine the breed of origin for products consisting of mixtures of several/many animals [80].

4. Conclusions

Pig traceability and authentication require accurate and reliable methods that can be used on a large scale in commercial laboratories, but also on a smaller scale in laboratories that do not work with large numbers of samples. Since the advent of omics technologies, many DNA-based methods have been developed for this purpose, the use of which depends on the objective of the study, the laboratory resources and the skills of the personnel performing the analyses. Recently developed metagenomic analyses offer the most promising solution for identifying unexpected species in food matrices and also for authenticating a particular product. However, they require a high level of expertise and are still costly and quite complicated for use in smaller laboratories. In those facilities, the use of other, simpler and less expensive molecular techniques is preferred.

Author details

Ivona Djurkin Kušec* and Kristina Gvozdanović
Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia

*Address all correspondence to: idurkin@fazos.hr

References

[1] Fiorino GM, Garino C, Arlorio M, Logrieco AF, Losito I, Monaci L. Overview on untargeted methods to combat food frauds: A focus on fishery products. Journal of Food Quality. 2018. Article ID: 1581746. DOI: 10.1155/2018/1581746

[2] Union E. Regulation (EC) No 178/2002 of the European Parliament and of the Coun cil of 28 January 2002 laying down the general principles and requirements of food law, establishing the European food safety authority and laying down procedures in matters of food safety. Official Journal of European Union L 031, 0001-0024. 01/02/2002. 2002

[3] Amaral J, Meira L, Oliveira MBPP, Mafra I. Advances in authenticity testing for meat speciation. In: Advances in Food Authenticity Testing. Amsterdam: Woodhead Publishing; 2016. pp. 369-414. DOI: 10.1016/B978-0-08-100220-9.00014-X

[4] Alves E, Ovilo C, Rodriguez MC, Silio L. Mitochondrial DNA sequence variation and phylogenetic relationships among Iberian pigs and other domestic and wild pig populations. Animal Genetics. 2003;**34**(5):319-324. DOI: 10.1046/j.1365-2052.2003.01010.x

[5] Boore J. Animal mitochondrial genomes. Nucleic Acids Research. 1999;**27**:1767-1780

[6] Caine L, Lima G, Pontes L, Abrantes D, Pereira M, Pinheiro MF. Species identification by cytochrome b gene: Casework samples. In: International Congress Series. Vol. 1288. Elsevier; 2006. pp. 145-147

[7] Karabasanavar NS, Singh SP, Kumar D, Shebannavar SN. Detection of pork adulteration by highly-specific PCR assay of mitochondrial D-loop. Food Chemistry. 2014;**145**:530-534. DOI: 10.1016/j.foodchem.2013.08.084

[8] Barakat H, El-Garhy HA, Moustafa MM. Detection of pork adulteration in processed meat by species-specific PCR-QIAxcel procedure based on D-loop and cytb genes. Applied Microbiology and Biotechnology. 2014;**98**(23):9805-9816

[9] Man YC, Aida AA, Raha AR, Son R. Identification of pork derivatives in food products by species-specific polymerase chain reaction (PCR) for halal verification. Food Control. 2007;**18**(7):885-889. DOI: 10.1016/j.foodcont.2006.05.004

[10] Zarringhabaie GE, Pirany N, Javanmard A. Molecular traceability of the species origin of meats using multiplex PCR. African Journal of Biotechnology. 2011;**10**(73):16461-16465. DOI: 10.3390/nu14224727

[11] You J, Huang L, Zhuang J, Mou Z. Species-specific multiplex real-time PCR assay for identification of deer and common domestic animals. Food Science and Biotechnology. 2014;**23**:133-139

[12] Spychaj A, Szalata M, Slomski R, Pospiech E. Identification of bovine, pig and duck meat species in mixtures and in meat products on the basis of the mtDNA cytochrome oxidase subunit I (COI) gene sequence. Polish Journal of Food and Nutrition Sciences. 2016;**66**(1):31. DOI: 10.1515/pjfns-2015-0051

[13] Djurkin Kušec I, Samac D, Margeta V, Radišić Ž, Vincek D, Kušec G. Efficiency of PCR-RFLP and species-specific PCR for the identification of meat origin in

dry sausages. Czech Journal of Food Science. 2017;**35**:386-391

[14] Roy DC, Akhter S, Sarker AK, Hossain MMK, Lyzu C, Mohanta LC, et al. Tracing the pig and cattle origin in processed food and feed products targeting mitochondrial 12S rRNA gene. Journal of Food Quality and Hazards Control. 2021;**8**(4):152-161. DOI: 10.18502/jfqhc.8.4.8256

[15] Zhou S, Zhong G, Zhou H, Zhang X, Zeng X, Wu Z, et al. A heptaplex PCR assay for molecular traceability of species origin with high efficiency and practicality in both raw and heat processing meat materials. Frontiers in Nutrition. 2022;**9**:890537. DOI: 10.3389/fnut.2022.890537

[16] Ali ME, Hashim U, Dhahi TS, Mustafa S, Man YBC, Latif MA. Analysis of pork adulteration in commercial burgers targeting porcine-specific mitochondrial cytochrome B gene by TaqMan probe real-time polymerase chain reaction. Food Analytical Methods. 2012;**5**:784-794

[17] Mutalib SA, Nazri WSW, Shahimi S, Yaakob N, Sani NA, Abdullah A, et al. Comparison between pork and wild boar meat (sus scrofa) by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Sains Malaysiana. 2012;**41**(2):199-204

[18] Erwanto Y, Abidin MZ, Sugiyono EYPM, Rohman A. Identification of pork contamination in meatballs of Indonesia local market using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Asian-Australasian Journal of Animal Sciences. 2014;**27**(10):1487. DOI: 10.5713/ajas.2014.14014

[19] Gargouri H, Moalla N, Kacem HH. PCR–RFLP and species-specific PCR efficiency for the identification of adulteries in meat and meat products. European Food Research and Technology. 2021;**247**(9):2183-2192

[20] Uddin SMK, Hossain MM, Chowdhury ZZ, Johan MR. Detection and discrimination of seven highly consumed meat species simultaneously in food products using heptaplex PCR-RFLP assay. Journal of Food Composition and Analysis. 2021;**100**:103938. DOI: 10.1016/j.jfca.2021.103938

[21] Farag WMH, Aljuaydi SH, Galal MK, Kassem GMA, Gouda EM. Efficient mitochondrial genes in the characterization of meat species ap-plying PCR-RFLP technique. Advances in Animal and Veterinary Sciences. 2022;**10**(1):8-13

[22] Natonek-Wiśniewska M, Piestrzynska-Kajtoch A, Koseniuk A, Krzyścin P. Current analytical methods and research trends are used to identify domestic pig and wild boar DNA in meat and meat products. Genes. 2022;**13**(10):1825. DOI: 10.3390/genes13101825

[23] Kralik P, Ricchi M. A basic guide to real time PCR in microbial diagnostics: Definitions, parameters, and everything. Frontiers in Microbiology. 2017;**8**:108. DOI: 10.3389/fmicb.2017.00108

[24] Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K. The real-time polymerase chain reaction. Molecular Aspects of Medicine. 2006;**7**:95-125. DOI: 10.1016/j.mam.2005.12.007

[25] Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5′—3′ exonuclease activity of Thermus aquaticus DNA polymerase. National Academy of Sciences of the United States of America. 1991;**88**:7276-7280

[26] Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous amplification and detection of specific DNA sequences. Biotechnology. 1992;**10**:413-417

[27] Orbayinah S, Widada H, Hermawan A, Sudjadi S, Rohman A. Application of real-time polymerase chain reaction using species specific primer targeting on mitochondrial cytochrome-b gene for analysis of pork in meatball products. Journal of Advanced Veterinary and Animal Research. 2019;**6**(2):260. DOI: 10.5455/javar.2019.f342

[28] Janudin AAS, Chin NA, Ahmed MU. An Eva green real-time PCR assay for porcine DNA analysis in raw and processed foods. Malaysian Journal of Halal Research. 2022. DOI: 10.2478/mjhr-2022-0005

[29] Burns M, Nixon G. Validation of two real-time PCR approaches for the relative quantitation of pork and horse DNA in food samples. Food and Nutrition Sciences. 2022;**13**(4):387-403. DOI: 10.4236/fns.2022.134029

[30] Al-Kahtani HA, Ismail EA, Ahmed MA. Pork detection in binary meat mixtures and some commercial food products using conventional and real-time PCR techniques. Food chemistry. 2017;**219**:54-60. DOI: 10.1016/j.foodchem.2016.09.108

[31] Nolte FS, Hill CE. Polymerase chain reaction and other nucleic acid amplification technology. McPherson RA, Pincus MR. Henrys Clinical Diagnosis and Management by Laboratory Methods. 2011;**21**:1239-1249

[32] Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research. 2000; **2000**(28):E63. DOI: 10.1093/nar/28.12.e63

[33] Ahmed MU, Hasan Q, Hossain MM, Saito M, Tamiya E. Meat species identification based on the loop mediated isothermal amplification and electrochemical DNA sensor. Food Control. 2010;**21**(5):599-605. DOI: 10.1016/j.foodcont.2009.09.001

[34] Yang L, Fu S, Peng X, Li L, Song T, Li L. Identification of pork in meat products using real-time loop-mediated isothermal amplification. Biotechnology & Biotechnological Equipment. 2014;**28**(5):882-888

[35] Ran G, Ren L, Han X, Liu X, Li Z, Pang D, et al. Development of a rapid method for the visible detection of pork DNA in halal products by loop-mediated isothermal amplification. Food analytical methods. 2016;**9**:565-570

[36] Lee SY, Kim MJ, Hong Y, Kim HY. Development of a rapid on-site detection method for pork in processed meat products using real-time loop-mediated isothermal amplification. Food Control. 2016;**66**:53-61. DOI: 10.1016/j.foodcont.2016.01.041

[37] Girish PS, Barbuddhe SB, Kumari A, Rawool DB, Karabasanavar NS, Muthukumar M, et al. Rapid detection of pork using alkaline lysis-loop mediated isothermal amplification (AL-LAMP) technique. Food Control. 2020;**110**:107015. DOI: 10.1016/j.foodcont.2019.107015

[38] Thangsunan P, Temisak S, Morris P, Rios-Solis L, Suree N. Combination of loop-mediated isothermal amplification and AuNP-oligoprobe colourimetric assay for pork authentication in processed meat products. Food Analytical Methods. 2021;**14**:568-580

[39] Ren J, Deng T, Huang W, Chen Y, Ge Y. A digital PCR method for identifying and quantifying adulteration of meat species in raw and processed food. PLoS One. 2017;**12**(3):e0173567. DOI: 10.1371/journal.pone.0173567

[40] Floren C, Wiedemann I, Brenig B, Schütz E, Beck J. Species identification and quantification in meat and meat products using droplet digital PCR (ddPCR). Food Chemistry. 2015;**15**(173):1054-1058. DOI: 10.1016/j.foodchem.2014.10.138

[41] Basanisi MG, La Bella G, Nobili G, Coppola R, Damato AM, Cafiero MA, et al. Application of the novel droplet digital PCR technology for identification of meat species. International Journal of Food Science & Technology. 2019;**55**(3):1145-1150. DOI: 10.1111/ijfs.14486

[42] Cai Y, Li X, Lv R, Yang J, Li J, He Y, et al. Quantitative analysis of pork and chicken products by droplet digital PCR. BioMed Research International. 2014:810209. DOI: 10.1155/2014/810209

[43] He C, Bai L, Chen Y, Jiang W, Jia J, Pan A, et al. Detection and quantification of adulterated beef and mutton products by multiplex droplet digital PCR. Foods. 2022;**11**(19):3034. DOI: 10.3390/foods11193034

[44] Ampaporn K, Phasuk Y, Duangjinda M. Droplet digital polymerase chain reaction assay for identifying and quantifying pork products. Animal Science Journal. 2021;**92**(1):e13595. DOI: 10.1111/asj.13595

[45] Xu H, Ma X, Ye Z, Yu X, Liu G, Wang Z. A droplet digital PCR based approach for identification and quantification of porcine and chicken derivatives in beef. Food. 2022;**11**(20):3265. DOI: 10.3390/foods11203265

[46] Cai YM, Dudley QM, Patron NJ. Measurement of transgene copy number in plants using droplet digital PCR. Bio-Protocol. 2021;**11**(13): e4075-e4075

[47] Wong EHK, Hanner RH. DNA barcoding detects market substitution in north American seafood. Food Research International. 2008;**41**(8):828-837. DOI: 10.1016/j.foodres.2008.07.005

[48] Thakur M, Javed R, Kumar VP, Shukla M, Singh N, Maheshwari A, et al. DNA forensics in combating food frauds: A study from China in identifying canned meat labelled as deer origin. Current Science. 2017;**112**:2449-2452

[49] Hajibabaei M, Singer GAC, Hebert PDN, Hickey DA. DNA barcoding: How it complements taxonomy, molecular phylogenetics and population genetics. Trends in Genetics Tig. 2007;**23**(4):167-172. DOI: 10.1016/j.tig.2007.02.001

[50] Xing RR, Hu RR, Han JX, Deng T, Chen Y. DNA barcoding and mini-barcoding in authenticating processed animal-derived food: A case study involving the Chinese market. Food Chemistry. 2019;**309**:125653. DOI: 10.1016/j.foodchem.2019.125653

[51] Crooijmans RPMA, San Cristobal M, Hui X, Li N, Groenen MAM. Biodiversity of pig breeds from China and Europe estimated from pooled DNA samples: Differences in microsatellite variation between two areas of domestication. Genetics Selection Evolution. 2008;**40**:103-128

[52] Duran C, Appleby N, Edwards D, Batley J. Molecular genetic markers: Discovery, applications, data storage and visualisation. Current Bioinformatics. 2009;**4**(1):16-27. DOI: 10.2174/157489309787158198

[53] Charoensook R, Gatphayak K, Brenig B, Knorr C. Genetic diversity analysis of Thai indigenous pig population using microsatellite markers. Asian-Australasian Journal of Animal Sciences. 2019;**32**(10):1491. DOI: 10.5713/ajas.18.0832

[54] Kannur BH, Fairoze MN, Girish PS, Karabasanavar N, Rudresh BH. Breed traceability of buffalo meat using microsatellite genotyping technique. Journal of Food Science. 2017;**54**(2):558-563. DOI: 10.1007/s13197-017-2500-4

[55] Sardina MT, Tortorici L, Mastrangelo S, Di Gerlando R, Tolone M, Portolano B. Application of microsatellite markers as potential tools for traceability of Girgentana goat breed dairy products. Food Research International. 2015;**74**:115-122. DOI: 10.1016/j.foodres.2015.04.038

[56] Yu GC, Tang QZ, Long KR, Che TD, Li MZ, Shuai SR. Effectiveness of microsatellite and single nucleotide polymorphism markers for parentage analysis in European domestic pigs. Genetics and Molecular Research. 2015;**14**(1):1362-1370. DOI: 10.4238/2015.February.13.15

[57] Zhao J, Li T, Zhu C, Jiang X, Zhao Y, Xu Z. Selection and use of microsatellite markers for individual identification and meat traceability of six swine breeds in the Chinese market. Journal of Food Science & Technology. 2018;**24**(4):292-300. DOI: 10.1177/1082013217748457

[58] Rębała K, Rabtsava AA, Kotova SA, Kipen VN, Zhurina NV, Gandzha AI, et al. STR profiling for discrimination between wild and domestic swine specimens and between Main breeds of domestic pigs reared in Belarus. PLoS One. 2016;**11**(11):e0166563. DOI: 10.1371/journal.pone.0166563

[59] Robert F, Pelletier J. Exploring the impact of single-nucleotide polymorphisms on translation. Frontiers in Genetics. 2018;**9**:507

[60] Negrini R, Nicoloso L, Crepaldi P, Milanesi E, Marino R, Perini D, et al. Traceability of four European protected geographic indication (PGI) beef products using single nucleotide polymorphisms (SNP) and Bayesian statistics. Meat science. 2008;**80**(4):1212-1217

[61] Fontanesi L, Ribani A, Scotti E, Utzeri VJ, Veličković N, Dall'Olio S. Differentiation of meat from European wild boars and domestic pigs using polymorphisms in the MC1R and NR6A1 genes. Meat Science. 2014;**98**(4):781-784. DOI: 10.1016/j.meatsci.2014.07.026

[62] Koseniuk A, Smołucha G, Natonek-Wiśniewska M, Radko A, Rubiś D. Differentiating pigs from wild boars based on NR6A1 and MC1R gene polymorphisms. Animals (Basel). 2021;**11**(7):2123. DOI: 10.3390/ani11072123

[63] Preckel L, Brünen-Nieweler C, Denay G, Petersen H, Cichna-Markl M, Dobrovolny S, et al. Identification of mammalian and poultry species in food and pet food samples using 16S rDNA Metabarcoding. Food. 2021;**10**(11):2875. DOI: 10.3390/foods10112875

[64] Ribani A, Schiavo G, Utzeri VJ, Bertolini F, Geraci C, Bovo S. Application of next generation semiconductor based sequencing for species identifi- cation and analysis of within-species mitotypes useful for authentication of meat derived products. Food Control. 2018;**91**:58-67. DOI: 10.1016/j.foodcont.2018.03.034

[65] Xing RR, Nan-Wang Hu RR, Zhang JK, Han JX, Chen Y. Application of next generation sequencing for species identification in meat and poultry

products: A DNA metabarcoding approach. Food Control. 2019;**101**. DOI: 10.1016/j.foodcont.2019.02.034

[66] Dobrovolny S, Blaschitz M, Weinmaier T, Pechatschek J, Cichna-Markl M, Indra A, et al. Development of a DNA metabarcoding method for the identification of fifteen mammalian and six poultry species in food. Food Chemistry. 2019;**272**:354-361. DOI: 10.1016/j.foodchem.2018.08

[67] Liu X, Liu Z, Cheng Y, Wu H, Shen W, Liu Y, et al. Application of next-generation sequencing technology based on single gene locus in species identification of mixed meat products. Journal of Food Quality. 2021;**2021**:1-5. Article ID: 4512536. DOI: 10.1155/2021/4512536

[68] Chaora NS, Khanyile KS, Magwedere K, Pierneef R, Tabit FT, Muchadeyi FC. A 16S next generation sequencing based molecular and bioinformatics pipeline to identify processed meat products contamination and mislabelling. Animals (Basel). 2022;**12**(4):416. DOI: 10.3390/ani12040416

[69] Cottenet G, Blancpain C, Chuah PF, Cavin C. Evaluation and application of a next generation sequencing approach for meat species identification. Food Control. 2020;**110**:107003. DOI: 10.1016/j.foodcont.2019.107003

[70] Akbar A, Shakeel M, Al-Amad S, Akbar A, Ali AK, Rahmeh R, et al. A simple and sensitive NGS-based method for pork detection in complex food samples. Arabian Journal of Chemistry. 2021;**14**(5):103124. DOI: 10.1016/j.arabjc.2021.103124

[71] Ripp F, Krombholz CF, Liu Y, Weber M, Schafer A, Schmidt B, et al. All-food-Seq (AFS): A quantifiable screen for species in biological samples by deep DNA sequencing. BMC Genomics. 2014;**15**:639. DOI: 10.1186/1471-2164-15-639

[72] Haiminen N, Edlund S, Chambliss D, Kunitomi M, Weimer BC, Ganesan B, et al. Food authentication from shotgun sequencing reads with an application on high protein powders. NPJ Science of Food. 2019;**3**:24. DOI: 10.1038/s41538-019-0056-6

[73] Crampton-Platt A, Yu DW, Zhou X, Vogler AP. Mitochondrial metagenomics: Letting the genes out of the bottle. GigaScience. 2016;**5**:15. DOI: 10.1186/s13742-016-0120-y

[74] Jiang M, Xu SF, Tang TS, Miao L, Luo BZ, Ni Y, et al. Development and evaluation of a meat mitochondrial metagenomic (3MG) method for composition determination of meat from fifteen mammalian and avian species. BMC Genomics. 2022;**23**(1):36. DOI: 10.1186/s12864-021-08263-0

[75] Margeta V, Kralik G, Dovč P, Jakšić D, Margeta P. A simple DNA based method for determination of pure black Slavonian pigs. Italian Journal of Animal Science. 2009;**8**(sup3):92-94. DOI: 10.4081/ijas.2009.s3.92

[76] Fontanesi L, Scotti E, Gallo M, Costa LN, Dall'olio S. Authentication of "mono-breed" pork products: Identification of a coat colour gene marker in Cinta Senese pigs useful to this purpose. Livestock Science. 2016;**184**:71-77. DOI: 10.1016/j.livsci.2015.12.007

[77] Gvozdanović K, Djurkin Kušec I, Margeta P, Salajpal K, Džijan S, Bošnjak Z, et al. Multiallelic marker system for traceability of black Slavonian pig meat. Food Control. 2020;**109**:106917. DOI: 10.1016/j.foodcont.2019.106917

[78] Moretti R, Criscione A, Turri F, Bordonaro S, Marletta D, Castiglioni B, et al. A 20-SNP panel as a tool for genetic authentication and traceability of pig breeds. Animals. 2022;**12**:1335. DOI: 10.3390/ani12111335

[79] Muñoz M, García-Casco JM, Alves E, Benítez R, Barragán C, Caraballo C, et al. Development of a 64 SNV panel for breed authentication in Iberian pigs and their derived meat products. Meat Science. 2020;**167**:108152

[80] Fontanesi L. Meat authenticity and traceability. In: Toldra F, editor. Lawrie's Meat Science. 19th ed. Amsterdam: Woodhead Publishing; 2022. pp. 627-665