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Detection of the Species Composition of Food Using Mitochondrial DNA: Challenges and Possibilities of a Modern Laboratory

Małgorzata Natonek-Wiśniewska and Piotr Krzyścin

Abstract

Monitoring food quality is an important and constant element of the food market. This need is connected with health issues, religious beliefs of consumers, and economic considerations. For analysis, mtDNA is most commonly used because it is resistant to physical factors such as temperature and pressure, which very often accompany food processing. Nowadays, scientific publications present a number of methods describing species identification from both farm animals and also less common animals. The most effective methods for determining species are based on PCR, real-time PCR, and sequencing. The methods are very sensitive, limit of detection (LOD) is 0.001% for many of them. An indispensable element of performing the described research is the strict application in the laboratory of several principles, which are intended to improve the work and make it safe for the lab technician, as well as guarantee the quality and effectiveness of the experiments carried out. The high work requirements set for the crew naturally shape the quality system from which the most popular is ISO/IEC 17025. Modern methods based on mtDNA are a good tool for food analysis, creating great opportunities for the researcher, at the same time causing challenges for the contemporary laboratory.

Keywords: mtDNA, quality system in the laboratory, PCR, real-time PCR, sequencing, species identification

1. Benefits of knowledge about possibility species identification

The reliability of food products available on the market, in terms of their origin, quantitative and qualitative composition, has long been the focus of consumers. Therefore, monitoring food quality is an important and constant element of the food products market. This need arises from health issues, consumers' religious convictions, and economic reasons. According to the WHO, in Europe, 8% of children and 4% of adults are allergic to bovine milk or hen eggs. While these products can be rapidly and easily identified in pure form, their presence in complex products may be much more difficult to detect. Knowledge of the species composition of these products, although unavailable without detailed analyses, is crucial for many

patients. Likewise, religious convictions of many communities provide a powerful incentive for monitoring real composition of the food. For example, Judaism prohibits the consumption of pork, so a large part of the followers of this religion avoid the meat of pigs and replace it with beef or sheep meat, which form a considerable part of the meat market in these countries. Unfortunately, for economic reasons, food products are often intentionally adulterated by replacing declared, more expensive components with cheaper substitutes (e.g., meat of lower quality or plant fillers). There are also cases when the quantitative share of an expensive component in a complex product is lowered. By way of example, poultry meat is on average several times cheaper than pork, which, in turn, is priced lower than beef or lamb meat. Similarly, beef is cheaper and more readily available than game meat. The price differences may induce some unfair producers to adulterate and place on the market products whose components differ from manufacturer specifications.

The declaration of meat products in the EU is mandated by the Commission Directive 2002/86/EC [1] stating that meat products have to be labeled with precise information about the species and its percentage in the product. Nevertheless, as experience shows, there are numerous examples of components being misrepresented to make a product more attractive, justify a higher price, or enter new markets. Here, it suffices to mention that in products like fast food 65% of adulteration is deducted [2, 3] and in preparations of game meat, the percentage of factually inaccurate labeling is less (30%) [4], but in sausage, this percent has grown to 90% [5]. Both food products and pet foods were found to be adulterated, and Okuma found 40% of foods for animals with meat of chicken to be falsified [6]. Based on the information reported above and day-to-day practice, it could be claimed that food adulteration is becoming a global problem, which attracts consumer attention at international level and increases public concern about the quality of food products. By way of example, in 2013, the horse-meat scandal revealed gaps in the food safety system and undermined trust between producers and consumers.

It is, therefore, essential to identify the methods for (quantitative and qualitative) determination of species composition of food ingredients to monitor the conformity of a product with the description provided by the manufacturer. Research in this area can better protect consumers from illegal and undesirable adulteration, for whatever reason.

It should be also mentioned that recent years have seen increasing awareness of the importance of food safety and quality, which increases public interest in this issue and leads to changes in legislation. This necessitates continuous development and improvement of analytical methods.

2. The scope of the species identification tests

The analysis most often uses mtDNA, although exceptions outlined below are permitted. The advantage of mitochondrial over genomic genome results from its resistance to the action of physical factors such as temperature and pressure, which very often accompany the processing of food. These characteristics of mtDNA contribute to a very high sensitivity of the analyses. In principle, the whole mitochondrial genome can be used for the analyses, but more frequent use is made of cytochrome B and D-loop. Cytochrome B is the most conservative of the entire mitochondrial genome. Its identification and creation of a bar code were the subject of projects aimed to describe all living organisms—both the most common and the most unique. In turn, D-loop is characterized by the highest variation between species, which enables the method to be quickly determined. The mitochondrial genome is very short compared to the body's entire genome and forms a very small

proportion of it. In animals, it is slightly over 16,000 bp, which means it is relatively easy to develop methods for identifying the panel of organisms chosen by a researcher. Current research papers present several methods from identify single farm species such as pigs [7, 8, 9], cattle [7, 8, 9, 10], sheep [7], horses [9, 11], chickens [9, 12], turkeys [9], ducks [8, 13], fish [14] to less common animals like kangaroos [15], snails [16], and marine animals like octopuses [17], shrimp [18] and sharks [19]. This is relatively the simplest method of analysis. With proper time investment, labor inputs, and funds, a laboratory is capable of identifying a concrete species. Such methods are generally very sensitive and enable determining adulterations as low as 0.001% [20, 21, 22], although this has little practical use because determinations below 1% are generally treated as artifacts. For this reason, the laboratories that commercially used methods most often set the limits of determination between 0.1 and 1% [23]. In certain cases, it is more beneficial to determine a whole group of animals rather than single species. These methods are more demanding because the reaction conditions have to be adjusted as to make the method specific for several DNA fragments that differ in sequences. The primers most commonly used are compatible with DNA of several species, which necessitates finding the most homologous fragments. Most often, however, the primers are homologous only in a certain percentage [19, 24]. Such analysis very often yields products of similar, indeed identical, length. Sometimes, it is, therefore, more beneficial to design one primer compatible with all species and another primer specific for single species, which gives products of different length [23, 25]. The choice of method depends on needs. Increasingly often laboratories face the challenge of discriminating between animal and plant DNA in a sample. This apparently easy task is in fact more complicated than identifying smaller groups of animals and impossible to perform based on mtDNA identification. Most often, animal DNA is identified using a DNA fragment that encodes myosin, a muscle protein; that is why myosin-based methods yield a positive reaction only for samples that contain muscles. This limitation may be a problem during analysis because the method allows no identification of matrices such as bones. Another limitation is the differentiation of animals with very similar mitochondrial genomes. This problem can be seen, for example, when distinguishing between pig (*Sus scrofa scrofa*) and wild boar (*Sus scrofa*) components. The mitochondrial genome of both species is 99% homologous (according to BLAST between these species), and there are only single point mutations, so they cannot be used for species identification. Research is underway to make differentiations based on MCR 1 [26, 27], which is a color-determining gene. In the context of food, this issue is important because of differences in taste, price, and availability of meat from these two species.

All the identified DNA fragments should be short, less than 250 bp. There is the rule that the more the food product is processed, the shorter the PCR product should be.

Extreme temperature and pressure cut DNA into short segments; for example, exposure to a pressure of 3.2 Ba results in approximately 100-bp segments and only such or short DNA fragments can be identified. Of course, in raw or cooked meat, DNA is not degraded so much, but the method involving short DNA fragments is more universal and enables determinations to be made whatever the degree of processing.

Molecular methods enable determination to be made in any matrix. In practice, DNA can be identified regardless of matrix form or earlier processing. We can freely determine species composition of both raw tissues and processed tissues in the form of meat, bones, blood, eggs, dairy products such as cheese, milk and butter, drinks, gelatin, lyophilized milk products, meat preparations, and egg products [7, 12, 27, 28, 29].

It often happens that the matrices in which DNA is sought have a form that prevents its biological origin to be clearly identified, and so it may become a source

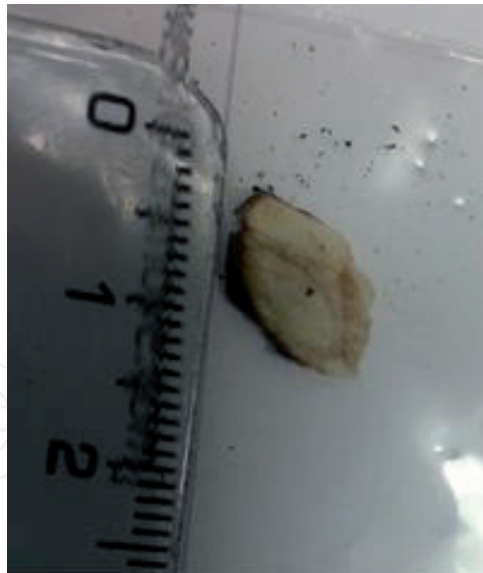


Figure 1.
Biological material found by a consumer in meatballs.

of potential problems. This is exemplified by a fragment of biological material found by a consumer in meatballs [13]. The object concerned, which was small in size and additionally resembled a human nail (**Figure 1**), was identified during the analysis as material coming from one of the breeding species, so its presence in food preparations was fully justified.

3. Used methods, possibility each of methods, their advantages, and disadvantages

The most effective methods of species identification are based on PCR technique. These methods use both conventional PCR and real-time PCR. Both methods can be used as monoplex or multiplex PCR. Detection in real-time PCR can use both probes and DNA-binding dyes (e.g. SYBR Green, Eva Green). A detailed schematic representation of the method is given in **Figure 2**.

Each method has its pros and cons. The simplest method, conventional monoplex PCR, is unbeatable when one concrete species is sought. These methods generally have a very high limit of determination, which is often so high that it has no practical application in commercial analyses. This figure, often below 0.001%, acquires real significance when determining undesirable trace substances or accidental artifacts.

Such methods are simplest but at the same time show the least potential, and only allow determining if a given substance contains the DNA of the species being identified.

Multiplex reactions not only offer more possibilities but also cause more problems. Since they require carrying out the reaction in one temperature, which is not necessarily optimal for all primers and as a result reactions may take place with different efficiencies, this may lead to false-negative reactions when the level of adulteration is low. Thus, although multiplex reaction unquestionably shortens the time of analysis and reduces its costs, when complex products are analyzed, the result for low content DNA can be subject to risk [30].

Another group of methods is restriction fragment length polymorphism (RFLP). This technique is based on amplification of a DNA fragment with different sequences, followed by its digestion with appropriate restriction enzymes,

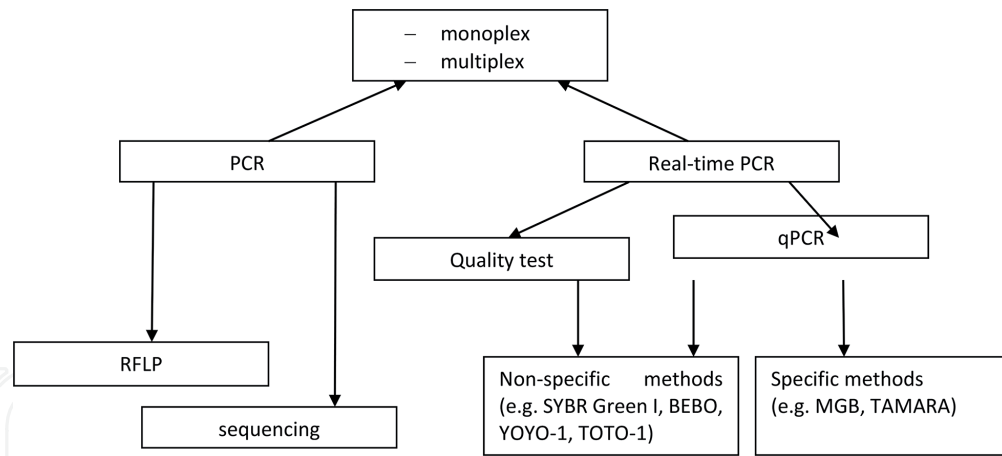


Figure 2.
Detailed schematic diagram of the methods used for species identification.

which enables even related species to be distinguished [31]. The method allows for identification of several to 25 animal species, although the latter requires the use of several restriction enzymes.

The PCR-RFLP method is simple, inexpensive, and easy to use for monitoring purposes. PCR-RFLP has been used for years and many researchers consider it outdated. However, this method works very well in the case of complex analyses, where we are interested in finding the potential presence, for example, of a group of species (e.g., birds, ruminants) and then their specific representatives. Similarly to the case of multiplex reaction, this method performs better for single-species samples, while their application for complex products may cause read errors, firstly because of similar restriction patterns for the analyzed species of animals, secondly due to the competitiveness of RFLP reaction. Another disadvantage of the PCR-RFLP method is that erroneous results may develop because of the occurrence of incomplete digestion of the restriction site or intraspecific differences, which may contribute to the removal or development of restriction sites [32].

When we analyze samples whose composition is completely unknown and has to be identified, Sanger sequencing is a very good solution. If we analyze a fragment homologous to several species, we can quickly and accurately determine its species origin. Again, this method is better applied to single-species samples and it is not a method of first choice for routine determination of specific species, if only because of higher price and the need to use more specialist equipment. However, it is an indispensable tool for analyses subject to greater uncertainty.

Another group of methods are quantitative determinations. They continue to be a major challenge for researchers because sample reactivity depends on processing method, type of matrix, and sometimes the animal. Therefore, production of the reference material that is later used to generate standard curves is subject to error of 10% or sometimes even 30%.

The production of reference material is an important issue when determining the type of meat. It should be noted that the certified reference material (CRM) is only available in the form of DNA, which in the case of quantitative tests does not work and is completely unsuitable because the mismatch of such material to the analyzed meat samples can be huge. That is why laboratories themselves produce reference materials. Usually, meat samples purchased commercially from the butcher or shop are used for this. It is important that they came from a few or several individuals. The material produced in this way is more precisely matched to the analyzed samples and has a lower risk that it will not completely match it. Before using the reference material so manufactured, it should be checked. First, the standard curve obtained from it must meet certain parameters such as slope,

y-intercept, R^2 value, and amplification efficiency (EFF%). The appropriate numerical values for these parameters guarantee the specificity and reaction efficiency of the standard curve used. The second necessary condition is the analysis against this curve of a sample with a guaranteed concentration of the species being determined. Such samples are most often obtained as residues from proficiency tests. It should also not be forgotten that the method of isolating DNA from reference material should be the same as the test samples [33]. Many authors use methods that match the largest number of food-related matrices, e.g. CTAB [33], although this depends on the experience and preferences of each laboratory.

Standard amounts of the material needed for the analyses range from 0.1 to 0.5 g because such amounts are most often recommended by the manufacturers of DNA isolation kits, but when determining microtraces in foods, we must often settle for a fraction of this weight. Since mtDNA is most commonly used, which allows for very sensitive analyses because it is present in every cell in many million copies, often trace amounts of material are sufficient to perform the analysis.

4. Ensuring the quality of analyses, quality systems in the laboratory, and certificates for laboratories

The high sensitivity of mtDNA-based PCR methods is a great advantage, but at the same time, this is associated with a serious risk of cross reactions. Therefore, the tests described above must be governed by a strict application of several rules, which, by design, should make the work more efficient and safe for the laboratory technician while ensuring the quality and effectiveness of the experiments.

The overriding rule is to perform most of the procedures in a laminar flow cabinet, in which air is constantly blown out to ensure sterile conditions. Prior to the commencement of work, it is a good practice to switch on the unit for more than 10 minutes, which will allow for a complete exchange of air, and to turn on the UV lamp, which is usually part of the unit, to make the work area sterile. The working area must be wiped with a DNA-removal solution. Before starting the job, make sure all necessary equipment and materials are ready at hand. At the same time, the working space must be divided into a "clean zone" (pipettes, centrifuges, vortex mixers, reagents, pipette tips) and a "dirty zone" (used tips and basket). These zones must be separated to avoid cases where a used pipette tip is carried over the reagents, test-tube stand, etc. Laboratory technicians working in a laminar flow cabinet should be adequately prepared for work. To ensure sterility, they should wear protective aprons and disposable gloves, additionally cleaned with a DNA-removal agent.

It is also important to separate workstations at which different stages of the analysis (sample preparation, DNA isolation, PCR, electrophoresis) are performed. Any change in workstation requires that the protective apron and disposable gloves be changed. One workplace must not overlap with another. Before starting and after completing the job, working surfaces must be cleaned with a DNA-removal agent. A laboratory sample should be moved in one direction only, in accordance with each successive stage of determinations. Test equipment must be regularly verified and calibrated.

An important aspect of work at a laboratory engaged in species DNA identification is validation of methods before they are introduced. An essential requirement for every research or scientific laboratory that performs commercial testing is to use reliable methods. The methods taken from ISO/IEC or recommended by umbrella organizations (e.g., EURL-AP) have already been validated, so it is enough to check their function in the laboratory. It should be noted, however, that in the DNA

research area concerned, many laboratories use their own methods. These have the advantage of being flexible and adaptable to the current needs of customers, which means that the laboratory can react quickly and optimally to the evolving market needs. Naturally, these methods have to be validated, which incurs additional charges for the laboratory:

- increased costs; before the method becomes profitable, the laboratory must usually pay high validation costs,
- time-consuming nature; there must be adequate time between the decision to introduce a method to its real application in the laboratory. The longer and more laborious the validation process, the longer the time needed,
- the need of training; it increases the costs and delays the practical implementation. However, this has a positive aspect for the laboratory in the form of better trained and more aware staff.

The high requirements placed on the personnel are naturally shaping the quality system, in which all employees are aware of their responsibilities, the work is safe, and ensures reliable results. Nowadays, most laboratories want to introduce a defined quality system. The most popular system is ISO/IEC 17025, which provides requirements for testing and calibration laboratories. Since its publication in 1999 by the International Organization for Standardization, the regulations in this document help to organize work in laboratories. Implementation of this standard certifies that all tests performed in the laboratory meet the standard and respect the chosen testing procedure. Because species identification is directly linked to food safety monitoring, introduction of the system provides measurable benefits in the form of growing prestige of the laboratory, increased efficiency, greater competencies of the managerial staff, clearly defined responsibilities and rights of the staff, increased testing accuracy, and higher number of commissioned tests.

The accreditation requirement most often results out of external pressure, from the customer or the regulatory authority [34], but sometimes it may result from the internal desire to increase the level of testing services [8] or even from institutional strategic planning [10]. However, decision to adopt ISO/IEC should consider (1) the organization's culture, (2) the actual need for pursuing accreditation—the accreditation requirement from the customer or the regulatory authority, (3) the time and the resources available, (4) the staff's knowledge and previous experience in quality, (5) the current conditions of the laboratory with reference to compliance with the standard, (6) use of standard test methods already established and known well by the laboratory staff, and (7) condition of equipment used for tests, in addition to involving appropriate costs of maintenance and calibration [34].

Modern methods based on mtDNA are a powerful tool for food analysis, creating great opportunities for the researcher, at the same time causing a number of challenges for the contemporary laboratory. The newly developed, commercially used methods are made taking into account the above-mentioned activities.

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