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Advantages, Possibilities, and Limitations of Mitochondrial DNA Analysis in Molecular Identification

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Review article

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Contemporary molecular biology provides information about entire genome sequences, which are used in fields such as medicine, pharmacogenetics, and nutrigenomics. Apart from the DNA located in cell nuclei, studies of the DNA in cell organelles, such as chloroplasts and mitochondria, are important as well. Analysis of selected mtDNA fragments has become the basis for molecular barcoding – species identification based on polymorphisms in the nucleotide sequence. Of particular importance in identification analyses are fragments encoding genes of the respiratory chain – cytochrome b and cytochrome oxidase, with intraspecific similarity but at the same time high interspecific variation, owing to which mtDNA analysis has found an application in many fields of science, such as forensic genetics and molecular anthropology. An unquestionable advantage of molecular methods over traditional ones is that identification can be based on trace quantities of material, including highly processed or degraded material (bone fragments, teeth, or fur). Moreover, bioinformatic tools supporting molecular analyses limit the need for testing of reference material, as the results can be compared to sequences deposited in databases. Given the increasing availability of molecular methods and the decrease in costs resulting from the development of the technology, species identification based on polymorphism in mtDNA may become a routine research method in the fields of ecology, molecular archaeology, molecular taxonomy, and forensics. This work is a review of areas in which mitochondrial DNA is used for species and individual identification. The analysis methodology and examples of the practical applications of mtDNA studies are discussed as well.

Key words: Forensic Genetics, Molecular Ecology, Food adulteration, SNP.

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Mitochondria are organelles that play a key role in cellular energy transformations. Respiratory chain reactions take place in their inner membranes, as a result of which energy obtained from substrate degradation is stored in high-energy bonds of adenosine triphosphate (ATP) (DROSE & BRANDT 2012). A characteristic feature of mitochondria is the fact that apart from the cell nucleus, they are one of only two organelles that contain DNA; for this reason they are referred to as semi-autonomous organelles.

The mitochondrial genome takes the form of a circular molecule of dsDNA. It is much smaller than the nuclear genome, with about 16,500 bp, and it encodes 37 genes, responsible for respiratory chain function (13 genes) and the activity of the mitochondrial translation apparatus (24 genes, coding for tRNA and rRNA) (BALLARD & WHITLOCK 2004).

Oxidative processes taking place in the mitochondrion and a lack of repair mechanisms lead to mutations in mtDNA. The sequences of genes coding for elements of the respiratory chain, such as cytochrome

b (Cytb) and cytochrome oxidase I (COI), vary between species due to the accumulation of specific mutations (BLAXTER 2003; KUMAR *et al.* 2019; LINACRE & LEE 2016). The organization of the mitochondrial genome makes it possible to design universal primers in conserved regions for a wide range of species, and the variation in the fragments flanked by them can be used for species identification of biological samples of unknown origin. Polymorphic nucleotides contained in mtDNA can also be used to analyze variation within a species and to identify variants characteristic of specific populations, which are classified into haplogroups (TORRES 2016). The most commonly analyzed mtDNA fragments include cytochrome b, ribosomal 12S RNA, 16S RNA, the D-loop (containing hypervariable regions), tRNA, and ATPase6/ATPase8 (HABZA-KOWALSKA *et al*. 2019).

Due to the group specificity of mtDNA sequences, the uniparental nature of inheritance, the large number of copies in cells, the double membrane surrounding mitochondria, the lack of recombination, and a relatively small genome, mtDNA is used in numerous branches of molecular biology (SALAS *et al.* 2007). These analyses are primarily used with severely degraded material. Examples of such areas of knowledge include forensic genetics (especially wildlife forensics) (JUST *et al.* 2004), molecular ecology (GALTIER *et al.* 2009; RUBINOFF 2006), and molecular archaeology and anthropology (BROWN *et al.* 2016; GLEIZE *et al.* 2016).

Molecular archaeology and anthropology

Genetic material isolated from contemporary donors can be used to analyze relationships between populations and to track migration, colonization of various geographic regions, and the shaping of demographic structure over the centuries. Owing to the haploid and uniparental nature of mtDNA inheritance, it is an excellent tool for recreating the history of individual haplogroups. The most commonly used mtDNA fragments in archaeological studies are hypervariable regions. Analysis of polymorphism in the hypervariable fragment HV1 of mtDNA has been used to investigate the origin of and relationships between people inhabiting Siberia (PAKENDORF *et al.* 2003), south-western North America (MALHI *et al.* 2003), and Romania (COCOS *et al.* 2017). An approach at the border of forensic genetics and molecular anthropology is the creation of mtDNA databases, which can be used to identify the perpetrators of crimes. For example, the results of analyses carried out in the Netherlands of control regions from 640 people from different parts of the country have been included in the international database EMPOP (CHAITANYA *et al.* 2016).

The methodology of mtDNA analysis is not limited to the study of the structure of contemporary populations. Analyses of polymorphisms in mtDNA are also increasingly used to test archaeological samples, in which the material usually consists of bones and teeth. Mitochondrial DNA was analyzed for historical research as early as the 1980s, to study Egyptian mummies. Interesting examples of the use of mtDNA analysis in molecular archaeology include the identification of remains found in an Egyptian tomb in the Valley of the Kings as Queen Nefertiti, based on polymorphism in HV1 (HABICHT *et al.* 2016), or the identification of remains of Nicholas II found among bodies in a mass grave (IVANOV *et al.* 1996).

Molecular methods are increasingly used to study the past, as confirmed by numerous studies, e.g. by ARIFFIN *et al.* (2007). The researchers analyzed the sequence of the D-loop in DNA isolated from bones found on the wreck of a ship sunk in the 17th century. Analysis of hypervariable regions in mtDNA has also been used to study mummies from the 12-13th centuries, found in western Siberia. The remains were shown to belong to five different haplogroups, indicating that the population of that time was a unique mixture of haplotypes specific to western regions and typically East Siberian regions (SLEPCHENKO *et al.* 2019). Interesting conclusions can be drawn from analysis of the mitochondrial DNA of Egyptian mummies. Analysis of 90 mitochondrial genomes from different periods (from before and during the Ptolemaic dynasty and from the Roman period) indicates that the population was more genetically similar to the contemporary population of the Middle East than to the modern inhabitants of Egypt. The genetic structure currently observed in the population may be due to a recent contribution to the genetic pool from people from sub-Saharan areas (SCHUENEMANN *et al.* 2017).

A study of the hypervariable region HVR1 was carried out for four sets of remains, dated 8-10 AD and found in the Province of L'Aquila in Italy. Two individuals were confirmed to belong to haplogroup H, dominant in Europe, while one sample was identified as a representative of haplogroup R0a (probably originating in the Arabian Peninsula), and one belonged to haplotype J1, typical of the Mediterranean. The results indicated high genetic similarity in mtDNA between inhabitants of L'Aquila and contemporary inhabitants of central-northern Italy. Analysis of evolutionary distances between the fragments obtained and other aDNA (ancient DNA) samples from databases confirmed the similarity of the sequences obtained in Italy to the German Lombard tribes. Some of the samples were shown to be closely related to sequences from inhabitants of Byzantium (POMA *et al.* 2019). Similar analyses have been carried out in Sweden and Norway, determining the similarity of samples from the Viking Age to contemporary populations (KRZEWINSKA *et al.* 2015, BUS *et al.* 2019), Oceania (NAGLE *et al.* 2017), and China (LI *et al.* 2017).

The role of mtDNA analysis in forensic genetics

Due to the possibility of testing the relationships between individual donors, the capacity for group identification, and variation in hypervariable regions, molecular analyses have a significant influence on contemporary forensic biology, allowing law enforcement authorities to obtain expert opinions with unprecedented power of evidence. Polymorphism resulting from differences in DNA sequences enables individual identification, determination of paternity, and identification of the species of test samples and can be applied both in case of samples derived from humans and animals (KOWALCZYK *et. al.* 2018; BUTLER 2012).

mtDNA in human forensic genetics

In the case of human forensic analysis, the primary focus is nuclear DNA and the highly polymorphic microsatellite sequences in it, which form the basis for molecular profiling and the formation of databases (BUTLER & HILL 2012). In many cases, genetic material found at the scene of an event is present in extremely small quantities, which may be due to the nature of the sample (single hairs, chewing gum, cigarette filters, contact traces, bones, or remains of victims of disasters and attacks) or to the high degree of DNA degradation, resulting from exposure to environmental factors and the time passed since the event (BUS *et al.* 2016). In the case of difficult material, it is not always possible to obtain a complete and reliable result for nuclear DNA. For this reason, a valuable addition to genetic analyses for forensic purposes is the analysis of mitochondrial DNA (mtDNA), which despite its much lower discriminatory power, can provide results from degraded, highly processed, or archaeological samples (GRELA *et al*. 2021; BUS *et al.* 2016).

Advantages of the use of mtDNA in forensic genetics include the high number of the organelles in the cell, high resistance to degradation due to the presence of a double membrane around the mitochondrion, and a smaller genome size. For these reasons, mtDNA is the best and often the only solution for analyzing highly degraded samples. The complete sequencing of a human mitochondrial genome was first published in 1981 (ANDERSON *et al.* 1981), followed by a corrected version in the late 1990s. The corrected Anderson sequence, also known as the Cambridge Reference Sequence (CRS), is a reference with which polymorphisms detected in test samples are compared (ANDREWS *et al.* 1999).

The mitochondrial genome, apart from genes coding for elements of the respiratory chain, also contains hypervariable regions (HV1, HV2, and HV3) forming what is known as a control region, also called a D-loop. Due to the higher variation compared to other mtDNA regions, HV regions are of some importance in individual identification (MOROVVATI *et al.* 2007). The HV1 and HV2 regions were used first, because they exhibit relatively high variation in a short region (PARSON & BANDELT 2007), but in accordance with the recommendations of the DNA Commission of the International Society of Forensic Genetics (ISFG), the entire control region is analyzed (PARSON *et al.* 2014). HV regions are the most variable fragment of mtDNA, because they are non-coding regions that evolve rapidly. One difficulty in mtDNA analysis is the phenomenon of heteroplasmy – the possibility of mutations in mtDNA, and thus the presence of two different mtDNA sequences. Therefore, in mtDNA analyses, at least two differences must be shown between the test sample and the reference material.

With the development of technology exploiting mini-STR sequences, the role of mtDNA in individual identification is decreasing and nowadays it is rather marginal. At the start of the 1990s, analysis of the mtDNA control region was used to identify the remains of victims of the Vietnam War (HOLLAND *et al.* 1993). A decade later, after the attack on the World Trade Center, victims were identified on the basis of mini-STR polymorphism (HOLLAND *et al.* 2003), with higher discriminatory power. Currently, mitochondrial genetic material is rarely used for individual identification, but in some cases, when the material is highly degraded and it is impossible to obtain a profile from nuclear DNA, mtDNA analysis remains the only chance of identification. Examples include the identification of remains from World War II found in Ukraine (DUDAS *et al.* 2019) and of victims of the Spanish Civil War (BAETA *et al.* 2019).

mtDNA in wildlife forensics

Forensic genetics use molecular markers not only to solve the cases involving human biological material but also in so called wildlife forensics. Both human and wildlife forensics use similar genetic tools, but often for different purposes. The popular trend in wildlife forensic genetics is species identification based on polymorphism in mtDNA, largely due to the presence of species-specific sequences in mtDNA. In forensic practice, sequences of the cytochrome oxidase I and cytochrome b genes are most often used for species identification. Owing to high interspecific variation and the availability of universal primer pairs, mitochondrial DNA analyses were rapidly adopted for the needs of forensic genetics dealing with crimes against protected species (wildlife forensics) but also in breeding and companion animals. There are many examples of crimes against animals (poaching, illegal slaughtering of farm animals, persecution and the illegal killing of wild animals, hunting out of season, illegal trade of protected species) in which mtDNA analysis can be applied to species identification and tracing the geographical origin of a sample (LORENZINI & GAROFALO 2021; IYENGAR 2014; JOHNSON *et al.* 2014).

Analysis of the cytochrome b sequence was applied by AN *et al*. (2007) to species identification of illegally hunted specimens. Authors testing hair and meat samples, confirmed that three out of six samples were obtained from roe deer, as hunting of roe deer is prohibited in Korea, results provided forensic evidence of illegal wild animal hunting. CANIGLIA *et al.* (2010), used a set of molecular markers (including the mtDNA control region) to identify the species origin of a tooth necklace and to solve the case of a suspect serial wolf killer. A mitochondrial cytochrome *b* sequence analysis prepared by GUPTA *et al.* (2005), confirmed that a wooden chopping block was used to chop the meat of a peafowl, which is considered to be an endangered bird in India. Trade in products containing tissues of protected animals is a serious problem for law enforcement authorities. Trade in this type of goods is estimated at $20 billion (https://www.interpol.int/Crimes/Environmental-crime/Wildlife-crime).

Combating this type of crime is impeded by the difficulty of identifying illegal components added to a product, which is often highly processed. Examples include traditional folk medicines, jewellery and ornaments made of bones and teeth, and animal furs. Molecular analyses often remain the only reliable method of determining the species of origin of a component. Sources of mtDNA analyzed in smuggling cases include rings, earrings, and guitar picks made of hawksbill sea turtle shells (FORAN & RAY 2016), ivory figurines (GUPTA *et al.* 2011), and pangolin scales (ZHANG *et al.* 2015).

Analysis of mtDNA to detect food adulteration

The possibility of species identification means that mtDNA analysis can be used to detect food adulteration. Growing consumer awareness, religious convictions, food allergies, and dishonest practices in food production confirm the need for testing to control and verify the composition of food products. Food adulteration has been an inseparable element of trade since ancient times, when water was added to wine, or spices were counterfeited. Modern molecular methods enable identification of the species composition of a product and its components. In this case as well, the main material used in the analysis is mtDNA, because most food products are processed and subjected to heat treatment or pressure. The mitochondrial genome is characterized by molecular stability in variable, unfavorable temperature and pressure conditions, which allows for the identification of products subjected to technological processes, and thus enables determinations in both raw material (meat, milk, bones, or blood) and processed material (e.g. sausages, cheese, whey, or animal feed) (HA *et al.* 2017).

As in the case of forensic genetics and species identification, the most conserved fragment is most often used, i.e. cytochrome b, cytochrome oxidase I (COI), and the D-loop. Adulterated products include those containing seafood, fish, and meat, which is linked to the high price of the raw material. Adulteration very often involves the use of meat of a cheaper species or breed (e.g. the addition of undeclared pork to beef products) (PRUSAKOVA *et al.* 2018).

HA *et al.* (2017) conducted a study on a method for detecting the adulteration of meat products with pork, using primers specific for pig mitochondrial DNA. The main sequence for which the primers were designed was the D-loop region of mitochondrial DNA, with a length of 294 bp. The primers were able to detect a 1% addition of pork in heat-treated meat products. A total of 35 meat products were purchased for the study in retail stores (14 patties, 8 nuggets, 8 meatballs and 5 sausages). The presence of undeclared pork was confirmed in three samples.

An example of the use of multiplex PCR methods for species identification from meat samples is the protocol proposed by PRUSAKOVA *et al.* (2018), enabling the simultaneous identification of ten species of meat. The authors used primers specific for five species whose meat is commonly consumed (including pork, lamb, and beef), as well as five species whose consumption is prohibited (such as mice, rats and dogs). Primers flanking the eighth ATPase subunit were used in the reaction. The use of the method to analyze commercially available products indicated adulteration in over 90% of samples. In most of them the adulteration involved replacing beef or turkey with cheaper substitutes, such as chicken, or detection of undeclared chicken meat in products such as salami or sausages. The effectiveness of multiplex PCR for the detection of food adulteration has also been confirmed by THANAKIATKRAI *et al.* (2019), who designed a reaction with five pairs of primers to detect dog, duck, goat, beef, and lamb meat. Species-specific primers were designed for the mitochondrial cytochrome oxidase I gene (COI). The amplification products differed in length, which made it possible to verify the species composition following separation on an agarose gel. The results confirmed that 26 of 117 tested meats and commercial products contained DNA from species that were not declared on the label by the manufacturer.

Another example of the use of molecular techniques relying on mtDNA analysis was a study by ILHAK & GÜRAN (2015), who analyzed the composition of Turkish sujuk sausage, which should contain only beef. Using multiplex PCR to test 50 sausages they determined that only 30 samples were made of 100% beef. The rest contained poultry meat, and in one case the presence of horse meat was detected. In a scandal in China, mutton in grilled shashlik was replaced with the meat of rats and mice, causing vomiting, nausea,

and symptoms of acute food poisoning in consumers. FANG & ZHANG (2016) used Real-Time PCR and specific primers complementary to cytochrome b in mtDNA to develop a method for detecting rat and mouse meat in samples.

Fish products are also subject to adulteration. KUNG *et al.* 2012 analyzed the species composition of 25 tuna sausages available in shops in Taiwan. They used multiplex PCR to identify the addition of meat of undeclared species, amplifying mitochondrial genes (12S rRNA, tRNA Val, and 16S rRNA). The addition of pork was detected in 20 sausages, and of poultry meat in one sausage. In addition, they used PCR-RFLP to determine the species of tuna the sausage was made from (analyzing polymorphism in the gene coding for cytochrome b). Three species of tuna were detected in the sausages – yellowfin (88%), albacore (4%), and Atlantic bluefin (4%), as well as one species from the marlin family – the Atlantic blue marlin, identified by DNA sequencing. Certain fish species, crabs, shrimp, and sushi are categorized as luxury products, and for this reason these products are often subject to adulteration (MONDAL & MANDAL 2020). A study conducted on seafood products in Greece found discrepancies between the declared and actual composition in over 12% of samples (MINOUDI *et al.* 2020).

The use of mtDNA in molecular ecology

The species specificity of the mtDNA sequence is exploited not only in forensic analyses and the detection of food adulteration. The rapid development of molecular biology techniques as well as overall technical advances and automation has been accompanied by significant development of most branches of modern biology, including ecology. Molecular ecology, through the use of genetic and bioinformatic analyses, makes it possible to study the relationships between individual species or populations at the level of DNA. The data obtained are important in many areas of science, from taxonomy to evolutionary biology.

The relatively simple structure of mtDNA (lack of repeated elements, transposons, pseudogenes, or introns) facilitates analysis, which in combination with nucleotide substitutions that arise relatively rapidly, makes it possible to analyze the history of individual species and the relationships between them. Moreover, the genes in mtDNA do not undergo recombination, and the mitochondrial genome corresponds to a single locus.

Mitochondrial DNA is an effective molecular marker in phylogenetic analyses. Conserved sequences of genes coding for proteins are used to study interspecific variation (BRUFORD *et al.* 2003), while the control region is a reliable source of information in intraspecific variation (GALTIER *et al.* 2009; BRUFORD *et al.* 2003). Individual protein-coding genes located within mtDNA are characterized by a different utility and can be classified into three groups of good (ND4, ND5, ND2, Cytb, and COI), medium (COII, COIII, ND1, and ND6), and poor (ATPase 6, ND3, ATPase 8, and ND4L) phylogenetic performers in recovering these expected trees among phylogenetically distant relatives (ZARDOYA & MEYER 1996). Within the first group, two genes: Cytb and COI proved to be the most commonly used in phylogenetic studies. Research concerning the reconstruction of mammalian phylogenies revealed that Cytb more accurately reconstructs phylogeny and known relationships between species in this group at the Super Order, Order, Family, and generic levels than the COI gene (TOBE *et al.* 2010). The mentioned genes are also useful in phylogenetic studies concerning other groups of vertebrates. In case of birds, Cytb and COI sequences were used for the phylogeny reconstruction of numerous wild species (DAI *et al* 2010; LEI *et al*. 2010) as well as to evaluate the genetic diversity of native chicken populations (DAVE *et al.* 2021). In relation to invertebrates, based on the example of insects, the Cytb and COI genes have been used in species identification and phylogenetic analysis, also based on different developmental (larval) stages of individuals (KAUR *&* SINGH 2021; RAKHSHANDEHROO *et al.* 2019). However, nowadays, with the increasing availability of next-generation sequencing methods, single-gene-based phylogenetic analyses are gradually being replaced by phylogenomics based on the sequence of whole mitochondrial genomes (DA SILVA *et al.* 2020; PHILLIPS *&* ZAKARIA 2021).

Mitochondrial DNA sequences can also be used in phylogeographic studies to compare the evolutionary relationships between genetic lines with their geographic distribution in order to understand the factors that influenced the current distribution of genes, populations, and species. Phylogeographic studies are based on the analysis of the type, frequency, and share of particular haplotypes in populations and, as in case of phylogenetic analyses, mainly use Cytb and COI sequences (WANG *et al.* 2021; ZHOU *et al.* 2021). However, also in this case, analysis of mtDNA fragments is replaced by analysis of the whole mitochondrial genomes (LI *et al*. 2021; REDING *et al.* 2021).

The usefulness of mtDNA in molecular ecology has been confirmed by the identification of a COI gene fragment as a reference on which molecular taxonomy and species identification should be based (HEBERT *et al.* 2003; DAWNAY *et al.* 2007). Identification and taxonomy are linked to DNA barcoding. This method was proposed in 2003 by the Canadian scholar Paul Hebert (HEBERT *et al.* 2003). It is based on analysis of a specific DNA fragment contained in the genome of every organism in a form that is similar but different enough to enable identification at the species level. While COI and less often Cytb sequences are commonly used to identify animals, in the case of plants or fungi, these sequences do not provide polymorphism enabling species identification. For

this reason it was necessary to find alternative markers, which were found in different semi-conserved organelles – chloroplasts. Several sequences have been proposed as potential markers in the species identification of plants. These include genes contained in cpDNA (chloroplast DNA), such as the large subunit of the RuBisCO gene (rbcL) and the maturase K gene (matK) located in chloroplasts (BELL *et al.* 2017). In 2009, the Consortium for the Barcode of Life (CBOL) Plant Working Group proposed the chloroplast gene rbcL and matK as the core barcodes of plant species, as well as intergenic sequence trnH-psbA and nuclear gene ITS as the supplement barcodes (CBOL PLANT WORKING GROUP *et al.* 2009). They were tested in a variety of plant types, from medicinal plants (SHINWARI *et al*. 2018) to tropical tree species (KANG *et al.* 2017; TRIPATHI *et al*. 2013). However, the use of DNA barcoding in plants remains a major challenge despite the use of different combinations of several markers simultaneously.

With the development of barcoding, many projects dedicated to this technology have emerged, such as iBOL (International Barcode of Life Project) and BOLD (Barcode of Life Data Systems), which deal with data storage and analysis (VERNOOY *et al.* 2010). The idea of DNA barcoding has generated a great deal of discussion, especially among taxonomists (EBACH & DE CARVALHO 2010). Most of the arguments concern a tendency towards an oversimplification of taxonomy (RUBINOFF *et al*. 2006), the possibility that the COI sequence will lack differences sufficient to distinguish individual species (KERR *et al*. 2007), and the risk associated with the interpretation of data from species that may be hosts for parasites causing male lethality, such as Wolbachia (JOHNSTONE & HURST 1996).

Despite the criticisms of barcoding, it has many advantages over classic taxonomy, which relies largely on comparative analyses. Unfortunately, the material obtained by laboratories is not always a complete individual; in practice it is often processed animal material. These may be insect fragments (the identification of insects, e.g. certain species of mantis or ants, often requires comparative analysis of body fragments that are difficult to isolate, such as the copulative apparatus of male mantises, or easily lost or destroyed, such as ant antennae); processed animal products (e.g. leather goods or figurines made of bone); or larval stages of invertebrates, which cannot be conclusively identified by classic methods, so that only molecular methods are effective. For this reason methods based on mtDNA analysis have found broad application in examining the degree of variation both within and between species (JOHNSON *et al.* 2018; CAMARGO *et al.* 2016; WANG *et al.* 2019; PENG *et al.* 2015).

Methodology of mtDNA analyses

Routine analysis includes DNA isolation, amplification of target fragments, sequencing of PCR products, and a bioinformatic analysis of the results (Fig. 1).

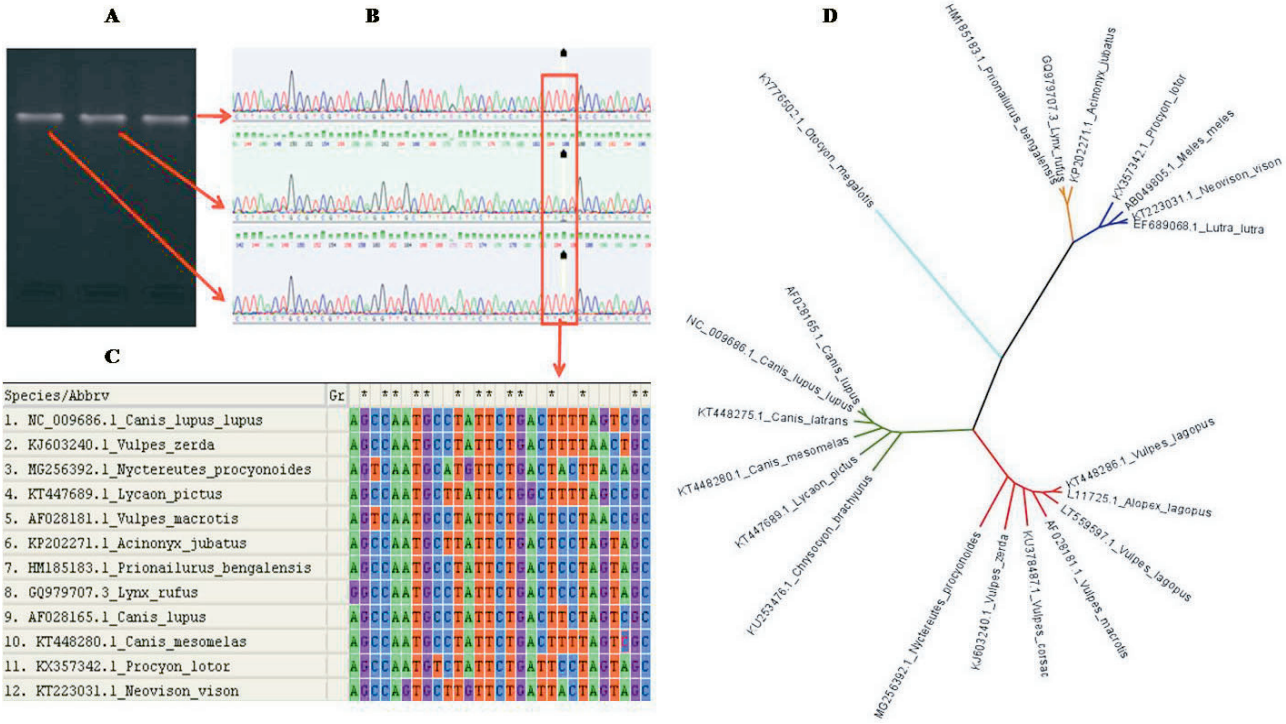

Fig. 1. Stages of mtDNA analysis: A – electrophoretic separation of PCR products, B – results of sequencing purified PCR products, C – bioinformatic analysis involving a comparison of the sequences obtained with database resources and analysis of polymorphisms between the test sequence and reference sequences, D – phylogenetic analysis based on the sequences obtained (our own results).

A correct and reliable analysis, especially in the case of analyses using universal primers and concerning human DNA, requires strict adherence to protocols, not only during the analysis itself, but also during the preparation stages. Of particular importance is the appropriate preparation of a sample and the place where analyses are to be carried out, as well as adherence to defined standards. The greatest threat to this type of analysis is the risk of contamination, which results in false positive results; for this reason, appropriate organization of the laboratory space is essential.

The most stringent restrictions apply to laboratories that analyze archaeological samples, but specific procedures to reduce the risk of contamination must also be followed in the case of other mtDNA analyses. One of the principles is a physical separation of the space where the genetic material is isolated and amplified and where post-PCR analysis is performed. In practice, the procedures should be carried out in separate rooms equipped with UV lamps to sterilize the surfaces. In the case of work with archaeological material, analyses should be carried out in rooms designated specifically for that purpose. In addition, airlocks with HEPA filters are often used. To verify the reliability of results, negative controls should be used at each stage (isolation and amplification) (BUS *et al.* 2019; KNAPP *et al.* 2012).

The first mtDNA analyses were based on polymorphism of the length of restriction fragments (RFLP – restriction fragment length polymorphism), obtained following digestion of the genetic material by restriction enzymes (BROWN 1980). Currently, methods relying on amplification of nucleic acids predominate. Species identification based on nucleotide polymorphism in mtDNA is possible owing to the use of the polymerase chain reaction technique (PCR). The technique, developed by Mullis in the 1980s, enables the amplification of a selected genome fragment flanked by primers specific to the template. The reaction products are separated on an agarose gel to confirm the specificity of the amplification and the quality of the products. When universal primers are used for species identification, PCR is not sufficient to provide a conclusive answer regarding the origin of the sample. Additional steps involving sequencing and a bioinformatic analysis of the results are necessary. The most commonly used sequencing method remains the Sanger method developed in the 1970s, whose indisputable disadvantage is the short length of the fragments analysed in a single reaction, i.e. 500-1000 bp, but this throughput is usually sufficient for the purposes of species identification.

Sanger sequencing is still widely used for analysis of mtDNA, but there are some limitations (time consuming capillary electrophoresis, relatively high labor intensivity, and the fact that a single run is focused on a small fragment of a whole mtDNA). The development of technology enables the usage of alternative methods with higher throughput, including next generation sequencing (NGS), which is used to analyze entire mitogenomes. Therefore, the application of NGS (known also as massively parallel sequencing – MPS) is becoming a more and more popular trend, which yields much more information than methods of the first and early second generation (454 sequencing technique). The technology is used in forensic genetics in cases in which the increased throughput, which makes the method more informative, is crucial for the power of the evidence. The launching of more sophisticated next-generation sequencing methods and platforms such as Illumina, Ion Torrent, or PacBio and MinION (belonging to third generation) vastly broaden the capability of analyses of both the nuclear and mitochondrial genomes. An example of the application of NGS is the use of Illumina technology to sequence an entire control region of mtDNA (BRANDHAGEN *et al.* 2020).

In the case of mtDNA, MPS has become an important tool, which provides enhanced sensitivity and resolution in comparison to traditional Sanger sequencing. Moreover, involving MPS in mtDNA analysis, it is possible to deal with such problems as detection of heteroplasmy (BRUIJNS *et al*. 2018). Due to its high throughput, MPS gives the opportunity to expand and accelerate mtDNA analysis, as an entire mitogenome can be sequenced in a single run, even from forensic quality samples (BALLARD *et al.* 2020; CHURCHILL *et al.* 2017). Therefore, the discriminatory power and reliability of analysis is considerably higher in comparison to Sanger sequencing technologies. The usefulness of MPS was considered in the case of forensic studies (see for example the sequencing of complete mitochondrial genomes from hair shaft samples – PARSON *et al.* 2015 or an analysis of heteroplasmy – GALLIMORE *et al.* 2018), ecology (the DNA-metabarcoding approach with COI in the assessment of species spectrum in a tested area – BONATO *et al.* 2021; EUCLIDE *at al.* 2021; GUEUNING *et al.* 2019), as well as in analyses of the origin and migration of individual populations based on whole mtDNA (LOPOPOLO *et al.* 2016).

The importance of NGS in ecology and taxonomy emerges from the barcoding – approach, especially useful in the detection and assessment of biodiversity from a wide variety of environmental (environmental DNA) and biological samples. Generally, mitochondrial metagenomics is a methodology for shotgun sequencing of total DNA from specimen mixtures followed by bioinformatic extraction and the analysis of mitochondrial sequences. Mitochondrial metagenomics plays an important role in modern ecology. This approach was used in the evaluation of the biodiversity and phylogenetic tracing of the relations of beetles in the Bornean rainforest (CRAMPTON-PLATT *et al.* 2015), to study mite specimens extracted from

forest and grassland soils in Iberia (ARRIBAS *et al.* 2020), as well as zooplankton diversity (REY *et al.* 2020), and to analyze and reconstruct carnivores' diets by sequencing samples obtained from feces (MONTERROSO *et al.* 2019).

Molecular analyses are significantly facilitated by bioinformatic databases such as NCBI or Ensembl, containing nucleotide sequences, as well as applications used to compare them. This means that there is no need to test reference samples in order to verify the species of samples of unknown origin; it is sufficient to compare the sequence obtained during sequencing with database resources, e.g. using the BLAST application, or solutions dedicated to mtDNA analysis, such as the EMPOP database (PARSON & DUER 2007).

Conclusions

Analyses of mtDNA are used in many branches of science, enabling studies at the border of forensic genetics and anthropology as well as analyses in molecular ecology, becoming the gold standard in these fields. Owing to the increasing throughput of molecular analyses, in conjunction with a growing bioinformatics infrastructure, analyses of mitochondrial DNA provide good support for analyses based on markers present in nuclear DNA, and are an interesting direction of research.

Author Contributions

Research concept and design: M.K.; Collection and/or assembly of data: M.K., A.S., K.K.; Data analysis and interpretation: M.K., A.S., K.K.; Writing the article: M.K., A.S., K.K., P.D., B.H.; Critical revision of the article: M.K., P.D., B.H.; Final approval of article: M.K., A.S., K.K., P.D., B.H.

Conflict of Interest

The authors declare no conflict of interest.

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