POLISH JOURNAL OF NATURAL SCIENCES
Abbrev.: Pol. J. Natur. Sc., Vol 36(1): 79–94, Y. 2021

MOLECULAR METHODS OF ANIMAL SPECIES IDENTIFICATION

***Tadeusz Malewski*[1], *Andrzej Dzikowski*[2], *Ireneusz Sołtyszewski*[3]**

[1] ORCID: 0000-0001-8061-435X
[2] ORCID: 0000-0002-3223-7542
[3] ORCID: 0000-0003-1128-8129
[1] Museum and Institute of Zoology
Polish Academy of Sciences, Warsaw, Poland
[2] Department of Pathophysiology, Forensic Veterinary Medicine and Administration
[3] Department of Criminology and Criminalistics
University of Warmia and Mazury, Olsztyn, Poland

Key words: species identification, DNA analysis, barcoding, veterinary medicine.

Abstract

Species identification of animals carried out with molecular biology methods plays an increasingly important role in laboratory practice. Currently, such methods are used, i.e., in phylogenetics, environmental protection, forensic medicine and forensic veterinary medicine, plant and animal breeding. DNA analysis is of particular importance in the identification of protected and endangered animal species being subject to illegal trade. Illicit commerce concerns both live animals and variety of products and objects made of them. In case of such products and objects, identification based on the morphological characteristics is not possible. It is, therefore, necessary to use animal DNA analysis. The paper analyses and presents molecular methods of animal species identification currently in use beginning from qualitative PCR-based methods: random amplification of polymorphic DNA – RAPD-PCR, restriction fragment length polymorphism PCR – RFLP-PCR, single-stranded conformational polymorphism PCR – SSCP-PCR, though quantitative: real-time PCR, high resolution melting PCR – HRM-PCR and finishing DNA barcoding and metabarcoding methods.

Introduction

In the 17th century, Carl Linnaeus began the identification of species on the basis of its morphology. Currently, the number of eukaryotic species is estimated at about 10 million. Identification of organisms on the basis of morphological features is tedious, time-consuming and often sta-

Address: Ireneusz Sołtyszewski, University of Warmia and Mazury in Olsztyn, 68 Warszawska Av., 10-719 Olsztyn, Poland, e-mail: ireneusz.soltyszewski@uwm.edu.pl

ge-dependent. This is the reason why only about 1.7 million of species have been described over the course of 300 years. The breakthrough came with the development of the polymerase chain reaction (PCR). Currently, molecular techniques are the most preferred methods in many fields, such as phylogenetics, nature protection, plant and animal breeding, and in – broadly understood – forensics.

Materials and Methods

The aim of this study is to present the methods of animal species identification currently in use. The literature review included data obtained by 2020 and presented in the PubMed and Web of Science databases. The authors employed the following keywords and their combinations concerning: species identification animals, DNA analysis, barcoding, veterinary medicine. The results presented in selected papers were used to describe the methodology of genetic testing for the purposes of individual identification of animals.

Results and Discussion

PCR-based Method

The primary method of the identification of animal species is the polymerase chain reaction. This procedure enables the amplification of any selected DNA sequence. The PCR reaction mix contains four components: a double-stranded DNA template, primers hybridizing with a template, deoxyribonucleotides (dATP, dCTP, dGTP, dTTP) and a thermostable DNA-dependent DNA polymerase. The thermal profile of the reaction consists of three stages: denaturation, primer annealing and synthesis of a new DNA fragment. During denaturation, the double-stranded DNA is split, which allows the primers to attach to complementary sequences on each strand of DNA when the temperature is lowered. Denaturation at a temperature of ca. 95°C takes from several to several dozen seconds. Lowering the temperature to 50–60°C allows for complementary matching and the attachment of primers to a single DNA strand. The temperature range is selected according to the primers used. After primers are attached, a new DNA fragment is synthesized with the help of DNA-dependent DNA polymerase. Each three-step cycle produces two daughter molecules from one parent DNA molecule.

Molecular tests identifying animal species use different variants of PCR reactions:

– random amplification of polymorphic DNA (RAPD-PCR),
– restriction fragment length polymorphism (RFLP-PCR),
– single-stranded conformational polymorphism (SSCP-PCR)
– real-time PCR
– high resolution melting PCR (HRM-PCR).

The technique of random amplification of polymorphic DNA was developed in 1990 (Williams et al. 1990, Welsh and McClelland 1990) and is considered to be one of the simplest methods of species identification.

The discussed method is a modification of the classic PCR and is based on using one primer with a randomly selected sequence (approx. 9–15 bp long). If the primer attaches in the correct orientation to opposite strands of DNA within several thousand base pairs of each other, the PCR reaction takes place (Bardakci 2001). The number and arrangement of these random primer-binding sites varies from species to species. After the separation of the amplification products by electrophoresis, a pattern of bands characteristic for individual species is obtained. The method does not require the knowledge of the DNA nucleotide sequence of the organism in question. This fact provides a great advantage of this method, as it enables the identification of organisms which have never been previously sequenced (Senthil Kumar and Gurusubramanian 2011). The method in question has some disadvantages, the most important of which are: the susceptibility to various changes in the conditions of the amplification reaction (e.g. changes in the concentration and origin of polymerase, fluctuations in the amount of the starting material) and low repeatability of the reaction (Al-Samari and Al-Kazaz 2015).

This method is most commonly used in microbiology (Jarocki et al. 2016, Lin et al. 2018, Mohkam et al. 2016, Tamang et al. 2009) and botany (Abdelsalam et al. 2020, Heubl 2010). In zoology, RAPD-PCR is successfully used to identify beetles of the *Oncocires* genus (Cordeiro et al. 2019), *Bemisia* whiteflies (Jiu et al. 2017), nematodes (Mattos et al. 2019) and others (Ramella et al. 2005, Marmiroli et al. 2007). It is also successfully used in detecting fraud in commerce (Sawicki 2009).

Restriction fragment length polymorphism method is based on amplifying a selected DNA fragment, followed by digesting the amplified fragment with restriction enzymes. Restriction enzymes recognize specific sequences (4–6 bp) and digest DNA specifically. The obtained DNA fragments are separated by electrophoresis. The analysis of the obtained fragments allows to assess their number and size, and to compare them with the patterns characteristic for the given species.

The advantages of the PCR-RFLP include: relatively cheapness, lack of technical overcomplication, suitability for routine analyses. Various regions of DNA are used to identify species, such as satellite – satDNA (GRENIER et al. 1997), mitochondrial – mtDNA (BOWLES et al. 1992, BOWLES et al. 1993) and ribosomal – rDNA (JACOBS et al. 1997, ZHU et al. 1999). Of these DNA regions, the rDNA sequences are often studied. These regions of DNA contain highly conserved sequences, which ensures high reaction sensitivity, while internal transcribed spacer regions – ITS1 and ITS2 rDNA show high interspecies variation and high intraspecific stability (WĘDRYCHOWICZ 2000).

The discussed method found application, e.g. in parasitology for the identification of nematode species. It has been used it to distinguish some species of *Ascaridoidea* – canine and feline parasites, including *Toxocara canis* and *Toxocara cati* (JACOBS et al. 1997). The amplified DNA fragments of the *T. canis* and *T. cati* parasites can be digested with the Rsa I enzyme, while it does not have the sequences recognized by Hinf I and is not cleaved by this enzyme. On the other hand, the DNA fragments of the closely-related nematodes, such as *Toxascaris leonina*, *Baylisascaris procyonis*, *Ascaris suum* and *Ascaris lumbricoides* are fragmented under the influence of the Hinf I enzyme. *Toxocara* spp. can be easily distinguished from other *Ascaridoidea* species that can occur in human tissues. Moreover, *T. canis* and *T. cati* can be distinguished from each other and unambiguously identified – due to the Rsa I enzyme, which cuts their amplified DNA fragments at various sites. Digestion of the *T. canis* amplicons allows 2 fragments (250 and 290 bp) and *T. cati* – 3 fragments (110, 160 and 280 bp) to be obtained. Reaction carried out with primers complementary to the ITS2 sequence of parasites does not amplify human, canine or feline DNA (*Ascaridoidea* larvae can be detected in biopsy material taken from humans and animals without the need to isolate the parasite, specific PCR can be performed directly on the tissue homogenate). RFLP-PCR is used to identify parasitic species such as *Taenia asiatica* (EOM et al. 2020), *Toxoplasma gondii* (IVOVIC et al. 2019), *Trypanosomatidae* (BENTO et al. 2018) and *Phlebotomus* (ZAHRAEI-RAMAZANI et al. 2017).

It has been shown that the PCR-RFLP is effective in the analysis of relatively closely-related species, as well as samples containing different species or subjected to various technological processes, including thermal sterilization (SAWICKI et al. 2009). For these reasons, the PCR-RFLP is often used in research to determine the species of fish and seafood. Over 70% of analyses related to the identification of fish species are performed with the discussed technique (RASMUSSEN et al. 2009, SAWICKI et al. 2009). The cytochrome B gene in mitochondrial DNA is the fragment most

frequently used in the identification of seafood and fish, e.g. sackcloth, cod, salmonids, flatfish (MARMIROLI et al. 2007, TELETCHEA et al. 2006). Although the PCR-RFLP technique is considered to be favorable and often used in species identification, it has also some drawbacks, e.g. the possibility of intra-species variability or the appropriate selection of restriction enzymes.

Single-stranded conformation polymorphism method enables detection of small DNA changes, such as: point mutations, small deletions and insertions, or micro-inversions. This technique is based on the phenomenon that each single-stranded DNA molecule adopts its own conformation during electrophoresis under non-denaturing conditions. This conformation depends, i.a. on its sequence. Replacement of even a single nucleotide in a few-hundred nucleotide strands effects in change of the spatial structure, which leads to differences in electrophoretic mobility of the particle (VORECHOVSKY 2005). The first stage of the SSCP analysis covers thermal denaturation of the double-stranded DNA in a buffer with denaturing agent (most often formamide, however urea or sodium hydroxide can also be used) and then rapid cooling to prevent renaturation of the separated fragments. In the second step of the discussed procedure, the obtained single-stranded molecules are separated electrophoretically in a non-denaturing polyacrylamide gel. The electrophoretic mobility of single-stranded DNA chain fragments depends on their size, charge and spatial structure. Under non-denaturing conditions, individual single-stranded DNA molecules assume a specific conformation determined by intramolecular interactions. The adopted structure depends on the length of the strand, the nucleotide sequence, as well as the location and number of regions within which the internal base pairing has occurred (GASSER et al. 2006).

The PCR-SSCP method is also suitable for testing heat-treated samples and for detection of mixed samples. Its conduct is more demanding than the RFLP-PCR. It can, however, detect differences in the DNA sequence regardless of their location in places recognized by restriction enzymes. PCR-SSCP has been used successfully to identify nematode species, such as *Toxocara vitulorum*, *Toxocara cati*, *Toxocara canis*, *Toxascaris leonina*, *Baylisascaris procyonis*, *Ascaris suum*, *Parascaris equorum* (ZHU and GASSER 1998), *Elaphostrongylinae – Protostrongylidae* (HUBY-CHILTON et al. 2006), *Parelaphostrongylus odocoilei* (BRYAN et al. 2010), protozoa (POWER et al. 2011), fish (REHBEIN et al. 1999, REHBEIN 2002, TISZA et al. 2016, SIVARAMAN et al. 2019), molluscs (ARANCETA-GARZA et al. 2011), mosquitoes (KOEKEMOER et al. 1999) and others (ZHU et al. 2001, GASSER 2013).

Contrary to the classic PCR reaction, which provides information about the presence or absence of the marked species in the tested material, the real-time PCR provides additional information on the quantita-

tive share of individual species. In order to detect sample, fluorochromes or fluorescently labeled probes are used. The level of emitted fluorescence is measured with a spectrofluorimeter coupled with a thermocycler specially adapted for this purpose (the stronger the fluorescence, the greater the number of DNA copies is). Probes may have a fluorescent reporter can be attached at one end of a probe and a fluorescence quenching molecule (TaqMan), a light-emitting molecular beacons (associated with the target DNA sequence), or a LightCycler (which uses two oligonucleotide probes – donor and receptor) at the other end (DORAK 2006). Simultaneous determination of the amount of DNA in a sample of several species is possible since the probes can be labeled with different stains. The combination of the RT-PCR with high resolution melting (HRM) curve analysis enhances the range of possibilities of this method. This combination allowed to identify, e.g., 15 species of *Calliphoridae* (*Diptera*) flies (MALEWSKI et al. 2010), 21 species of fish (BEHRENS-CHAPUIS et al. 2018), 13 species of farm animals (ISHIDA et al. 2018), or 9 species of beetles (MALEWSKI et al. 2019).

The RT-PCR allows short amplicons to be analysed. Therefore, this method can be and is widely used to identify animal species in processed products, e.g. in research on food adulteration, control of illegal trade of wild animals belonging to endangered species. In such trade, prohibited by the particular and international law (CITES 1973), the object of a crime is often a highly processed part of animal body. It is thus impossible to recognize the species on the basis of tradictional keys for species recognition. Illicit trade in live and dead animals is a crucial and emotive problem. According to a report by the World Wide Fund for Nature – WWF, the value of such trade is estimated at $ 19 billion annually. It is the third most-profitable black market in the world, after arms and drug trafficking (MCGARTH 2012).

For example, the RT-PCR method has been successfully applied to detect and quantify material from the elephants (*Loxodonta africana* and *Elephas maximus*) and the woolly mammoth (*Mammuthus primigenius*) in highly processed samples. It provides reproducible results in samples containing only 100 copies of template DNA (WOZNEY and WILSON 2012). There is also a multiplex set of probes and primers developed, which detects nine of the twelve species of sharks listed by the CITES (CARDEÑOSA et al. 2018). It can be also used to determine the species of animals in processed meat products, as even small pieces of DNA left over from heat treatment can be amplified and identified. RT-PCR has been successfully used for species verification and DNA quantification for many species of fish and canned meat products, such as Atlantic cod (*Gadus morhua*) in a mixed sample (HERRERO et al. 2010), hake (*Merluccius merluccius*)

(Sánchez et al. 2019), or multiplex identification of Atlantic cod, salmon (*Salmo salar*) and flounder (*Pleuronectes platessa*) (Hird et al. 2012). In addition, RT-PCR is successfully used to quantify DNA from cattle, pigs, horses, sheep (Natonek-Wiśniewska and Krzyścin 2015), chickens, ducks and geese (Natonek-Wiśniewska and Krzyścin 2016).

DNA marker technology represents a promising means for determining the genetic identity and kinship of an animal. Compared with other types of DNA markers, single nucleotide polymorphisms (SNPs) are attractive because they are abundant, genetically stable, and amenable to high--throughput automated analysis. In cattle, a set of 32 SNPs has sufficient power to identify breeds and crossbred populations (Heaton et al. 2002). SNP arrays were succesfully used in salmonid species identification (Wenne et al. 2016), mussels (Peñarrubia et al. 2019), mule deer, white-tailed deer (Russell et al. 2019) and others (Yang et al. 2013).

DNA Barcoding

PCR-based methods can determine if a sample belongs to a certain species, but cannot determine what the species is. Assignation of a sample to a species is possible by DNA sequencing methods. The most powerful of them is the DNA barcoding.

Paul Herbert from the University of Guelph, Ontario, Canada, inspired by barcodes placed on products in the store, gave rise to the idea of DNA barcoding. This method is based on analysis of a region of DNA, which is present in all organisms and differs sufficiently to distinguish species. The received and processed data are placed in the reference database, which then enables unique identification of an organism and its assignment to certain species.

A genetic marker suitable for DNA barcoding needs to meet a several criteria. Firstly, it needs to be sufficiently variable between species, but sufficiently conserved within than between species. Secondly, priming sites need to be sufficiently conserved to permit a reliable amplification. Thirdly, its amplification and sequencing should be as robust as possible (Hebert et al. 2003, Vences et al. 2005).

Different genes can be used to identify different groups of organisms via DNA barcoding. The most commonly used barcode region for animals and some protists is a portion of the cytochrome C oxidase I (COI or COX1) gene. COI have two important advantages: the universal primers for this gene are very robust, enabling recovery of its 5‘ end from representatives of most, if not all, animal phyla (Folmer et al. 1994; Zhang and Hewitt 1997); COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene.

Additionally, with thousands of copies per cell, mitochondrial COI is readily amplified by polymerase chain reaction, even from very small specimens. It was proposed that a 648 bp fragment of COI could permit species-level resolution among all animals. Initial tests of DNA barcoding using COI on animals reported near-100% accuracy (Hebert et al. 2003, Hebert et al. 2004a, Barrett et al. 2005).

Differences in COI sequences between closely related species in birds were 19–24 times greater in magnitude than the differences within species. On the basis of these data it was found that the majority of avian species can be discriminated via DNA barcoding. A standard sequence threshold of ten times the mean intraspecific variation to flag genetically divergent taxa as provisional species can be proposed (Hebert et al. 2004b). The actual threshold value varies depending upon group of organisms. The presence of this 'barcode gap' (Meyer et al. 2005) has been observed in many animal taxa (Ward et al. 2005, Hajibabaei et al. 2006, Waugh 2007, Bucklin et al. 2011). DNA barcoding studies on animals provide an ideal source of data. Analysis of these data have established two important patterns:

– more than 95% of animal species examined possess a diagnostic COI sequence,

– and COI divergences rarely exceed 2% within species, while members of different species typically show higher divergence (Ratnasingham et al. 2013).

One of goals of the DNA barcoding is to build a publicly accessible reference database with species-specific DNA barcode sequences. This goal has been achieved by creation of the Barcode of Life DataSystems – BOLD (Ratnasingham et al. 2007). BOLD, established in 2005, is a web platform that provides an integrated 'environment' for assembling and usage of the DNA barcode data. The on-line database for the collection and management of specimens covers distributional and molecular data, as well as analytical tools to support their validation. Currently BOLD contains sequences for ~317,000 formally described species (~8.8 million specimens).

The possibilities and merits of the DNA barcoding can be well illustrated by entomofauna studies conducted in Germany. During two major DNA barcoding campaigns starting at 2009: the 'Barcoding Fauna Bavarica' and the 'German Barcode of Life', the DNA barcode library for 5,200 German flies and midges (*Insecta – Diptera*) has been assembled (Morinière et al. 2019). It should be noted that – despite this impressive achievement – the barcode coverage varies from only about 10% in marine invertebrates to 70% freshwater fish (Weigand et al. 2019).

DNA Metabarcoding

The DNA barcoding simplifies only the taxonomic aspect by establishing the list of species present in an ecosystem. It does not help to reduce the sampling effort. The development of next generation sequencing – NGS offers an opportunity to solve this problem.

Next generation sequencing technologies allow considerably greater numbers of nucleotides to be characterized, The DNA barcodes can be, therefore, generated for thousands of taxa in parallel (SHOKRALLA et al. 2015). DNA metabarcoding refers to an automated identification of multiple species from a single environmental sample containing degraded DNA (soil, water, faeces, etc.). It can be implemented for both modern and ancient environmental samples. By relaxing the need for an intensive sampling effort, the DNA metabarcoding opens the door to high-throughput biodiversity assessment for plants and animals (TABERLET et al. 2012).

It should be observed, that the COI region (used for the DNA barcoding) is too long to be applied for most commonly used NGS platforms, such as Illumina, or Ion Torrent. Instead of COI, mini-barcodes must be used (BRANDON-MONG et al. 2015). Nevertheless, research has shown that COI barcode of smaller size, such as 250 bp (MEUSNIER et al. 2008), and even 135 bp (HAJIBABAEI et al. 2006), can distinguish majority of animal species. Ability to identify species by mini-barcodes opens the opportunity to work with environmental samples in which DNA is usually degraded.

The metabarcoding approach provides a significant step-change in the analysis of foodwebs. For a diet analysis it is necessary to identify not only animals, but also plants, fungi and microorganisms. While the COI is an almost perfect marker for animals, it is not preferable for plants and fungi, and not applicable for bacteria. For fungi identification an internal transcribed spacer – ITS of the ribosomal DNA is recommended as a barcode (SCHOCH et al. 2012). For plants – it is the ribulose 1,5-bisphosphate carboxylase gene (*rbcL*), combined with a fragment of the maturase gene (*matK*) (CBOL PLANT WORKING GROUP 2009). For identification of microorganisms 16S ribosomal DNA is widely used (KLINDWORTH et al. 2013).

The metabarcoding can be used not only to identify species. It can also be applied to estimate relative abundances within a sample (HARRISON et al. 2020, LIN et al. 2019; LOU et al. 2018). Several aforementioned advantages provide the multifaceted application of the metabarcoding in biological research (COBLE et al. 2019, DREINER et al. 2017, DUARTE et al 2020, POPESCU et al. 2018, SACCÒ et al. 2019, WILLIS and GABALDÓN 2020).

Several short-read NGS platforms are currently available, each having unique pros and cons, allowing one to generate increasing quantities of data at decreasing costs (Reuter et al. 2015). Although the Illumina MiSeq instrument is capable of sequencing maximum 2 x 300 bp, it is the most expensive platform for short-read sequencing and still insufficient to cover the entire 16S rRNA gene which is approximately 1500 bp long. More recently, a new generation of NGS technologies was revealed, capable of producing much longer reads. One of them has been offered by Pacific Biosciences since 2010 (Eid et al. 2009, Korlach et al. 2010) and successfully been used to generate long-read DNA barcode data (Hebert et al. 2018, Tedersoo et al. 2018). It was reported that the long-read DNA metabarcoding provides better taxonomic resolution than any single marker (Heeger et al. 2018). Prohibitive expensiveness of PacBio instruments hampers broad introduction of this sequencing platform for species identification.

A highly promising alternative solution for long read sequencing has been offered by nanopore-based sequencing technologies (ONT – Oxford Nanopore Technologies). In this technology, single-strand of DNA passes through a protein nanopore, resulting in changes in the electric current that can be measured. The DNA polymer complex consists of double-stranded DNA and an enzyme that unwinds the double-strand and passes the single-stranded DNA through the nanopore. As the DNA bases pass through the pore, there is a detectable disruption in the electric current, and this allows the identification of the bases on the DNA strand (Lu et al. 2016). ONT sequencer is of a small size, lightweight, inexpensive and allows for sequencing of several gigabases of DNA on a single flow cell.

Quality of nanopore sequencing significantly increased over the last few years. Together with the development of appropriate software, consecutive sequence error rates decreased to 0.1% (Zhang et al. 2020). Moreover, nanopore sequencing has been recently used for metagenomics in clinical, environmental and agricultural settings (Latorre-Pérez et al. 2020, Petersen et al. 2019, Pomerantz et al. 2018, Stewart et al. 2019), as well as in monitoring of the current state of genetic biodiversity in higher Eukaryotes (Krehenwinkel et al. 2019).

Existing molecular methods offers many possibilities of animal species identification. Simple PCR-based methods (RAPD-PCR, RFLP-PCR) can easily be implemented in testing laboratories. More demanding - real-time PCR, high resolution melting PCR, DNA barcoding are today widely used in everyday scientific work. Appearance of Next Generation Sequencing platforms revolutionised animal identification. It has become possible not only to identify individual specimens but also to determine what species are present in soil, water or other environmental samples. Pendrive size

NGS sequencer of Oxford Nanopore Technologies democratized metabarcoding and made it possible to perform in every laboratory as well as in non-laboratory environment in field studies.

Accepted for print 10.12.2020

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