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# Use of DNA analysis of histopathological specimens in species identification for purposes of forensic veterinary medicine

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Summary

**In some legal proceedings, the species identification of animal on the basis of fragments of biological material is extremely difficult. This applies both to closely-related and to distant species characterized by similar morphological features. In such circumstances, methods of molecular biology are used, whose evidential value is definitely not in doubt. Histopathological scraps may also have to be used for identifying tests. The aim of the present study was to verify the possibility of using DNA analysis in determining the species of animals on the basis of biological material contained in archival histopathological samples. The examined material consisted of twenty-eight histopathological preparations stained with hematoxylin and eosin. The samples had been prepared from the liver, kidney, spleen, and skeletal muscles. Their age varied from one to seventeen years. Specimens (from twelve species) were identified by inputting sequences in the Barcode of Life Database species identification tool on the basis of the similarity percentage figure from the BOLD report. It was found that genetic tests can effectively identify animal species through the analysis of biological material from histopathological samples.**

**Keywords: animal species affiliation, DNA analysis, histopathology, forensic veterinary medicine**

Over the recent years, there has been a systematic increase in the number of expert opinions in forensic veterinary medicine admitted as evidence by procedural authorities (2, 10, 12, 15, 16). In some cases, it is necessary to determine the animal species (15). In the case of a live animal or a complete animal corpse, identification procedures are not a problem. With regard to dead animals, species identification is carried out on the basis of morphological characteristics, such as external appearance or unique anatomical features (5). For example, the identification of a Eurasian wild boar (*Sus scrofa*) is possible on the basis of bristles, covering both the head and the distal parts of the limbs. In addition, this species can be identified by the color of its hooves, which are black, unlike the hooves of domestic pigs, which are white (13). In the case of fragments of an animal corpse, the determination of the species is based primarily on differences in anatomical structure. For example, in the wild boar's humerus there is the supratrochlear foramen (13). Bone histology offers a potentially valuable tool for species identification through a microscopic comparison of bone cross-section when only fragments or portions of bones remain (14).

If macroscopic or microscopic determination is impossible, it is necessary to perform laboratory tests. The first group of tests consists of immunoassays based on a specific reaction of species-specific antigens with appropriate antibodies (17), for example, precipitation tests. A serum directed against the protein of a specific animal species causes a reaction resulting in the precipitation of the antigen-antibody complex. The

precipitation is performed in agar gel. A disadvantage of this method is the possibility of cross-reactivity, or a positive reaction of antibodies to antigens derived from closely related species, such as wild boars and pigs (11). However, the ELISA test (enzyme-linked immunosorbent assay) makes it possible to detect proteins in biological material with the use of species-specific polyclonal or monoclonal antibodies, conjugated with an appropriate enzyme (8).

In many cases exact identification based on fragments of biological material is extremely difficult. This applies both to closely related species and to distant species characterized by similar morphological features (5). Then, molecular biology methods should be used. Their evidential value is not in doubt (1, 6, 7, 21, 23, 24).

Molecular tests identifying animal species can use various techniques: Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) (1), and real-time PCR (19). Currently, these methods are rarely used due to their low reproducibility and interpretation problems. However, the development of molecular biology techniques has created new possibilities for the identification of species and individuals (4, 24, 25). It should be noted that a 648 bp fragment of the gene encoding cytochrome oxidase (COI, coxI), located in the mitochondrial genome (DNA barcoding), is currently used for this purpose. This fragment is short enough for sequencing, while showing low intraspecific and high interspecific variation (18). The obtained and processed data are stored in a special public BOLD database (20). It is confirmed that 98% of species currently classified can be distinguished using this method (9). Another advantage of DNA barcoding is the possibility of conducting tests on a degraded material (6).

The aim of the present study was to verify the possibility of using DNA analysis to determine the species of animals on the basis of material from archival hisopathological samples.

## Material and methods

The material consisted of histopathological samples prepared in a time interval of one to seventeen years before the present study. All tests were carried out at the Department of Pathophysiology, Veterinary Forensic Medicine and Administration, University of Warmia and Mazury in Olsztyn, Poland. The samples had been used in research conducted for public institutions and private entities, as well as in scientific experiments. Samples of the liver, kidney, spleen and skeletal muscle sections of twelve animal species were analyzed (Tab. 1). Sections of these organs were fixed in buffered formalin, and 10 µm scraps, after typical preparation, were stained with hematoxylin and eosin (3).

**DNA extraction.** Genomic DNA from archived histopathological specimens was extracted using a NucleoSpin FPPE Kit (Macherey-Nagel, Düren), following the manufacturer's instructions. For each sample, DNA was eluted in 100 µl $H_2O$. Extracted DNA was stored at –20°C.

**Tab. 1. List of specimens analyzed**

| Ordinal No | Specimen No | Species | Organ | Year of collection |
|---|---|---|---|---|
| 1. | 5871 | *Silurus glanis* | liver | 2001 |
| 2. | 8987 | *Oncorhynchus mykiss* | liver | 2011 |
| 3. | 8896 | *Oncorhynchus mykiss* | kidney | 2001 |
| 4. | 7148 | *Acipenser sturio* | liver | 2006 |
| 5. | 7184 | *Acipenser sturio* | kidney | 2006 |
| 6. | 7137 | *Acipenser sturio* | spleen | 2006 |
| 7. | 7270 | *Cyprinus carpio* | liver | 2006 |
| 8. | 7719 | *Cyprinus carpio* | liver | 2007 |
| 9. | 6739 | *Vulpes vulpes* | liver | 2005 |
| 10. | 6288 | *Gallus gallus domesticus* | liver | 2003 |
| 11. | 6200 | *Meleagris gallopavo* | liver | 2003 |
| 12. | 6954 | *Meleagris gallopavo* | liver | 2005 |
| 13. | 6208 | *Meleagris gallopavo* | muscle | 2003 |
| 14. | 6202 | *Meleagris gallopavo* | spleen | 2003 |
| 15. | 6210 | *Meleagris gallopavo* | kidney | 2003 |
| 16. | 8630 | *Felis silvestris domesticus* | liver | 2009 |
| 17. | 12097 | *Felis silvestris domesticus* | liver | 2016 |
| 18. | 12098 | *Felis silvestris domesticus* | spleen | 2016 |
| 19. | 12296 | *Felis silvestris domesticus* | spleen | 2017 |
| 20. | 12298 | *Felis silvestris domesticus* | muscle | 2017 |
| 21. | 12100 | *Felis silvestris domesticus* | kidney | 2016 |
| 22. | 8262 | *Canis lupus familiaris* | liver | 2006 |
| 23. | 11408 | *Canis lupus familiaris* | liver | 2009 |
| 24. | 11086 | *Canis lupus familiaris* | liver | 2014 |
| 25. | 12272 | *Canis lupus familiaris* | liver | 2017 |
| 26. | 12306 | *Sus scrofa domestica* | muscle | 2017 |
| 27. | 7456 | *Apodemus flavicollis* | liver | 2006 |
| 28. | 7857 | *Apodemus agrarius* | liver | 2007 |

**Amplification and sequencing.** A fragment of the COI gene was amplified using two sets of primers: LTyr – COI907aH2 (22) and MHemF – dgHCO-2198 (4). LTyr – COI907aH2 primer set amplified a fragment of about 1000 bp, while MHemF – dgHCO-2198 amplified about 400 bp of the COI gene. The PCR primers were supplied by Sigma-Aldrich (Milwaukee, WI, USA). The amplification of the LTyr – COI907aH2 fragment was performed as previously described (22). In short, 2.0 µl DNA template was added to 20.0 µl RedTaq Ready Mix (Sigma-Aldrich, Milwaukee, WI, USA) and 8.0 µl of the corresponding primer (5.0 µM), and then $H_2O$ was added to a total volume of 40.0 µl. All PCR reactions were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) as follows: an initial denaturation step of 94°C for 5 min, followed by 40 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 60 s, with a final incubation for 5 min at 72°C.

The amplification of the MHemF – dgHCO-2198 fragment was performed as previously described (4). The

reaction set was the same as for the LTyr – COI907aH2 fragment. The thermal profile was as follows: an initial denaturation step of 94°C for 3 min, followed by 5 cycles of 94°C for 30 s, 45°C for 20 s, and 72°C for 30 s; next 40 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 105 s, with a final incubation for 10 min at 72°C.

Amplicons were visualized by UV illumination after gel electrophoresis and Midori Green (Nippon Genetics Europe, Duren, Germany) staining. Excess dNTPs and unincorporated primers were removed from the PCR product with a Clean-Up Purification Kit (A&A Biotechnology, Gdynia, Poland). As a final purification step, the purified DNA was eluted in 40 µl $H_2O$.

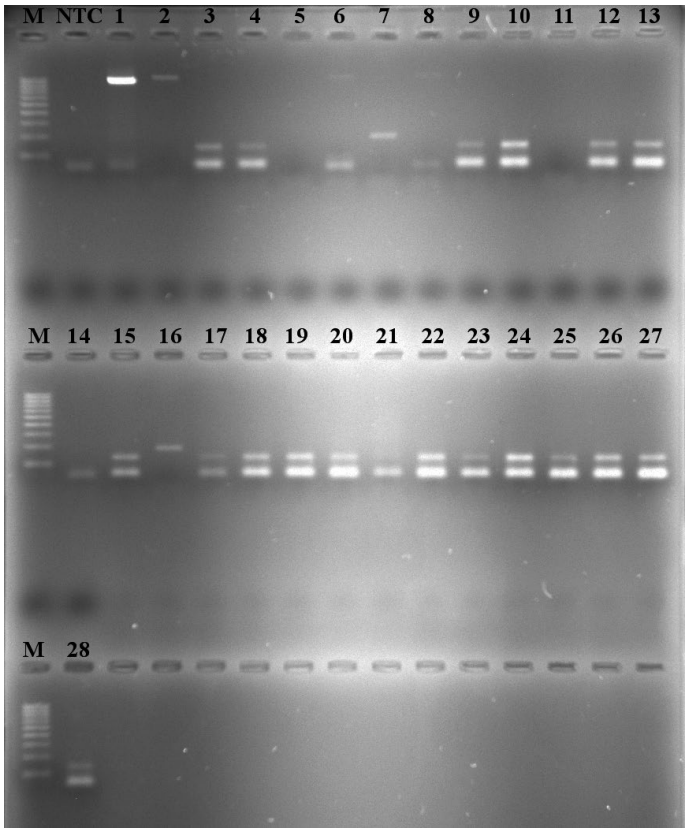



**Fig. 1. Amplification of DNA samples with LTyr – COI907aH2 primers**
Explanations: M – mass marker (100 bp); NTC – non-template control; 1-28 – DNA samples (*vide* Tab. 1)

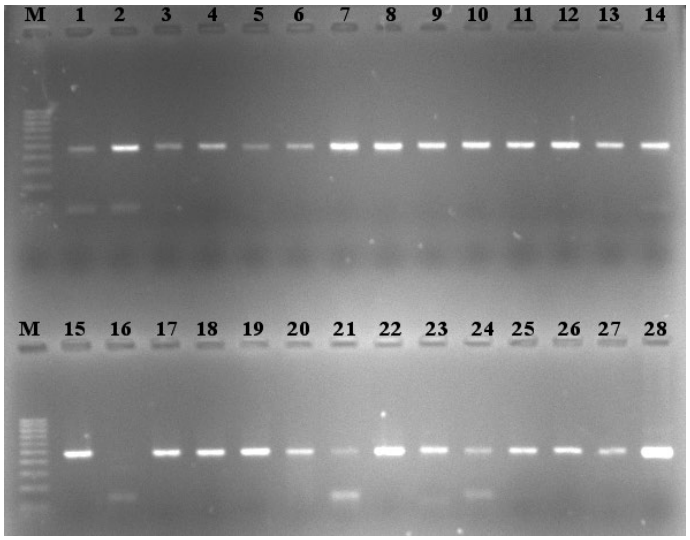



**Fig. 2. Amplification of DNA samples with MHemF – dgHCO--2198 primers**
Explanations: M – mass marker; 1-28 – DNA samples (*vide* Tab. 1)

Sequencing PCR reactions involved 1.0 µl BigDye Terminator v. 3.1 Ready Reaction Mix (ThermoFisher Scientific, Waltham, MA, USA), 2.0 µl BigDye sequencing buffer (ThermoFisher Scientific, Waltham, MA, USA), 1.0 µl (5 µM) forward or 1.6 (10 µM) reverse primer, and $H_2O$ to a total volume of 10 µl. The thermal profile for sequencing reactions consisted of an initial denaturation step of 96°C for 1 min followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 105 s. COI fragments were sequenced with an ABI 3500xL genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Identification procedures consisted of inputting sequences in the Barcode of Life Database species identification tool http://www.barcodinglife.org (20). Specimen identification was based on the similarity percentage figure from the BOLD report.

## Results and discussion

To examine the suitability of the COI sequence as a diagnostic tool for identification of animals involved in traffic accidents, twenty-eight samples (belonging to fish, birds, and mammals) were analyzed. The DNA extracted using the FFPE showed an amplification success rate of only about 10% for the LTyr – COI907aH2 amplicon ~ 1000 bp (Fig. 1) and 96% for the amplicon MHemF – dgHCO-2198 ~400 bp (Fig. 2). As the amplicon length decreased from ~1000 to ~400 bp, amplification success increased to almost 100%. The age (1-17 years) and tissue type of the specimens did not affect amplification success.

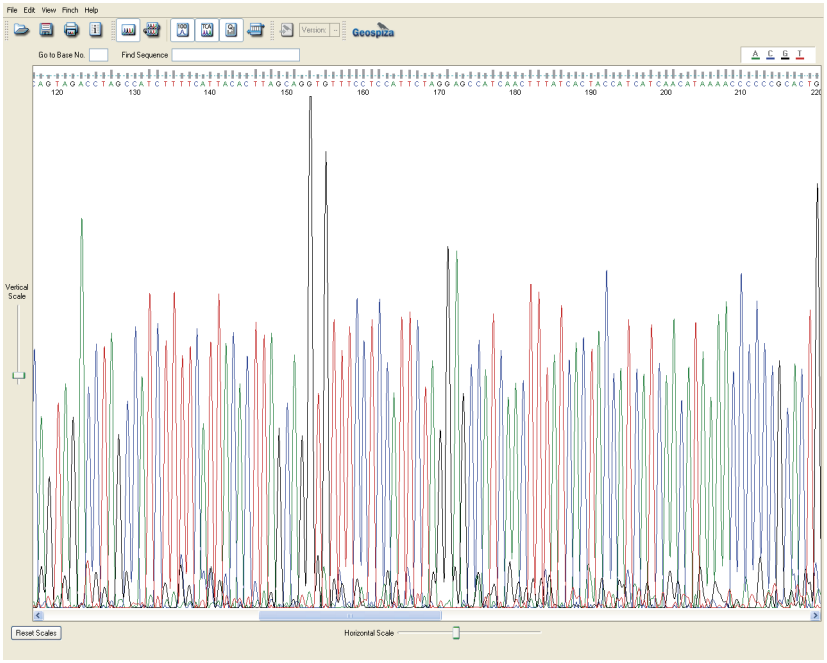
The quality of DNA obtained using MHemF – dgHCO--2198 barcoding primers for four different animal specimens was suitable for genetic (PCR) analysis (Fig. 2), which suggests that the protocol is applicable to animal tissues. Further, we used the amplified DNA in a sequencing reaction to identify specimens collected in the field. Because COI sequences of the investigated species are already available in the BOLD and GenBank databases, the sequences obtained were not deposited in these databases. By sequencing this amplicon and searching the BOLD database, we successfully identified the scientific names of the specimens (Fig. 3).

Summing up the results of the present research, it can be concluded that, in cases where the animal species cannot be identified by macroscopic examination, genetic tests should be performed. Such tests are capable of effective and reliable animal species identification, even on the basis of degraded biological material, such as histopathological preparations.

## References

1. *Alford L. R., Caskey C. T.*: DNA analysis in forensics, disease and animal/plant identification. Curr. Opin. Biotechnol. 1994, 5, 29-33.
2. *Babińska I., Kusiak D., Szarek J., Lis A., Gulda D., Felsmann M. Z., Łyko A., Maciejewska M., Popławski K., Szweda M.*: Veterinary expert opinions on conflicts involving dogs and cats in Poland. Int. J. Forensic Sci. Pathol. 2017, 5, 347-351.

A



B

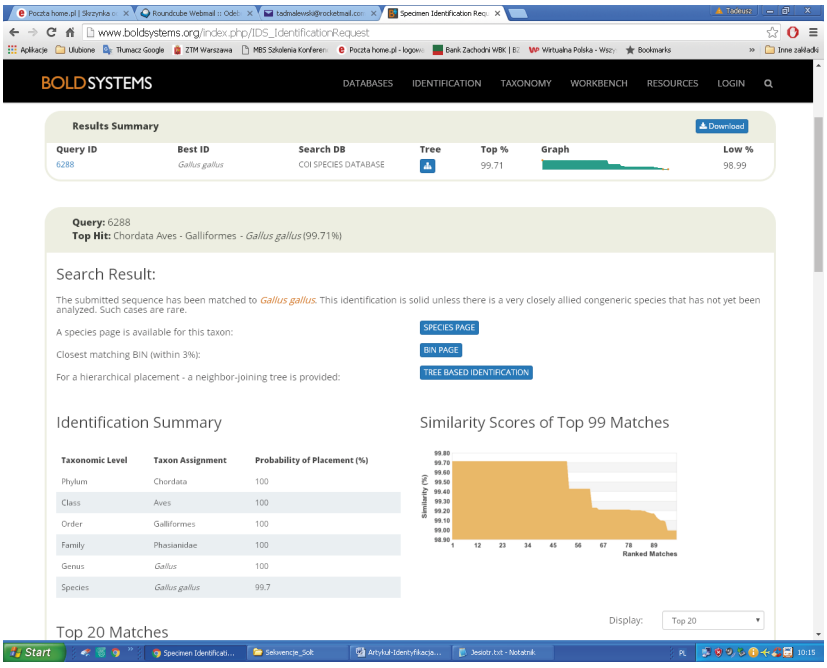



**Fig. 3. Specimen identification by the BOLD identification tool: A. Fragment of the COI sequence of specimen 6288 (*vide* Tab. 1); B. Screenshot of specimen identification by the BOLD search engine**


3. *Bancroft J. D., Gamble M.*: Theory and Practice of Histological Techniques. Elsevier, Churchill – Livingstone 2002.
4. *Beng K. C., Tomlinson K. W., Shen X. H., Surget-Groba Y., Hughes A. C., Corlett R. T., Slik J. W.*: The utility of DNA metabarcoding for studying the response of arthropod diversity and composition to land-use change in the tropics. Sci. Rep. 2016, 6, 24965.
5. *Boroń A., Szlachciak J., Kirtiklis L.*: Cechy taksonomiczne zwierząt i metody ich analizowania, [in:] Boroń A., Szlachciak J. (ed.): Podręcznik metodyczny. Różnorodność i taksonomia zwierząt. vol. 1. Charakterystyka i systematyka zwierząt. Wydawnictwo Mantis, Olsztyn 2013, p. 22-25.
6. *Girish P. S., Anjaneyulu A. S., Wiswas K. N., Anand M., Rajkumar N., Shiwakumar B. M., Bhaskar S.*: Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. Meat Sci. 2004, 66, 551-556.
7. *Holland M. M., Parson T. J.*: Mitochondrial DNA sequence analysis – validation and use for forensic casework. Forensic Sci. Rev. 1999, 11, 21-50.
8. *Lequin R. M.*: Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). Clin. Chem. 2005, 51, 2415-2418.
9. *Koroiva R., Pepinelli M., Rodrigues M. E., Roque F. O., Lorenz-Lemke A. P., Kvist S.*: DNA barcoding of odonates from the Upper Plata basin: database creation and genetic diversity estimation. PLoS One 2017, 12, e0182283, doi: 10.1371/journal.pone.0182283.
10. *Listos P., Gryzinska M., Kowalczyk M.*: Analysis of forensic veterinary opinions produced in a research and teaching unit. J. Forensic Leg. Med. 2015, 36, 84-89.
11. *Marek Z., Jaegermann K., Turowska B.*: Oznaczanie gatunkowej przynależności białek przy pomocy precypitacji w polu elektrycznym w żelu agarowym (elektroimmunoprecypitacja). Folia Med. Cracov. 1964, 6, 83-91.
12. *McEwen B. J.*: Trends in domestic animal medico-legal pathology cases submitted to a veterinary diagnostic laboratory 1998-2010. J. Forensic Sci. 2012, 57, 231-233.
13. *Mayer J.*: Biology of wild pigs: wild pig physical characteristics, [in:] Mayer J. J., Brisbin I. L. (ed.): Wild pigs: biology, damage, control techniques and management. Savannah River National Laboratory, Aiken 2009, p. 31-50.
14. *Morris Z. H.*: Quantitative and spatial analysis of the microscopic bone structures of deer (*Odocoileus virginianus*), dog (*Canis familiaris*), and pig (*Sus scrofa domesticus*). LSU Master's Theses. Louisiana, USA 2007.
15. *Newbery S. G., Cooke S. W., Martineau H. M.*: A perspective on veterinary forensic pathology and medicine in the United Kingdom. Vet. Pathol. 2016, 53, 894-897.
16. *Ottinger T., Rasmusson B., Segerstad C. H., Merck M., Goot F. V., Olsen L., Gavier-Widen D.*: Forensic veterinary pathology, today's situation and perspectives. Vet. Rec. 2014, 175, 459.
17. *Pauling L., Campbell H. D., Pressman D.*: The nature of the forces between antigen and antibody and of the precipitation reaction. Physiol. Rev. 1943, 23, 203-219.
18. *Pečnikar Ž. F., Buzan E. V.*: 20 years since the introduction of DNA barcoding: from theory to application. J. Appl. Genetics 2014, 55, 43-52.
19. *Pegels N., González I., Fernández S., Garcia T., Martin R.*: Sensitive detection of porcine DNA in processed animal proteins using a TaqMan real-time PCR assay. Food Addit. Contam. Part A 2012, 29, 1402-1412.
20. *Ratnasingham S., Hebert P. D. N.*: BOLD: the barcode of life data system. Mol. Ecol. Notes 2007, 7, 355-367.
21. *Tartaglia M., Saulle E., Pestalozza S., Morelli L., Antonucci G., Battaglia P. A.*: Detection of bovine mitochondrial DNA in ruminant feeds: a molecular approach to test for the presence of bovine-derived materials. J. Food Prot. 1998, 5, 513-518.
22. *Tavares E. S., Allan J., Baker A. J.*: Single mitochondrial gene barcodes reliably identify sister-species in diverse clades of birds. BMC Evol. Biol. 2008, 8, 81.
23. *Unseld M., Beyermann B., Brant P., Hiesel R.*: Identification of species origin of highly processed meat products by mitochondrial DNA sequences. PCR Methods Appl. 1995, 4, 241-243.
24. *Weiner A., Paprocka I., Gołębiowska A., Kwiatek K.*: Zastosowanie metody Real-time PCR do identyfikacji DNA przeżuwaczy w surowych produktach pochodzenia zwierzęcego. Med. Weter. 2018, 74, 272-275.
25. *Yang L., Tan Z., Wang D., Xue L., Guan M. X., Huang T., Li R.*: Species identification through mitochondrial rRNA genetic analysis. Sci. Rep. 2014, 4, 4089.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