DOI 10.32900/2312-8402-2025-134-128-137
UDC 575:595:592/599:631.4

GENETIC IDENTIFICATION OF EARTHWORMS FROM THE FAMILY *LUMBRICIDAE*: *EISENIA FETIDA* AND *DENDROBAENA VENETA*

Konstantin POCHERNYAEV, Doctor of Agricultural Sciences, Senior Researcher
https://orcid.org/0000-0001-9973-6429
Institute of Pig Breeding and Agro-Industrial Production of NAAS,
Poltava, Ukraine

The effectiveness of vermiculture using earthworms largely depends on the species. In Ukraine, the cultivation of the red California worm, commonly recognised as Eisenia fetida, is becoming increasingly popular. Although this name typically refers to this specific species, Dendrobaena veneta is sometimes included as well. While both species are utilised in vermiculture for industrial production, they are indeed different types of worms. The sale of brood stock, regardless of the name used, relies primarily on physical characteristics assessed through visual inspection. However, trying to identify earthworm species by their physical features alone is often a guessing game, since these creatures lack the intricate and distinctive structures found in other animals. Tracing the origins of products made from such earthworms becomes an even greater challenge. To reduce potential errors and prevent misuse, a genetic method has been developed to identify two specific earthworm species using single-nucleotide polymorphisms (SNPs) in the mitochondrial COI gene. Research was performed on four species of earthworms from the Lumbricidae family at the genetics laboratory of the Institute of Pig Breeding and AIP of the NAAS. Forward primers and reverse primers targeting the cytochrome c oxidase (COI) region were developed for the mitochondrial genome of E. fetida, D. veneta, and other representatives of the Lumbricidae family. In the forward primer sequences of E. fetida and D. veneta, one nucleotide substitution was observed in the reverse three. Despite this, specific PCR products were obtained using these primer pairs for the species E. fetida, E. andrei, D. veneta and Lumbricus terrestris. Nevertheless, specific PCR products were successfully obtained using these primer pairs for the species E. fetida, E. andrei, D. veneta, and L. terrestris. The size of the amplification products, which is 253 base pairs, matches the length predicted from the nucleotide sequence. An analysis of restriction fragments from amplified mitochondrial COI gene DNA samples of four species from the Lumbricidae family revealed three distinct sets of bands. As expected, the restriction fragments for E. fetida and E. andrei were identical. In contrast, the restriction fragments of D. veneta exhibited different characteristics, as predicted by bioinformatic analysis. Furthermore, it was confirmed that DNA from L. terrestris is suitable for use as a negative control. Therefore, the proposed method for genetic identification of the earthworm species E. fetida and D. veneta using PCR-RFLP may be useful for routine analyses.

Keywords: DNA, PCR-RFLP, SNP, *Eisenia andrei*, *Eisenia fetida*, *Dendrobaena veneta*, *COI*.

ГЕНЕТИЧНА ІДЕНТИФІКАЦІЯ ДОЩОВИХ ЧЕРВ'ЯКІВ РОДИНИ *LUMBRICIDAE*: *EISENIA FETIDA* ТА *DENDROBAENA VENETA*

Костянтин ПОЧЕРНЯЄВ, д. с.-г. н., с. н. с.
https://orcid.org/0000-0001-9973-6429
Інститут свинарства і агропромислового виробництва НААН
м. Полтава, Україна

Ефективність вермикультури дощових черв'яків значно залежить від виду. В Україні набирає популярності вирощування дощових черв'яків відомих під назвою червоний каліфорнійський черв'як. Хоча під цією тривіальною назвою фахівці розуміють Eisenia fetida інколи до цього включають і Dendrobaena veneta. Звичайно це різні види черв'яків, хоча обидва використовуються в вермикультурі для промислового виробництва. Продаж материнського поголів'я під тією чи іншою назвою базується виключно на фізичних характеристиках, які досліджуються за допомогою візуального огляду. Однак спроба ідентифікувати види дощових черв'яків лише за їхніми фізичними ознаками часто є грою вгадування, оскільки цим істотам бракує складних та характерних структур, характерних для інших тварин. Відстеження походження продуктів, виготовлених з таких дощових черв'яків, стає ще більшим завданням. Щоб виключити потенційні помилки та зловживання, було розроблено генетичний метод ідентифікації двох видів дощових черв'яків заснований на визначені SNP мітохондріального гена COI. Дослідження чотирьох видів дощових черв'яків родини Lumbricidae виконані в лабораторії генетики Інституту свинарства і АПВ НААН. Для мітохондріального геному E. fetida, D. veneta та інших представників родини Lumbricidae було розроблено послідовності прямих та праймерів для області цитохром-с-оксидази (COI). У послідовностях прямих праймерів E. fetida та D. veneta спостерігалася одна нуклеотидна заміна у зворотних три. Незважаючи на це, з використанням цих пар праймерів для видів E. fetida, E. andrei, D. veneta та Lumbricus terrestris отримані специфічні ПЛР-продукти. Розмір продуктів ампліфікації 253 пар основ відповідає довжині, розрахованій за нуклеотидною послідовністю. Аналіз рестрикційних фрагментів з ампліфікованого мітохондріального гена COI зразків ДНК чотирьох видів родини Lumbricidae виявив три різні набори смуг. Як і очікувалося, рестрикційні фрагменти для E. fetida та E. andrei були ідентичними. Натомість, рестрикційні фрагменти D. veneta демонстрували різні характеристики, що було передбачено біоінформаційним аналізом. Крім того, було підтверджено, що ДНК з L. terrestris є придатною для використання як негативний контроль. Таким чином, запропонований спосіб генетичної ідентифікація видів дощових черв'яків E. fetida та D. veneta методом ПЛР-ПДРФ може бути корисним для рутинних аналізів.

Ключові слова: ДНК, ПЛР-ПДРФ, SNP, *Eisenia andrei, Eisenia fetida, Dendrobaena veneta, COI.*

Introduction. Organic waste management is a growing issue due to the unsustainable practices of its disposal. Sewage treatment plants are designed to treat wastewater to produce a safe effluent. However, one of the by-products, the sewage sludge which is disposed of in landfill or used as fertilizer in agricultural operation is high in pathogens. Sustainability can be achieved by Vermicomposting of organic matter which involves accelerated cycling of nutrients though a closed cycle whereby waste

products are put to productive end use. Vermicomposting and vermifiltration are natural waste management processes relying on the use of worms to convert organic wastes to stable soil enriching compounds. Domestic wastewater management can be accommodated through these processes in a sustainable manner. A considerable reduction in pathogens has been noticed in the end product to a level that it can be safely applied to land. This paper provides an overview of the system characteristics of management systems utilising vermiculture, to manage wastewater. The process can be used in a small scale for household waste treatment to rural or urban waste management (Bajsa O. et al., 2023).

The soil ecosystem is one of the most vital ecological systems on Earth, playing a crucial role in maintaining and regulating biogeochemical cycles. This is largely due to the presence of various organisms, including earthworms, microorganisms, and fungi, which function as reducers or sinks in these cycles. Additionally, the soil serves as a critical infrastructure for agricultural ecosystems, supporting many economically important crop production systems that rely on its fertility and nutrients (Dhakane R. & Shinde A. 2020).

Although earthworms are biologically and economically significant, the taxonomic status and evolutionary relationships of most *Lumbricidae* families continue to be debated. Furthermore, earthworms demonstrate considerable cryptic diversity, which adds complexity to this issue (Latif R. et al., 2020). In addition, there are hybrids, such as *E. fetida* and *E. andrei*, which require species-specific sequences of both the maternal mitochondrial *COI* gene and the maternal-paternal nuclear *28S* gene for identification (Jaskulak M. et al., 2022).

The importance of the correct identification of earthworm species lies in the differences in response to different chemical and physical factors. For example, vermicomposting is applied to different sewage sludges. *D. veneta* in experimental conditions shows much lower resistance. Their body weight and total number of circulating immune cells are reduced in the most polluted conditions. Eisenia sp. worms showed the highest ability to accumulate heavy metals (Suleiman H. et al, 2017).

Also, pharmaceuticals released into aquatic and soil environments can be taken up by plants and soil organisms, potentially leading to the formation of unknown metabolites that may adversely affect these organisms or contaminate the food chain. A study by Fučík J, et al. (2024) determined that the earthworms *E. fetida* and *Lactuca sativa* showed the highest intensities of metabolic reactions, followed closely by roots, and the leaves showed the lowest intensities. Common metabolic reactions observed included hydroxylation, decarboxylation, acetylation and glucosidation, with metabolites related to ketoprofen being the most abundant (Fučík J. et al., 2024).

Vermicompost, commonly referred to as bio humus, is a valuable organic fertiliser created during the decomposition of organic waste by earthworms, primarily *E. foetida* and *D. veneta*, along with other soil microorganisms. As it turned out, this organic fertiliser can also be used as a bio additive to pig feed. Fomichenko M., (2025) found that the introduction of vermicompost obtained using *E. foetida* into the diet of weaned piglets had a positive effect on their growth energy and survival. In terms of live weight, weaned piglets exceeded control counterparts at the age of 60 days by 13, 11 and 6.96%, and at 90 days by 14.2 and 11.52%, respectively (Fomichenko M. 2025).

Finally, earthworms are attracting increasing attention as potential next-generation protein production systems, especially for biopharmaceuticals and animal proteins. For example, Akazawa S.I. et al. (2021) reported the development of the earthworms *E. fetida* and *E. andrei* for the production of a new generation of animal protein. They developed a transfection method for *E. fetida* and *E. andrei* using

microinjection and electroporation systems. The maximum survival and transfection efficiency were 79.2% and 29.2% for *E. fetida*, and 95.8% and 50.0% for *E. andrei*, respectively. As a result, human erythropoietin was detected in the transformed earthworm fragments by enzyme-linked immunosorbent assay. These results contribute to the development of a potentially novel animal protein production system based on earthworms (Akazawa S.I. et al. 2021). An analysis of the publications allows us to conclude that a key characteristic of vermiculture products is the species of earthworm used. There is a clear need for straightforward and dependable methods to identify earthworms, which we aimed to address in this study.

Thus, this study aims to develop a PCA-RFLP method for identifying the earthworm species *E. fetida* and *D. veneta*. This will involve a bioinformatic analysis establishing specific molecular genetic characteristics by determining the single-nucleotide polymorphisms in the mitochondrial COI gene. Additionally, we aim to assess the effectiveness of this method for the genetic identification of these earthworm species using control DNA samples.

Materials and methods. Bioinformatic analysis of six species of the genus *Eisenia*, namely – *E. andrei* (KM823569), *E. fetida* (MT271119), *E. nordenskioldi pallida* (KU708411), *E. nordenskioldi* (JX531495), *E. nordenskioldi* (JX531494) and *E. japonica* (AB542698), as well as species of four other families – *D. veneta* (KM823561), *Aporrectodea caliginosa* (KX400650), *Hormogaster huescana* (HQ621980), *Haplotaxida sp.* (HM400434) were performed by aligning nucleotide sequences using the BLAST program: Basic Local Alignment Search Tool (Altschul S.F. et al., 1990). The primer sequences specific to *D. veneta* and *E. fetida* were designed using the Primer-BLAST program (Ye J., et al. 2012). The primers EISF: GGTGGATTTGGAAACTGACT and EISR: CTAAAATTGAGGAGGCACCTG were synthesised (Metabion International AG, Germany). The search for restriction enzymes for SNP analysis was performed using the GenScript Biotech Corporation online resource (GenScript Biotech Corporation).

Control DNA samples of *E. fetida* and *E. andrei* were obtained from Dr. Jörg Römbke at ECT Oekotoxikologie in Flörsheim am Main, Germany, and Professor Barbara Plytycz from the Department of Evolutionary Immunobiology at the Institute of Zoology, Jagiellonian University, Kraków, Poland. Live samples of *D. veneta* and *E. fetida* were sourced from the entrepreneur N.L. Fomichenko, Zaporizhia, Ukraine. The common earthworm (*L. terrestris*) served as a negative control, with samples collected in Poltava, Ukraine (GPS coordinates 49.631625, 34.549721).

Before isolating DNA from live worms, they were euthanised by freezing at -20°C for 3 to 4 hours. DNA was extracted from the worm tissue using the DNA-sorb-B nucleic acid isolation kit following the manufacturer's instructions (InterLabServis-Ukraina, LTD).

DNA amplification was performed on a programmable thermostat (Biometra GmbH), with annealing of primers under conditions (t = 60°C). Restriction digestion of PCR products was performed using *Tas*I endonuclease (Thermo Scientific™) under the manufacturer's conditions.

The restriction digestion products were separated by electrophoresis on an 8% polyacrylamide gel in 1×TBE buffer, using pUC19/*Msp*I DNA (Thermo Scientific™) as a molecular weight marker. The gel was stained with ethidium bromide solution (10 mg/cm^3). The results were documented using the MicroDOC Gel Documentation System (Cleaver Scientific).

Research results. Forward primer sequences were developed for the cytochrome c oxidase (*COI*) region of the mitochondrial genome for the species *E. fetida*, *D. veneta*,

and other members of the family *Lumbricidae*. A single nucleotide substitution was observed in the forward primer sequences of *E. fetida* and *D. veneta*. Additionally, the forward primer sequences for *E. fetida* and *E. andrei* were found to be identical (Tab. 1).

Table 1.

Sequence of forward primers for *Eisenia fetida, Dendrobaena veneta* and other members of the family *Lumbricidae*

An earthworm species	Nucleotide accession from NCBI Datasets	Forward primer sequences
Eisenia fetida	MT271119	GGTGGATTTGGAAACTGACT
Dendrobaena veneta	KM823561	* GGAGGATTTGGAAACTGACT
Eisenia andrei	KM823569	GGTGGATTTGGAAACTGACT
Lumbricus terrestris	PV871429	* * GGCGGGTTTGGAAACTGACT
Eisenia nordenskioldi pallida	KU708411	* * GGGGGTTTTGGAAACTGACT
Eisenia japonica	AB542698	* * * GGGGGGTTTGGAAATTGACT
Hormogaster huescana	HQ621980	* * * GGGGGATTTGGAAATTGATT
Aporrectodea caliginosa	KX400650	* * * * GGCGGCTTCGGAAATTGACT

Note. Nucleotide mismatches with the Eisenia fetida sequence (MT271119) are indicated with an asterisk.

Three nucleotide substitutions were identified in the reverse primer sequences of *E. fetida* and *D. veneta*. The forward primer sequences of *E. fetida* and *E. andrei* were identical, similar to the forward primers (Tab. 2).

Table 2.

Sequence of forward primers for *Eisenia fetida, Dendrobaena veneta* and other members of the family *Lumbricidae*

An earthworm species	Nucleotide accession from NCBI Datasets	Reverse primer sequences
Eisenia fetida	MT271119	CAGGTGCCTCCTCAATTTTAG
Dendrobaena veneta	KM823561	* * * CAGGGGCCTCATCAATCTTAG
Eisenia andrei	KM823569	CAGGTGCCTCCTCAATTTTAG
Lumbricus terrestris	PV871429	* * * * CAGGTGCATCCTCTATTCTGG
Eisenia nordenskioldi pallida	KU708411	* * * * ** * CTGGAGCTTCATCAATCCTTG
Eisenia japonica	AB542698	* * * * * * CTGGAGCCTCATCTATTCTTG
Hormogaster huescana	HQ621980	* * * CAGGAGCCTCATCTATTTTAG
Aporrectodea caliginosa	KX400650	* * * * * ** * CTGGAGCTTCATCTATCCTTG

Note. The sequence of the reverse primers is provided for the plus strand of DNA; nucleotide mismatches with the Eisenia fetida (MT271119) sequence are indicated with an asterisk.

Amplification of a fragment of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene from DNA samples of four species within the *Lumbricidae* family resulted in PCR products measuring 253 base pairs in size. The size of the amplified products matches the calculated size derived from the nucleotide sequence. This finding indicates that the developed primer pair is effective for studying at least four species of earthworms in this family (Fig. 1).

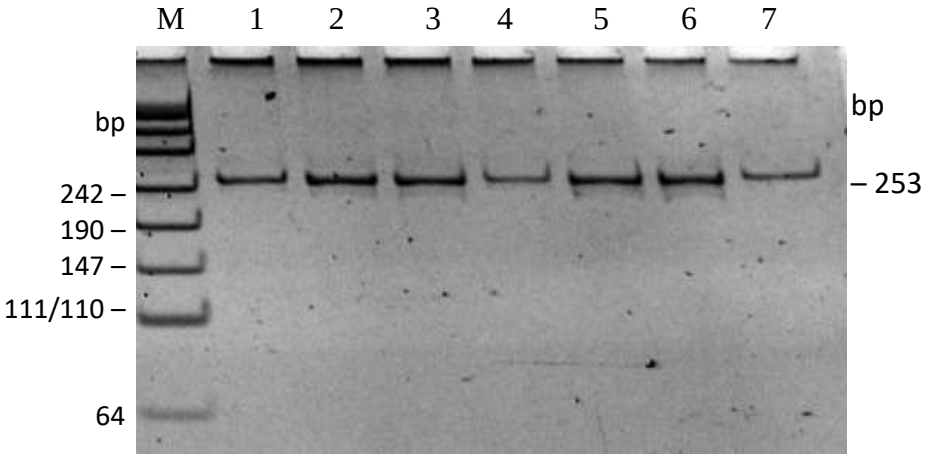

Fig. 1. ***Amplification products of a 253 bp fragment of the mitochondrial COI gene: M – pUC19/MspI DNA marker; 1,2,3,4 – DNA from Eisenia fetida; 5 – DNA of Lambricus terrestris 6 – DNA from Dendrobaena veneta; 7 – DNA of Eisenia andrei.***

An analysis of the restriction fragments of the amplified mitochondrial COI gene of DNA samples of four species from the *Lumbricidae* family revealed three distinct band sets. As anticipated, the *Tas*I restriction fragments for *E. fetida* and *E. andrei* were identical. In contrast, the *Tas*I restriction fragments for *D. veneta* displayed a clear and distinct identity, confirming the expected made through bioinformatic analysis. The *Tas*I restriction fragments of DNA from *E. fetida* and *E. andrei* measured 144, 101, and 8 bp. The sizes of the restriction fragments obtained correspond precisely to those calculated from the nucleotide sequence (Fig. 2).

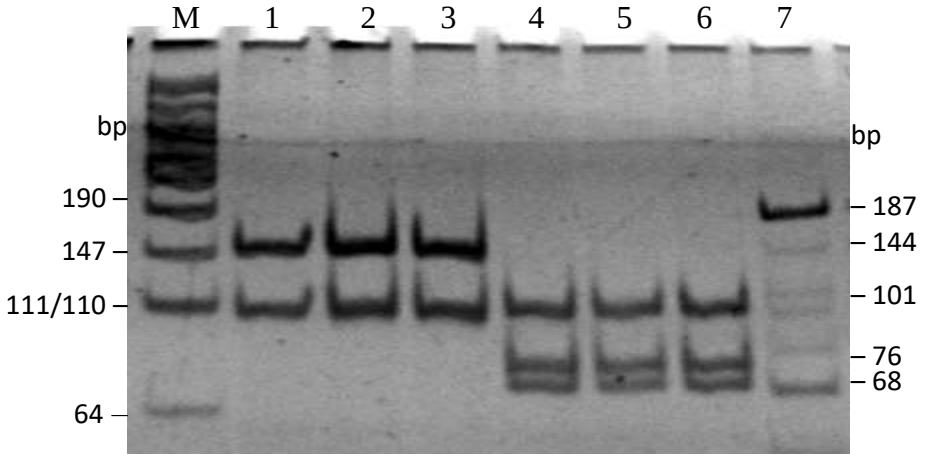

Fig. 2. ***Tas1 electrophoresis of restriction DNA fragments of earthworms of the Lumbricidae family: M – pUC19/MspI DNA marker; 1,2, – DNA Eisenia fetida; 3 – DNA Eisenia andrei; 4,5,6 – DNA Dendrobaena veneta; 7 – DNA Lambricus terrestris***.

The *Tas*I restriction fragments of *D. veneta* DNA measured 101, 76, 68, and 8 bp in size. However, the observed fragment sizes did not completely align with the expected

sizes based on the nucleotide sequence KM823561. According to the calculations derived from the nucleotide sequence KM823561, with have expected one fragment of 101 base pairs and two fragments of 76 bp.

The difference from what was expected is the incorporation of the reverse primer EISR: CAGGTGCCTCCTCAATTTTAG into the PCR product (the sequence is provided for the plus strand of DNA). This insertion creates an artificial *Tas*I (AATT) recognition site in the *D. veneta* DNA PCR product, resulting in the formation of restriction fragments of 76 and 8 base pairs.

L. terrestris DNA is confirmed as a suitable negative control. The application of an 8% polyacrylamide gel permits effective separation of TasI restriction fragments via vertical electrophoresis.

Discussion. Both *E. fetida* and *D. veneta* are epigean (surface) worms. These species live in organic-rich places such as manure piles, compost pits, and decaying plants. However, *D. veneta* is capable of penetrating deeper into the soil.

D. veneta, also known as *Eisenia hortensis*, is a species of earthworm in the family *Lumbricidae*. Daniele Rosa (1857-1944), the renowned Italian invertebrate zoologist celebrated for his pioneering studies of oligochaetes, was the first to describe *D. veneta* (Rosa, 1886). While the Latin word "veneta" conjures images of sea blue or blue-green, the name pays homage to its discovery site, Campo di Marte in Venice. Rosa described its colouration as matching that of *E. fetida*: a ruddy hue streaked with yellow bands between the segments, making these two species frequent sources of confusion. However, *D. veneta* stands apart with its broader setae, which glow clearly under blue light, and a clitellum that is typically pale and whitish, unlike the more ruddy clitellum of *E. fetida*. (Blakemore R.J. et al., 2022).

Composting earthworms of the genus *Eisenia* play an important role in soil ecosystems. However, the taxonomic classification of this genus, especially the sibling species *E. fetida* and *E. andrei*, is complicated due to their morphological similarity. C. Csuzdi et al. (2022) assessed the utility of the complete mitochondrial genome (mitogenome) for identification and differentiation of the two species. The complete mitogenomes of *E. andrei* and *E. fetida* were 15,714 and 16,560 bp, respectively. They contained 37 genes, comprising 13 protein-coding genes (PCGs), two rRNA genes, 22 tRNA genes, and a putative non-coding region, as observed in other earthworms. Sequence comparisons based on the complete nucleotide sequences, excluding the non-coding region, showed 85.8% similarity, whereas the predicted amino acid sequences of the 13 PCGs were 92.7% similar between the two species. The extended sequence showed significant differences between the two species and other known earthworm species, suggesting its potential as a feasible molecular marker for species identification (Csuzdi C. et al., 2022).

Nucleotide sequences, in particular a fragment of the mitochondrial *COI* gene, have been used for species identification of earthworms. This sequence is also involved in DNA barcoding, a common method of species identification. The practicality and reliability of this method were assessed by cross-validation organised by an international group of scientists, the Eisenia Barcoding Initiative (EBI), from four public institutions and two private laboratories. Coded samples of *E. fetida* and *E. andrei* were provided to 28 ecotoxicology laboratories from 15 countries on four continents. Five laboratories in Belgium, Canada, Germany and Spain tested the blank samples by DNA barcoding. The results of this cross-validation were submitted to the OECD and ISO for standardisation of DNA barcoding. This study also revealed nucleotide sequences of *Eisenia fetida* 1 and *Eisenia fetida 2*, which were assigned to different cryptic species (Römbke J. et al., 2016).

A simultaneous hermaphrodite is an organism that possesses both male and female reproductive organs, allowing it to produce both types of gametes: sperm and eggs. While self-fertilisation is possible, it is not the primary method of reproduction. In many species, isolated virgin earthworms tend to produce very few viable offspring or may even result in sterile cocoons.

To investigate the existence of hybridisation between *E. fetida* and *E. andrei*, Plytycz B. et al. (2018) crossed virgin individuals of both species in the laboratory. A hybrid offspring was identified using two methods: first, they were recognised by species-specific haploid mitochondrial DNA sequences of maternal origin, specifically the COI gene. Second, hybrids were identified using diploid nuclear DNA sequences from the 28s rRNA gene of maternal and paternal origins. The results obtained show that the *E. andrei* and *E. fetida* species are easily cultivated under laboratory conditions, making them ideal models in biomedicine and ecotoxicology. Fertile hybrids of the species *E. andrei* and *E. fetida* were discovered during this study, highlighting their potential for investigating the molecular basis of interspecific barriers, introgression mechanisms, and speciation (Plytycz B. et al., 2018) Experimental confirmation of the existence of fertile hybrids *E. andrei* and *E. fetida* revealed the insufficient suitability of standard methods for the differentiation of these species. For this purpose, another method of genetic identification was proposed.

Another method of genetic identification is the use of microsatellite markers. Thus M. Jaskulak et al. (2022) used Illumina technology (Nano 2×250 v2 - MiSeq) and a *de novo* assembly strategy. A total of 4,258 microsatellites were identified in the genomic DNA of the earthworm species *E. fetida* and *E. andrei*. The utilisation of microsatellite markers was essential for analysing genetic diversity and studying the population genetics of these species, particularly due to the presence of interspecific hybrids (Jaskulak M. et al., 2022)

Currently, molecular methods are preferred for accurately classifying earthworm species. However S. Andleeb et al. (2021) note that these methods can be labour-intensive and costly. To address this issue, a model has been proposed that uses digital images and machine learning to identify earthworm species. In all evaluation conditions, this model exhibited the highest performance, validating its use for studying earthworm taxonomy (Andleeb S. et al., 2021).

The most important reason for the lack of a nationwide and standardized survey of soil organisms in Germany is probably the laborious and expensive identification of soil invertebrates. To address this problem, S. Jänsch et al. (2025) sampled earthworms and soil at 25 sites. The animals were identified morphologically and by metabarcoding of community DNA (comDNA) and environmental DNA (eDNA). A comparison of the results showed that comDNA detected more species (on average 3.6) than eDNA (3.0) and morphological identification (2.8). In contrast, eDNA, on average, detected a similar number of species as morphological identification. However, some species seem to have a different probability of being detected by eDNA than others, depending on their abundance, behavior, biology or body size. All three identification methods can distinguish between sites with different species composition, and the degree of separation can vary depending on the identification method. The relative fraction of eDNA reads shows potential as a surrogate for relative abundance/biomass for endogeic but not for anecetic species. The overall goal of the MetaSOL project (from which this contribution emerged) was to develop guidelines for effective and routinely implemented monitoring of soil fauna. The results showed that genetic identification methods are suitable for earthworms. S. Jänsch et al. (2025) emphasize that before genetic identification methods can be implemented in official practice, key prerequisite issues, such as comprehensive,

well-curated and quality-controlled DNA databases and standardization of methods, need to be addressed.

In summary, it is important to acknowledge the points discussed. Although there are various methods for identifying earthworms, the PCR-RFLP method can be advantageous for laboratories that utilise it.

Conclusions.

1. The studies allowed us to utilise the PCR-RFLP method by selecting the optimal pair of primers and restriction enzymes for the genetic identification of the earthworm species *E. fetida* and *D. veneta*;

2. An artificial *Tas*I recognition site is created in the PCR product of *D. veneta* DNA by incorporating the reverse primer EISR into the PCR product: CAGGTGCCTCCTCAATTTTAG (sequence presented on the plus strand of DNA);

3. *Lambricus terrestris* DNA can serve as a negative control in genetic studies due to its notable differences in nucleotide sequences compared to *E. fetida* and *D. veneta*;

4. The genetic differentiation of *E. fetida* and *E. andrei* requires more complex analysis for several reasons. First, there is considerable genetic similarity between these species. Second, earthworms exhibit high cryptic diversity. For example, *E. fetida* 1 and *E. fetida* 2 have been assigned to different cryptic species. Finally, interspecific hybrids of *E. fetida* and *E. andrei* exist.

Acknowledgments. We would like to thank Ms. Natalia Fomichenko from FLP Fomichenko in Zaporizhzhia, Ukraine, for providing samples of live *Dendrobaena veneta* worms. We thank Dr. Jörg Römbke from ECT Oekotoxikologie in Flörsheim am Main, Germany, and Professor Barbara Plytycz from the Department of Evolutionary Immunobiology at the Institute of Zoology, Jagiellonian University in Kraków, Poland, for providing samples of *Eisenia fetida* and *Eisenia andrei* in ethanol. The author wishes to sincerely thank Tetyana Buslyk, Pig Breeding Institute and AIP NAAS, Poltava, for her technical assistance during this research.

References

Akazawa SI, Machida Y, Takeuchi A, Wakatsuki Y, Kanda N, Kashima N, Murayama H. (2021). Development of a novel heterologous gene expression system using earthworms. Sci Rep., 11(1):8190. https://doi.org/10.1038/s41598-021-87641-w.

Altschul S.F., Gish W., Miller W., Myers E.W., Lipman D.J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Andleeb S., Abbasi W.A., Ghulam Mustafa R., Islam G.U., Naseer A., Shafique I, Parween A, Shaheen B, Shafiq M, Altaf M, Ali Abbas S. (2021). ESIDE: A computationally intelligent method to identify earthworm species (E. fetida) from digital images: Application in taxonomy. PLoS One, 16(9), e0255674. https://doi.org/10.1371/journal.pone.0255674.

Bajsa O, Nair J, Mathew K, Ho GE. (2003). Vermiculture as a tool for domestic wastewater management. *Water Sci Technol.* 8(11-12):125-32. https://doi.org/10.2166/wst.2004.0821.

Blakemore R.J., Takehiro Sato, Vasnick Ch., Lim Shu. (2022). First Records from Japan of European Vermi-composter Dendrobaena veneta (Rosa, 1886) and of "Classical" Lumbricus terrestris Linnaeus, 1758 (Annelida, Oligochaeta, Megadrilacea, Lumbricidae). *Bull. Kanagawa Pref. Mus. (Nat. Sci.)*, 51, 89-94. https://www.researchgate.net/publication/373019158.

Csuzdi C, Koo J, Hong Y. (2022). The complete mitochondrial DNA sequences of two sibling species of lumbricid earthworms, *Eisenia fetida* (Savigny, 1826) and

Eisenia andrei (Bouché, 1972) (Annelida, Crassiclitellata): comparison of mitogenomes and phylogenetic positioning. *Zookeys,* 1097,167-181. https://doi.org/10.3897/zookeys.1097.80216.

Dhakane R. & Shinde A. (2020). Eisenia fetida and Eisenia andrei delimitation by Automated Barcode Gap Discovery and neighbor-joining analyses: A review. *Journal of Applied Biology & Biotechnology*, 8(6), 93–100. https://doi.org/10.7324/JABB.2020.80615.

Fomichenko M. (2025). New methods of manure disposal and use of processing products in pig breeding. *The Scientific and Technical Bulletin of the Institute of Animal Science NAAS of Ukraine,* 133, 4-18. https://doi.org/10.32900/2312-8402-2025-133-4-18.

Fučík J., Fučík S., Rexroth S., Sedlář M., Gargošová H.Z., Mravcová L. (2024). Pharmaceutical metabolite identification in lettuce (Lactuca sativa) and earthworms (Eisenia fetida) using liquid chromatography coupled to high-resolution mass spectrometry and in silico spectral library. *Anal. Bioanal. Chem.*, 416(28): 6291-6306. https://doi.org:10.1007/s00216-024-05515-2.

GenScript Biotech Corporation. https://www.genscript.com/tools/restriction-enzyme-map-analysis.

Jänsch S, Alves D, Cunha L, Krogh PH, Natal-da-Luz T, Rojo V, Römbke J, Sapkota R, Scheffczyk A, Schmelz RM, Scopel L, Sousa JP, Vierna J, Vizcaíno A. (2025). Comparison of Morphological and DNA-Based Identification Methods to Assess Earthworm (Clitellata: Lumbricidae) Diversity at 25 Permanent Soil Monitoring Sites in Germany. Ecol Evol., 15(4), e71155. https://doi.org/10.1002/ece3.71155.

Jaskulak M, Vandenbulcke F, Rorat A, Pauwels M, Zorena K, Grzmil P, Płytycz B. (2022). Data on the identification of microsatellite markers in *Eisenia fetida* and *Eisenia andrei*. Data Brief, 45, 108612. https://doi.org/10.1016/j.dib.2022.108612.

Jaskulak M., Rorat A, Vandenbulcke F., Pauwels M., Grzmil P., Plytycz B. (2022). Polymorphic microsatellite markers demonstrate hybridization and interspecific gene flow between lumbricid earthworm species, *Eisenia andrei and E. fetida. PLoS One,* 17(2), e0262493. https://doi.org:10.1371/journal.pone.0262493.

Latif R, Malek M, Aminjan A.R., Pasantes J.J., Briones M.J.I., Csuzdi C. (2020). Integrative taxonomy of some Iranian peregrine earthworm species using morphology and barcoding (Annelida: Megadrili). *Zootaxa,* 4877(1), https://doi.org:10.11646/zootaxa.4877.1.7

Plytycz B, Bigaj J, Osikowski A, Hofman S, Falniowski A, Panz T, Grzmil P, Vandenbulcke F. (2018). The existence of fertile hybrids of closely related model earthworm species, *Eisenia andrei and E. fetida. PLoS One,* 13(1), e0191711. https://doi.org/10.1371/journal.pone.0191711.

Römbke J., Aira M., Backeljau T. et al. (2016). DNA barcoding of earthworms (Eisenia fetida/andrei complex) from 28 ecotoxicological test laboratories. *Applied Soil Ecology,* 104, 3-11. https://doi.org/10.1016/j.apsoil.2015.02.010.

Suleiman H., Rorat A., Grobelak A., Grosser A., Milczarek M., Płytycz B., Kacprzak M., Vandenbulcke F. (2017). Determination of the performance of vermicomposting process applied to sewage sludge by monitoring of the compost quality and immune responses in three earthworm species: Eisenia fetida, Eisenia andrei and Dendrobaena veneta. *Bioresour. Technol.,* 241, 103-112. https://doi.org:10.1016/j.biortech.2017.05.104.

Ye J., Coulouris G., Zaretskaya I., et al. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics,* 13, 134. https://doi.org/10.1186/1471-2105-13-134.