XXIV. "GENETIKAI MŰHELYEK MAGYARORSZÁGON"

Uncovering Invisible Traces: Forensic Potential of Environmental DNA Revealed by Long-Read Sequencing

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Introduction

Environmental DNA (eDNA), defined as genetic material obtained directly from environmental samples (e.g., soil, water, air) without the presence of obvious biological source material, is a powerful tool widely used in biomonitoring. In forensic science, traditional methods relying on morphological identification are often limited in their ability to detect low-concentration or non-human biological traces, such as plant fragments, pollen, or insect remains. Here, eDNA offers a complementary approach with the characterization of multiple species from single source and complex mixtures to establish links between suspects, victims and a crime scene, or determine sample provenance (Antil et al., 2023; Boggs et al., 2019; Meiklejohn et al., 2018).

The emergence of long-read sequencing technologies, particularly the Oxford Nanopore Technologies (ONT) can revolutionize eDNA profiling for forensic applications. These platforms offer portability, real-time analysis and enhanced capabilities for analyzing degraded and/or mixed samples overcoming limitations of traditional Sanger and Illumina sequencing. Short-reads are accurate and high-throughput, but may not provide full-length barcode resolution to identify degraded traces effectively (Ogden et al., 2021; Santos et al., 2020).

By targeting universal DNA barcode markers, including mitochondrial markers like cytochrome c oxidase subunit I (COI), *cytb* gene, 12S and 16S rRNA genes for animals and trace evidence along with chloroplast markers like rbcL, matK, trnL and nuclear ribosomal markers like ITS2 for plants, species-level taxonomic identification and metagenomic profiling can be achieved from even the most challenging eDNA samples (Chen & He, 2021; Ferreira et al., 2025; Young & Linacre, 2021).

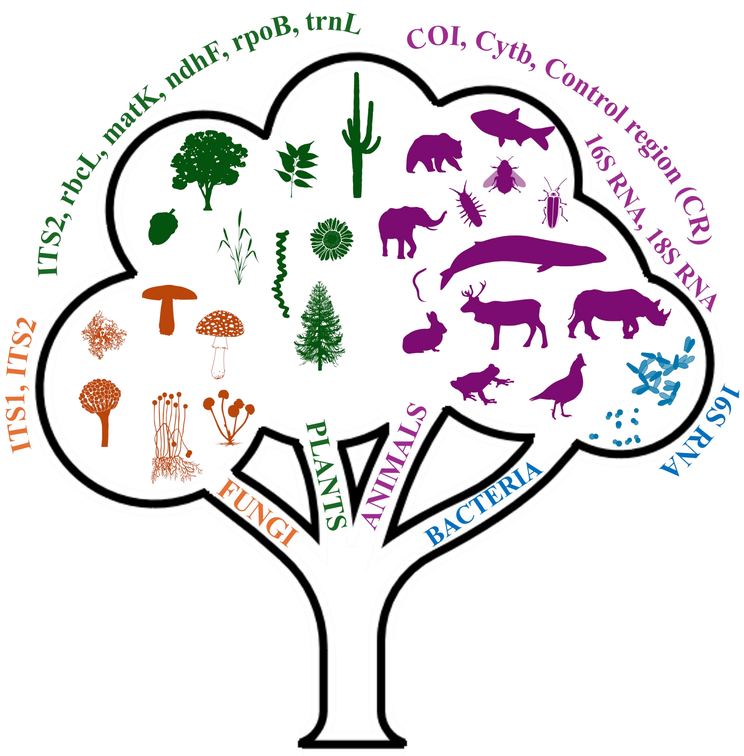

Figure 1. *Illustration on genetic markers and target regions applicable for eDNA profiling and taxonomic identification in forensic science across major taxa (based on Meiklejohn et al., 2021)*

Objectives

Our research aims to develop validated protocols for forensic environmental DNA profiling focusing specifically on eukaryotic species. We exclude fungi because of their higher genomic complexity and due to the lack of consistent universal barcoding markers in this taxon, that can complicate the robust analysis in mixed forensic samples. We utilize mock evidence experiments and persistence/decay studies to establish comprehensive, species-level eDNA profiles from trace biological materials. Our ultimate objective is to integrate the newly developed eDNA sequencing method into the routine forensic laboratory practice.

Materials & Methods

A 10-month indoor/outdoor mock evidence study was initiated using cotton T-shirts stained by purified genomic DNA from dog, horse, and goat, as well as "touch DNA" from direct animal contact. Samples were collected monthly, and DNA was extracted using the GeneJet Genomic DNA Purification Kit.

Target mitochondrial loci ***(16S** ~500 bp, **CytB** ~990 bp, **12S–16S** ~2000 bp)* were PCR-amplified and sequenced on an Oxford Nanopore MinION Mk1C using ligation-based amplicon sequencing with Native Barcoding Kit 96 V14. Bioinformatic analyses were performed to generate species-level eDNA profiles.

A.: Outdoor setup **B.:** Stained T-shirt outdoor **C.:** Stained T-shirt indoor

Figure 2. *Mock evidence setup with cotton T-shirts stained with purified genomic DNA from dog, horse, goat and a dog + horse mixture*

Preliminary Results

Cotton T-shirts were stained with 1 ng/µL purified genomic DNA from dog, horse, and a dog + horse mixture and placed either outdoors at the *ELTE Botanical Garden* and indoors under stable laboratory conditions. Samples were collected monthly by cutting, DNA extracted and quantified using a Qubit 4.0 fluorometer with the 1× dsDNA High Sensitivity Assay. Outdoor samples showed a progressive accumulation of DNA over time reaching >10 ng/µL by month 4, whereas indoor samples remained consistently low (≤0.2 ng/µL) reflecting the absence of eDNA accumulation and DNA degradation under controlled conditions.

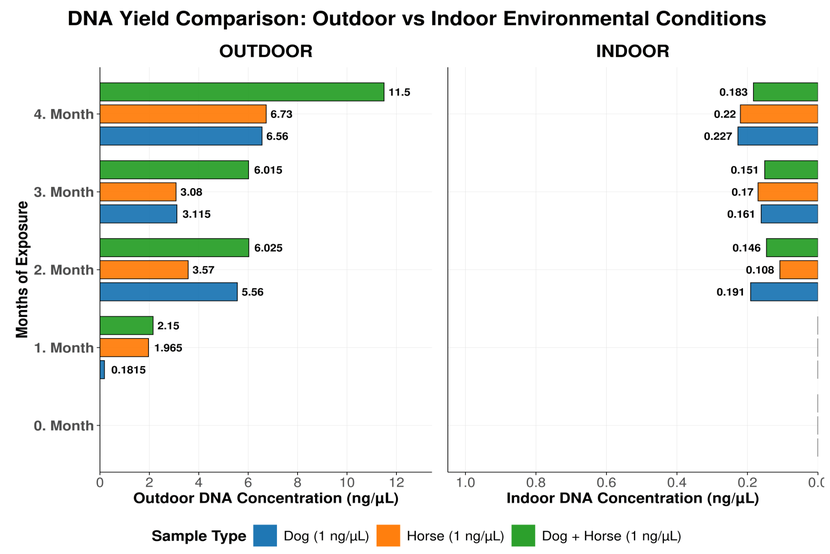

Figure 3. *DNA yield from outdoor and indoor mock evidence (T-shirts) over a 4-month sampling period*

Amplicon libraries from five representative samples (dog, goat, fish controls, and two mock T-shirt samples) were sequenced on the ONT MinION platform and species-level taxonomic profiles were generated using Kraken2 for taxonomy assignment. The positive controls (Dog, Goat, Paradise fish) largely matched their expected taxa, with fish showing the strongest recovery (~40%). In contrast, the mock evidence samples (Indoor Touch DNA T-shirt, Outdoor stained T-shirt) yielded only weak or mixed signals, frequently accompanied by human DNA. While conserved mitochondrial markers provided reliable resolution for distant taxa such as fish, their discriminatory power was more limited among closely related mammals (Figure 4, Table 1).

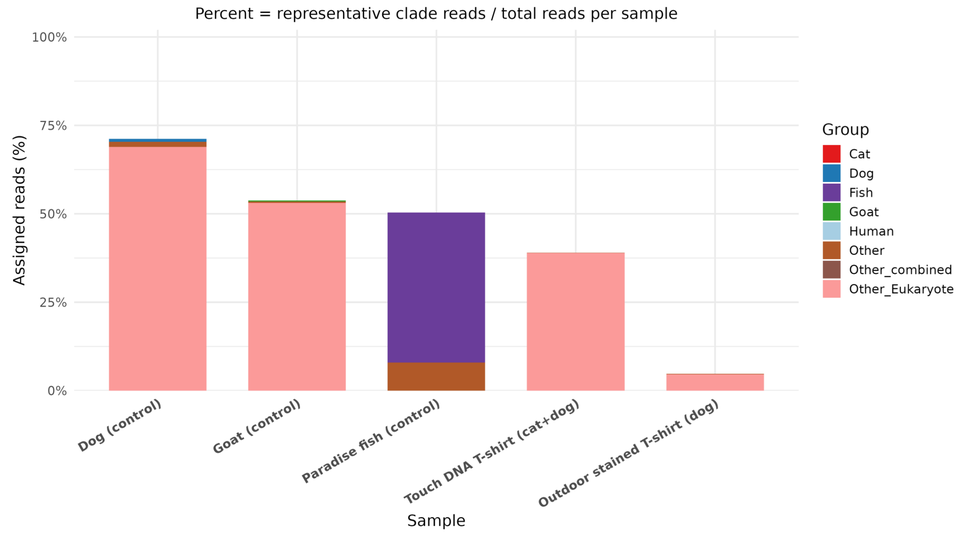

Figure 4. *Sequence read assignments by Kraken2 taxonomic profiling*

Samples	Expected species	Dominant detected group (Kraken2)	Assigned reads (%)	Results
Dog (control)	*Canis lupus familiaris*	*Canis*	0.8%	✓ Expected species / genus detected
Goat (control)	*Capra hircus*	Bovidae / Caprinae	0.16%	✓ Expected species / family detected
Paradise fish (control)	*Macropodus opercularis*	*Actinopterygii* / Teleostei	42.4%	✓ Fish DNA detected
Indoor Touch DNA T-shirt (cat + dog)	*Felis catus*	*Canis* / *Felis*	<0.1%	Weak mixed signals + Human DNA present
Outdoor stained T-shirt (dog)	*Canis lupus familiaris*	*Canis*	0.03%	Weak dog DNA + Human DNA present

Table 1. *Results of ONT long-read sequence evaluation from the control and mock evidence samples*

Conclusion & Future work

Our pilot study demonstrates the potential of ONT long-read amplicon sequencing in forensics with enabling complex taxonomic profiling of challenging eDNA evidence samples. However, our preliminary results emphasize the requirement of optimized workflows, strict contamination controls, dedicated and well-curated reference databases. Future work will design optimized reaction setup, extend assays to plant genomic markers and build dedicated databases. Combined with improved validation and bioinformatics, these efforts aim to deliver accurate, reproducible, and forensically robust metagenomic profiling — ultimately integrating this approach into routine forensic practice.

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References

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