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Environmental DNA Analysis: A New Approach to Monitoring Species Reduction

Analiza okolišne DNA: novi pristup praćenju redukcije vrsta

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ABSTRACT

Environmental DNA (eDNA) is defined as genetic material taken from an environmental sample that contains distinguishing characteristics of the original organisms that are or were present in the environmental sample and includes extracellular and intracellular DNA. Its presence and degradation depend on various abiotic conditions, and possible sources of eDNA include all biological materials of organisms. The analysis of eDNA is simple and cheap. It is of great importance for the study of environmental biodiversity because it provides quantitative estimates of the abundance and biomass of individuals and species. Changes in environmental conditions affect eDNA, which is why eDNA analysis is often used in ecology. In addition to climatic changes, there are also seasonal changes in abiotic factors that significantly affect eDNA abundance. In this study, the research methods and application of this genetic material in different research areas are presented.

Keywords: biodiversity, biomonitoring, PCR, sequencing

SAŽETAK

Okolišna DNA (eDNA) definirana je kao genetski materijal uzet iz okolišnog uzorka koji sadrži razlikovne karakteristike izvornih organizama koji su prisutni ili su bili prisutni u okolišnom uzorku i uključuje izvanstaničnu i unutarstaničnu DNA. Njegova prisutnost i razgradnja ovise o različitim abiotskim uvjetima, a mogući izvori eDNA uključuju sve biološke materijale organizama. Analiza eDNA je jednostavna i jeftina. Od velike je važnosti za proučavanje bioraznolikosti okoliša jer daje kvantitativne procjene brojnosti i biomase jedinki i vrsta. Promjene u uvjetima okoliša utječu na e DNA, zbog čega se eDNA analiza često koristi u ekologiji. Osim klimatskih promjena, postoje i sezonske promjene abiotskih čimbenika koji značajno utječu na obilje eDNA. U ovom radu prikazane su metode istraživanja i primjena ovog genetskog materijala u različitim područjima istraživanja.

Ključne riječi: bioraznolikost, biomonitoring, PCR, sekvenciranje

INTRODUCTION

Nowadays, due to increasing environmental pollution, there is a negative trend of species reduction in biodiversity and possible extinction, and there is an increasing need for monitoring (Needham et al., 2007). However, for monitoring to be successful, there is the need to collect complete individuals of animals, which may further endanger their population. Another problem is the intensive fieldwork and the availability of taxonomic experts for specific groups of organisms. In such an approach, the problem is inevitably a large number of experts in taxonomy and collaborators, in general. Because of that, environmental DNA (eDNA) analysis methods have been developed that allow ecosystem components to be analyzed in a much more efficient and less invasive way (Rees et al., 2014). Any genetic material obtained directly from environmental samples such as soil, air, sediment, or water, without the original biological material, i.e., the organisms or parts of organisms from which it is derived, refers to environmental DNA. Higher organisms usually release their DNA into the environment where it can be collected and analyzed. It is in the form of shed cells and tissues such as hair and skin, through secretions such as urine and feces, or the decomposition of dead organisms (Beng and Corlett, 2020). The process of DNA analysis from the environment consists of several main components: field work, processing of samples in the laboratory, and bioinformatic analysis. The results after the whole process are obtained as sequences of specific DNA sequences, which can then be determined to which species they belong based on the data available in the reference databases. This approach to biodiversity research provides a relatively quick and easy estimate of the number of species in the flora and fauna of a habitat. With appropriate modifications and optimization of methods, environmental DNA analysis can be an important tool in conservation biology, ecology, biomonitoring and palaeontology (Barnes and Turner, 2016). In this paper, we describe the potential applications of DNA analysis, the sampling methods, the main laboratory and bioinformatics methods and the advantages and problems of the aforementioned methods.

The use of eDNA in biological research

Detection of invasive species using eDNA analysis can be done in two different ways. A combination of active and passive monitoring of areas where there is a risk of an invasive species occurring could help control the spread of invasive species. In active detection, only one type of DNA that is suspected to be or may be invasive in a given area is amplified by highly specific starters. In passive detection using active metabarcoding, multiple species are analyzed at once so that a species that is not expected to occur in that area can be detected. One drawback of eDNA analysis in invasive species detection is false positives and negatives, as they can cause unnecessary responses, time and economic losses, or damage to the environment (Sepulveda et al., 2020). Bioindicator species are organisms that can be easily monitored and whose presence (or absence) reflects the state of the environment in which they occur (Carew et al., 2013). Using metabarcoding of environmental DNA samples, the extent and impact of pollution in an area can also be assessed by monitoring the community composition of bioindicator species.

Because DNA analyzes do not require invasive sampling methods they are also suitable for research on endangered and sensitive species. Detecting and monitoring the presence of endangered species using eDNA analysis makes it easier to monitor endangered species in protected areas, and the presence of multiple species can be monitored simultaneously. However, the problem with eDNA analysis occurs for monitoring sensitive species where eDNA cannot determine whether DNA is from a living or dead individual and cannot determine the age or abundance of individuals. Analysis of ancient DNA (aDNA) is the only method for analyzing ecosystems from the past. Using more detailed analyzes of aDNA, it is possible to understand how species became extinct in the past. This knowledge can be applied to better understand the unpredictable dynamics of populations and the role of humans in these processes (Pedersen et al., 2015).

Analyzes of eDNA in ecology can contribute significantly to knowledge about the food webs, mutual interactions among organisms and abundance of species in the ecosystem. Although often the results we get using eDNA on the number of species in an area is not significantly different from classical methods. But, by metabarcoding insight into the full composition of species across multiple taxonomic groups (bacteria, fungi, plants or animals) in an area in a very short time is easily provides (Valentini et al., 2016). Analysis of eDNA from feces or stomach contents can provide greater insight into the dietary habits of the species under study in a short period of time. This type of research can reveal dietary diversity and characteristics that would be difficult or impossible to detect using traditional methods. These studies are important because they can point to previously unknown relationships among organisms and suggest more appropriate actions to protect and conserve biodiversity (Ando et al., 2020). Figure 1 shows a large number of scientific papers are represented in the Web of science and Scopus scientific databases till Feburary 2024 in which certain groups of organisms were studied using eDNA.

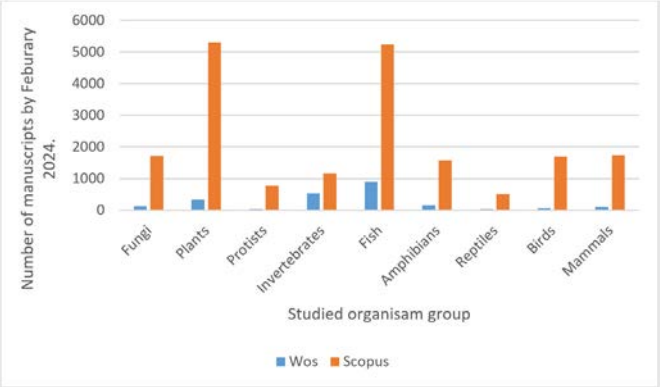

Figure 1. Use of eDNA in scientific papers represented in the Web of Science and Scopus scientific databases by specific groups of organism

Methods of extracting and analysing eDNA

eDNA from different environment

Due to environmental conditions (ultraviolet radiation, pH, temperature, salinity, presence of oxygen) and the presence of microorganisms and enzymes in the environment, eDNA will degrade sooner or later. In different types of environments, eDNA is degraded at different rates, such that degradation is much faster in aquatic environments than in soils or sediments. Environmental DNA is found in different types of substrates such as air, sediment, soil, terrestrial and marine water, and permafrost, and each of these substrates has its sampling method and subsequent processing of the collected samples. The substrate from which samples are collected also depends on the type and area of research.

Airborne eDNA is usually collected using various air filters or adhesive tapes. Analysis of airborne eDNA is based on the extraction of DNA found in pollen, dust, sand and other particles in the air. Analyzes of airborne eDNA were first used for analyses of pollen that can cause allergic reactions (Rowney et al., 2021). It has been discovered that airborne eDNA can also be used for the analysis of microorganisms in hospitals and that it provides more information about viruses, bacteria, archaea and fungi in the air than previously used cultivation methods in cultures (Gao et al., 2018). Salinity is the reason why collecting seawater samples is more challenging than collecting samples from terrestrial waters. Also, ocean currents accelerate eDNA degradation and a much larger sample volume is required due to the greater dilution of eDNA in the ocean. Notwithstanding the difficulties mentioned above, eDNA from seawater can be used to collect valuable data on the biodiversity of marine microorganisms, fish, and marine mammals. Samples can also be collected from marine sediments. Although such sampling requires specialized drilling equipment and is a major logistical challenge, eDNA from these samples can provide information about species diversity and community composition of benthic organisms (Pawlowski et al., 2021). Some specific examples of how eDNA analysis of seawater samples is being used are, for example, monitoring the recovery of the Great Barrier Reef from coral bleaching, tracking the movement of North Atlantic right whales, identifying new areas for marine protected areas, detecting the presence of invasive Asian carp in the Great Lakes, assessing the impact of oil pollution on marine ecosystems (Cristescu and Hebert, 2018; Hansen et al., 2018; Rees et al., 2018; Thomsen et al., 2016).

It has been observed that the number of fish species determined by eDNA analysis from surface water samples agrees better with the results of conventional methods (morphological determination and collection of whole individuals) than the number of species determined by eDNA analysis from the upper sediment layer (Turner et al., 2015). In freshwater ecosystems, samples can be collected from the water column by filtration or as the top layer of sediment to analyze the biodiversity of aquatic invertebrates and vertebrates. In the upper sediment layers, eDNA accumulates over a long period, and further research is needed to accurately determine the age of the eDNA (Thomsen and Willerslev, 2015). When collecting samples from streams or rivers, the results of eDNA analysis provide insight into the biodiversity of a much larger area than samples from standing waters and lakes. Due to water flow in rivers and streams, eDNA can be transported over much greater distances than in lakes (Deiner and Altermatt, 2014). In addition to eDNA in lakes and rivers, eDNA can also be collected from subsurface water and has previously been used for analyzes of bacterial and fungal diversity (Barnes and Turner, 2016). Overall, eDNA is a powerful tool that can be used to monitor and manage fish stocks, to detect invasive fish species, and to assess the impact of overfishing on marine and freshwater ecosystems.

Samples of aquatic and terrestrial sediments are used for ancient DNA (aDNA) analyzes, from which information about community composition and ecosystems from the past is obtained. They are usually collected at greater depths with larger tools and drills that can reach depths of tens of meters, and care must be taken to ensure that samples from different strata do not contaminate each other (Epp, 2019). Environmental DNA from surface soil samples is used to analyze the community composition and biodiversity of plants, fungi, subterranean annelids, invertebrates, and vertebrates (Guerrieri et al., 2021). Although there is no standard protocol for soil sampling, in most studies a circle of a specific diameter is chosen as the sampling location within which multiple soil samples are collected from different depths, depending on the depth at which the organisms being studied are most abundant. Sampling is usually done with different soil augers, which must be washed with water and sterilized with a flame after each sampling to prevent cross-contamination of the different samples. Homogenized soil samples can be stored in the laboratory in a solution of 96% ethanol or containers in a freezer at -40 °C (Lakay et al., 2007).

Environmental DNA analysis also includes stomach contents, honey, feces, saliva, and blood meals from mosquitoes leeches and spider webs (Blake et al., 2016). For example, by analyzing eDNA from honey, it is possible to determine which plants, animals, and pathogens bees interact with (Ribani et al., 2020). Such research provides an interesting insight into the feeding habits of individual species and interactions between organisms that are difficult or impossible to detect using conventional methods.

Methods of eDNA isolation

There are several different methods for isolating eDNA from a collected sample, depending on the substrate from which the DNA is extracted. Because of their ease of use and relatively efficient extraction, commercial DNA isolation kits are the most commonly used. These methods usually work on the principle of enzymatic degradation of cellular components with the enzyme proteinase K and, in the later steps of isolation, binding of DNA to special columns from which proteins, lipids, and other unwanted components of the solution are first washed out with the help of special elution solutions and, finally, pure DNA is washed out with columns (Eichmiller et al, 2016b). In addition to commercial kits, methods that use organic solvents for DNA isolation (liquid phase separation method) are commonly used for eDNA isolation, which can also efficiently extract DNA. The disadvantage of the methods that use organic solvents is that they require special care in performance and disposal because they use toxic chemicals such as chloroform and phenol. Several studies are using modified isolation protocols and comparing them to commercial kit protocols. Many of them achieve more successful results (higher eDNA yield) than commercial protocols, so it is sometimes desirable to use modified protocols tailored to specific sample types.

Polymerase chain reaction

The polymerase chain reaction or PCR (Mullis et al., 1986) is an indispensable method for amplification of specific DNA fragments. It is very important to select suitable primers for successful amplification of the desired fragments. The type of primers chosen depends largely on the nature of the research, i.e. whether only one species is being studied or whether several groups are being studied at once using metabarcoding. Research on one type of organism is the most common type of eDNA research (Barnes et al., 2014), which allows detection of invasive, endemic and endangered species in an area and monitoring. Such research is based on the amplification of a selected DNA fragment with highly specific primers. Research that uses metabarcoding uses a large number of primers designed specifically for a group of organisms based on a particular marker that ideally allows different species within the group to be distinguished.

Standard DNA markers, known as DNA barcodes, are DNA sequences usually longer than 500 bp that can be used to effectively distinguish individual groups of organisms down to the species level. In animals, the most commonly used DNA barcode region is the mitochondrial gene for cytochrome c oxidase subunit I (COI) (Kress et al., 2015). Alternative markers used in eDNA research are the mitochondrial gene for cytochrome b (Cytb), the D-loop (noncoding region of mtDNA), and the mitochondrial ribosomal genes for the 12S and 16S rRNA subunits (Tsuji et al., 2019). Alternative markers are often used in eDNA research because the degree of degradation of eDNA sometimes makes it impossible to amplify complete standard DNA barcode regions of longer length (Smith et al., 2009). Because eDNA is relatively short due to some degree of degradation, it is necessary to design short DNA markers large enough to distinguish one species from another or to use several different markers in research (Strickler et al., 2015).

Various types of PCR reactions are used for product detection and reaction success control. The most commonly used reactions include standard PCR, quantitative PCR (qPCR), and digital PCR (dPCR), as well as product detection by gel electrophoresis. Electrophoresis is a method that uses the natural negative charge of the DNA molecule to drive it into an electric field in which the DNA moves toward a positively charged electrode. It determines the presence and length of DNA fragments in samples. Standard PCR and gel electrophoresis do not require expensive equipment and chemicals, but accurate quantification of the amount of DNA in the electrophoresis gel after the PCR reaction is not possible, and electrophoresis is used only to confirm the presence of a sufficient amount of DNA in samples, which is sufficient for certain research. fluorescent dyes that bind to DNA allow visualization of DNA in an electrophoresis gel. However, it is possible to accurately determine the amount and confirm the presence of a particular DNA fragment using quantitative PCR. Accurate quantification of DNA in the sample is achieved by qPCR using DNA probes (probe-based qPCR) instead of dyes (dye-based qPCR). Like qPCR, digital PCR allows accurate quantification of DNA in the sample and is a more precise method than qPCR because reaction inhibitors do not affect dPCR results as they do with qPCR. Although dPCR is a more accurate method, the equipment required to perform this method is expensive, so qPCR is still the most commonly used (Conte et al., 2018).

Sequencing

Before sequencing, it is necessary to purify the DNA. This is usually done according to standard protocols using commercial kits for DNA purification in which residual nucleotides, primers, enzymes, and PCR products that are too short are removed from the reaction solution. The sequencing method determines the sequence of nucleotides in the DNA molecule. Next-generation sequencing (NGS) methods, which can sequence several hundred thousand to tens of millions of DNA sequences in parallel, are most commonly used for environmental DNA research. eDNA samples often contain sequences of a large number of species, and Sanger sequencing would be slow and inefficient in these cases (Wang et al., 2021). Nowadays, some several services and institutions provide different sequencing services. GenBank is a

comprehensive and publicly accessible database that serves as a repository for genetic sequence data. It is operated by the National Center for Biotechnology Information (NCBI), which is part of the United States National Institutes of Health (NIH). GenBank contains a vast collection of DNA and RNA sequences, including those from a wide range of organisms, such as bacteria, plants, animals, and viruses. Researchers from around the world contribute their genetic sequence data to GenBank (Benson et al., 2012), making it an invaluable resource for scientific research. This approach is especially useful for monitoring and studying biodiversity and ecosystems. Researchers can deposit eDNA sequences into GenBank, allowing others to access and analyze this data, aiding in the identification of species, tracking changes in ecosystems, and understanding the environmental impact of various factors, such as pollution or climate change.

GenBank is a collaborative partner of the International Nucleotide Sequence Database Collaboration (INSDC), which is a collaboration of DNA DataBank of Japan (DDBJ) (Ogasawara, Kodama, Mashima, Kosuge, & Fujisawa, 2019), the European Nucleotide Archive (ENA), and GenBank at National Center for Biotechnology Information (NCBI). One of the most well-known companies that contribute to these databases is Macrogen Inc. which provides reliable, fast, and relatively inexpensive sequencing and can reduce the cost of purchasing expensive sequencing equipment and make sequencing widely available. One of the most widely used platforms for high-throughput sequencing is the Illumina Genome Analyzer, which uses the principle of sequencing by synthesis.

Bioinformatics of eDNA

Bioinformatics processing is an important part of eDNA analysis for the correct interpretation of results. Therefore, after sequencing, sequences that are too short, sequences with poor read quality, and ends of sequences that are often poor reads in sequencers are removed.

Sequences are then classified into molecular operational taxonomic units (MOTUs) using a reference database. Reference databases are an important part of this expression. They contain data on which sequence of a particular marker belongs to which species. They already exist or need to be created as part of the research if the species being studied are not included in existing databases. Using the DNA barcoding method, new reference databases are created so that the species of interest can be identified morphologically. After isolating and sequencing a specific barcode region of their DNA, the corresponding species name is added to the sequenced sequences. The BOLD (Barcode of Life Data System) database is the best-known database of sequences of standard barcode regions (Ratnasingham and Hebert 2007). The sequences of many other genes are stored in the GenBank database (Benson et al., 2012). Several programs use sequence similarity comparison methods using sequence alignment (BLAST) or Markov chain models (jMOTU), machine learning, phylogenetic analyzes, or a combination of the above methods to classify sequences into the appropriate taxonomic group (Bazinet and Cummings 2012, Deiner et al., 2017). For eDNA and metabarcoding purposes, more robust programs are often needed that can analyze multiple sequences at once and determine taxonomic grouping by comparing sequences to those in databases.

DISCUSSION AND CONCLUSION

It is possible to discover a species that does not occur at all in a particular habitat but left DNA there in the past that has been preserved. Thus, determining the time interval after which the organism released its DNA into the environment is problematic because eDNA has different degradation rates under different conditions (Eichmiller et al., 2016a). The development of more accessible bioinformatics methods would also contribute to eDNA research, as many analyzes require the help of computer scientists. One drawback of eDNA analysis is problems in selecting DNA markers and primers, because the ability to distinguish species within a group depends on the selection of an appropriate marker, and some markers cannot effectively distinguish species within the same taxonomic group. Because of the relatively short fragments of eDNA amplified by the PCR reaction, there

is a problem with the nonspecific binding of primers to templates and the amplification of a DNA species that is not of research interest (Kumar et al, 2020). Incomplete reference databases that contain little or no information for specific markers are also a problem and sometimes contain sequences of some markers that are too short or incorrectly associated. Non-standard sampling methods are also a problem, as they can lead to non-reproducible and erroneous research results if poorly performed and inadequately described in research. Because DNA can be transferred in the environment in a variety of ways (wind, water, currents, feces), it is difficult to determine whether a species lives at the sampling site or a much more distant location (Deiner and Altermatt, 2014).

The major advantage of eDNA analysis is that sampling methods are non-invasive, which is not the case with traditional methods that require individuals to be physically removed for further analysis. Since whole organisms do not have to be collected, it facilitates the study of hard-to-reach and inaccessible habitats such as subsurface systems and deep sea. There are already several studies that show great species diversity and potentially new species in these inaccessible habitats (e.g., Beaver et al, 2021; Gorički et al, 2018; Reinhardt et al, 2019). The advantages of eDNA analysis are the accuracy and the amount of data that can be obtained in a short time compared to conventional methods. eDNA analyses cannot replace conventional methods, but they can greatly complement them by providing insight into the hidden diversity of an environment and directing future research toward the discovery of species not yet found physically in an area but recorded in eDNA. In addition, eDNA analyses make it possible to observe greater taxonomic and genetic diversity in the environment and the presence of rare, endemic, or cryptic species. Given the speed of eDNA degradation in aquatic ecosystems (several hours to several days), an effective assessment of the current state of microbiota and fauna composition in lakes, farms, oceans, and rivers is possible (Saito and Doi, 2021). Recent advances in sequencing technology and PCR reactions have made accurate quantification of environmental DNA possible (Hoshino et al., 2021), but future research should find a link between the amount of eDNA and the biomass of organisms so that eDNA can be used to more accurately estimate the number of species in a given area.

Although the process of analyzing environmental DNA consists of many steps that may seem complicated at first glance, as research progresses, the methods become increasingly standardized and more accessible to researchers just entering the field of environmental DNA research. Environmental DNA analyzes are increasingly being used in various areas of biology where, in addition to existing traditional methods, they can complement and enhance the way certain aspects of the environment are studied. Today, DNA analysis is gradually finding applications in invasive species biology, palaeontology, ecology, conservation biology and monitoring, where it is opening new opportunities for research and ecosystem conservation. In the future, by optimizing the process and solving methodological problems, environmental DNA analysis can improve existing methods and change the way we study the environment. eDNA enables large-scale monitoring of biodiversity but is still a partially limited method. We do not know enough about the genome of many organisms, so certain species are difficult to identify due to the lack of available sequences. Even the widely used genetic markers are sometimes not suitable for all species/groups of organisms. However, it is a relatively cheap and fast method that represents a turning point in the study of biodiversity around the world, and further work should be done to optimise it.

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