Review scientific article / Pregledni znanstveni članek

HIGH-THROUGHPUT SEQUENCING IN FOREST HEALTH SURVEILLANCE: OPPORTUNITIES AND CHALLENGES

VISOKOZMOGLJIVO SEKVENCIRANJE PRI SPREMLJANJU ZDRAVJA GOZDOV: PRILOŽNOSTI IN IZZIVI

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ABSTRACT

Regular phytosanitary border control and local surveillance are increasingly important for the early detection of novel plant pests and pathogens. High-throughput sequencing (HTS) is a promising tool that can complement visual inspections, morphological identification and targeted molecular tests. HTS can be applied to a wide array of sample matrices, enabling the analysis of bulk samples while simultaneously targeting multiple taxa. The workflow includes multiple preparation steps and extensive post-sequencing bioinformatic analyses. It needs to be optimised and carefully documented to minimise and control the various biases introduced at each step. The results should be carefully interpreted and, if a species of concern is detected, it currently still needs to be confirmed by an independent method. In this article, we discuss the usefulness of HTS in the context of forest protection, considering different sample types, technical considerations and potential synergies with established methods.

Keywords: forest health, metabarcoding, pests, fungal pathogens, high-throughput sequencing

IZVLEČEK

Redna fitosanitarna kontrola na mejah ter programi preiskav postajajo čedalje pomembnejši pri detekciji novih škodljivcev in povzročiteljev bolezni drevja. Kot eno izmed orodij za podporo vizualnim pregledom, morfološkim metodam identifikacije in tarčnim molekularnim metodam je tudi visokozmogljivo sekvenciranje. Uporablja se lahko na različnih tipih vzorcev ter omogoča simultano iskanje različnih taksonomskih skupin ter analizo velikega števila vzorcev hkrati. Visokozmogljivo sekvenciranje zahteva številne zaporedne korake, tako priprave na sekvenciranje kot bioinformacijsko zahtevne analize rezultatov. Koraki morajo biti optimizirani in skrbno dokumentirani, da lahko zmanjšamo in nadzorujemo vpliv, ki ga imajo na rezultat analize. Rezultate je prav tako treba skrbno tolmačiti ter v primeru detekcije vrste, ki ima karantenski značaj, tudi potrditi z alternativno metodo. V članku razpravljamo o uporabnosti visokozmogljivega sekvenciranja v okviru varovanja gozdov ter premišljujemo o različnih vrstah vzorcev, tehničnih vidikih ter možni sinergiji z že uveljavljenimi metodami.

Ključne besede: zdravje gozda, meta analiza črtnih kod, škodljivci, glive povzročiteljice bolezni, visokozmogljivo sekvenciranje

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1 INTRODUCTION
1 UVOD

Globalisation and the rise in international trade increase the risks of introducing novel plant pests and pathogens. Additionally, changes in environmental conditions can alter the geographical range of organisms harmful to plants. Consequently, interactions between pests, pathogens and their potential new plant hosts are increasing (Tedersoo et al., 2019), which makes regular phytosanitary border controls and local surveillance more important than ever, as early detection is key to timely risk assessment and damage mitigation.

High-throughput sequencing (HTS) theoretically enables the sequencing of any genetic material present in a sample (FAO, 2019). It also allows for the simultaneous processing of large batches of samples, targeting multiple organisms at the same time (Morinière et al., 2016; Olmos et al., 2018; Piper et al., 2019; Tremblay et al., 2019a; Butterwort et al., 2022). In combination with different DNA extraction protocols, it represents a powerful tool for analysing a wide range of input materials (Butterwort et al., 2022). The method includes multiple steps, from sampling to wet-lab sample and library preparation, to extensive post-sequencing bioinformatics and analysis (Figure 1).

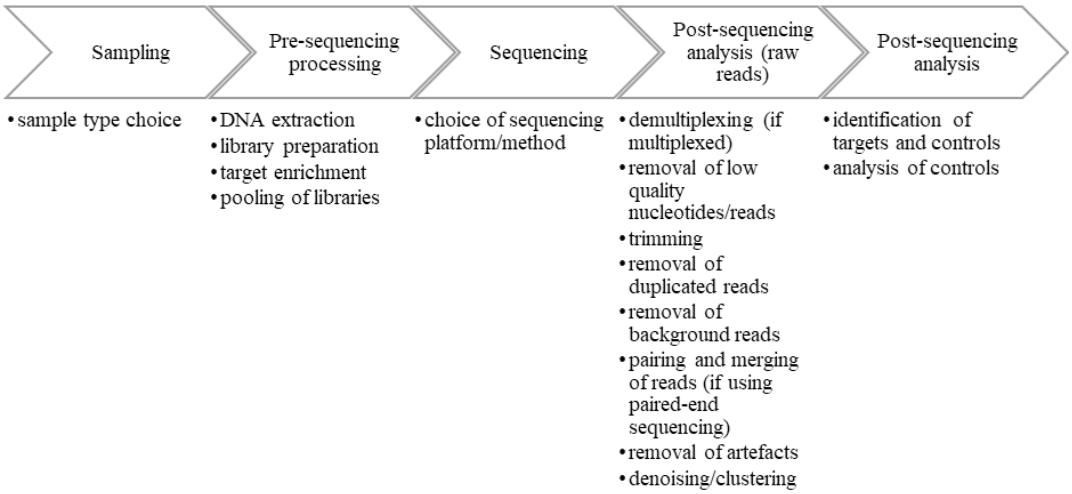

Fig. 1: Possible steps in the HTS process. The number of steps used in a specific application depends on the sequencing method and the end goal of the procedure.

Slika 1: Možni koraki pri postopku visokozmogljivega sekvenciranja. Število korakov, uporabljenih v posamezni analizi, je odvisno od uporabljene metode sekvenciranja in končnega cilja postopka.

In the context of increasing global trade and environmental change, early detection of forest pests and pathogens is essential for effective risk assessment and timely intervention. Surveillance systems must evolve to meet these challenges, and HTS is a useful tool for integration into established monitoring frameworks. By enabling broad-spectrum detection from diverse sample types, HTS can complement traditional surveillance methods and enhance the capacity for early warning, especially in forested areas where visual symptoms may be delayed or difficult to observe.

2 HTS AS A TOOL FOR OFFICIAL LABORATORIES IN FOREST PLANT HEALTH

2 VISOKOZMOGLJIVO SEKVENCIRANJE KOT ORODJE ZA URADNE LABORATORIJE NA PODROČJU ZDRAVJA GOZDA

The high-throughput sequencing era began with the publication of the pyrosequencing method by Ronaghi et al. (1996). Since then, the methodology has been constantly developing and diversifying and has seen increased use in different research and diagnostic sectors. On 4 February 2026, a Web of Science

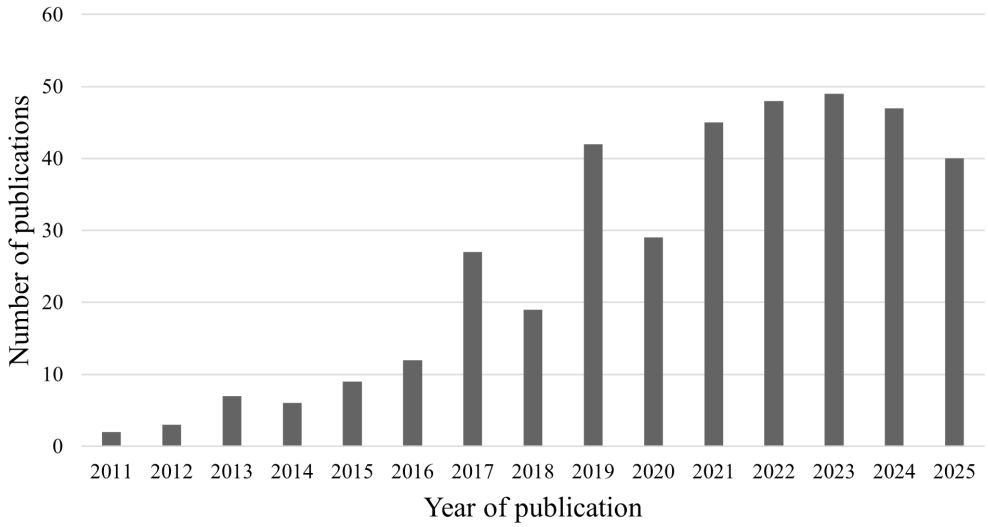

Fig. 2: Number of publications per year listed in Web of Science for the topic search using the terms "forest*" in connection with "high-throughput sequencing" or "next generation sequencing" and "disease*" or "pest*" or "phyto/pathogen/s". The search was performed on 4 February 2026, and results from the year 2026 and all medical and veterinary subject areas were excluded.

Slika 2: Število objav na leto na platformi Web of Science – iskanje po temah (angl. topic) z iskalniki "forest*" v povezavi s "high-throughput sequencing" ali "next generation sequencing" in "disease*" ali "pest*" ali "phyto/pathogen/s". Iskanje je bilo opravljeno 4. februarja 2026, iz rezultatov pa smo izločili rezultate iz leta 2026 ter z vseh področij medicine in veterine.

(www.webofscience.com) topic search in the CORE collection (all 10 indexes), using the search terms "forest*" in connection with "high-throughput sequencing" or "next generation sequencing" and "disease*" or "pest*" or "phyto/pathogen/s", and excluding results from 2026 and all medical or veterinary subject areas, produced 389 results. Starting in 2011, the number of publications slowly increased, peaking at 49 publications in 2023 (Figure 2).

This gradual increase in publications suggests a growing interest in applying HTS to forest health-related topics. The rising numbers point to a steady uptake of HTS-based techniques within forest pathology and pest research (Table 1), indicating their emerging role in complementing traditional diagnostic approaches.

There are several types of information that HTS can provide as a detection tool in the context of pests and fungal pathogens that target forest and ornamental trees. Traditionally, the time and specialist expertise needed severely limited the number of organisms that could be identified to the species level during phytosanitary surveys, consequently forcing national plant protection organisations (NPPOs) to focus their surveys on a smaller number of high-risk potential pests and pathogens. The nature of HTS methodology enables the targeting of numerous different species while processing large batches of diverse samples at the same time (Piper et al., 2019; Tremblay et al., 2019a), potentially vastly increasing the capacity and possible scope of yearly phytosanitary surveys (Piper et al., 2019).

Compared with agricultural or urban settings, the detection of symptoms in forests often takes longer, as forests are less frequented by people and are sometimes harder to access, which lowers the chance of visual detection. In such environments, traditional monitoring methods may be limited by logistical constraints and the low visibility of early-stage infections or infestations. A sampling strategy that can cover a large area with a single sample is ideal for such inaccessible forested regions (Figure 3). Detection by HTS in these samples can then represent the first sign of a newly introduced or previously undetected organism, which can be useful for directing further targeted sampling and specific morphological or molecular analyses (Olmos et al., 2018; Tremblay and Bilodeau, 2022). For that purpose, HTS could be employed as a tool during regular yearly regional surveys or used by border officials for either quarantine testing or routine monitoring of imported commodities (Olmos et al., 2018; FAO, 2019; EPPO, 2022).

In addition to its diagnostic potential, HTS can contribute to establishing or refining the baseline of organisms typically present in a given area (Olmos et al., 2018; Piper et al., 2019; Piombo et al., 2021). This baseline can serve as a reference for detecting shifts in community composition or the emergence of novel organisms. For species already known to occur in the region, HTS may reveal previously undocumented host associations or identify new potential insect vectors of fungal pathogens. Such information is valuable for long-term monitoring of biodiversity changes and for the early detection of unusual or emerging patterns in forest ecosystems.

If employed for more targeted sampling of diseased plants or plant tissues, HTS can be used for the detection of regulated pathogens and pests or even provide information on the identity of previously unknown

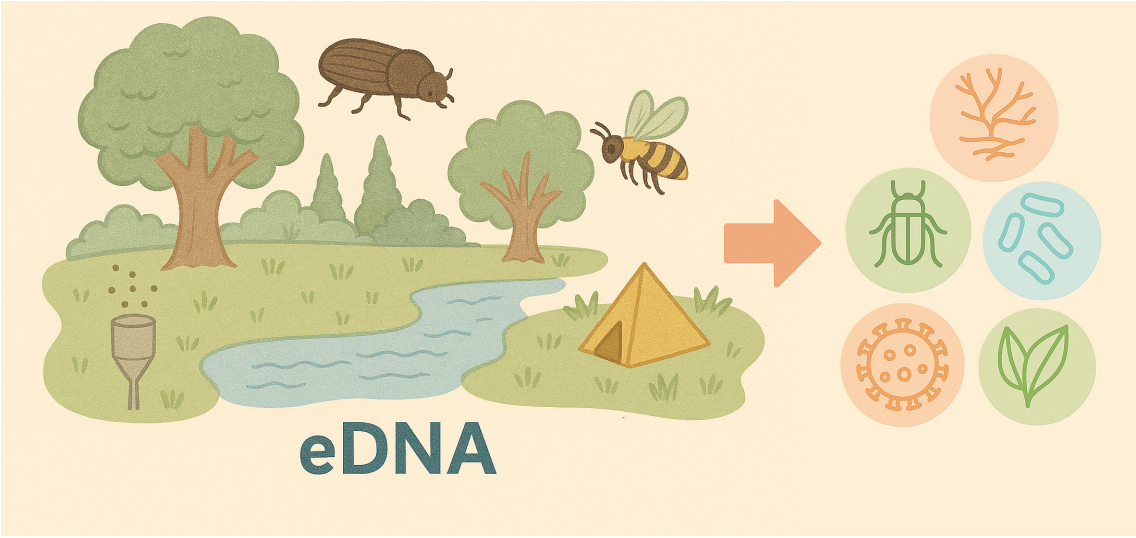

Fig. 3: Examples of sample types that can serve as sources of eDNA when using high-throughput sequencing for forest health protection (figure generated using AI).

Slika 3: Primeri vzorcev, ki so lahko vir eDNA za uporabo visokozmogljivega sekvenciranja pri varovanju zdravja gozda (slika ustvarjena s pomočjo umetne inteligence).

Table 1: Some examples of studies utilising HTS in combination with environmental DNA (eDNA) that specifically target or include forest pests or pathogens.

Preglednica 1: Nekaj primerov raziskav, kjer so s pomočjo visokozmogljivega sekvenciranja v kombinaciji z okoljsko DNA (angl. environmental DNA, eDNA) usmerjeno iskali ali našli organizme, škodljive gozdu.

Target taxa	Source of eDNA	Sampling location(s)	Publication
Plant pathogens			
Phytophthora de Bary	soil samples	chestnut orchards and forests	Vannini et al. (2013)
Phytophthora	soil and water samples	different types of forests	Català et al. (2015)
Phytophthora	water and soil samples	forest and ornamental nurseries	Bačová et al. (2024)
Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz & Hosoya	plant material (leaves)	forest stand	Cross et al. (2016)
Heterobasidion annosum (Fries) Brefeld, *Hymenoscyphus fraxineus* and *Erysiphe alphitoides* (Griffon & Maublanc) Braun & Takamatsu	spore samplers	different types of forest	Chandelier et al. (2021)
Fusarium Link	soil samples	field	Karlsson et al. (2016)
bark beetle-associated fungi, including phytopathogens	bark beetle specimens from insect traps	not provided	Miller et al. (2016)
bark beetle-associated fungi, including phytopathogens	non-native wood-boring beetles from insect traps	harbours	Malacrinò et al. (2017)
fungi, including tree pathogens	active spore traps	rural and urban areas	Nicolaisen et al. (2017)
exotic forest pathogens	spore traps, insect trap conservation fluid, rotary arm samplers, soil samples	commercial and industrial zones, urban forest, municipal green-waste disposal facilities	Tremblay et al. (2018)
phytopathogens, including several tree pathogens	insect trap conservation fluid	industrial and commercial zones, landfills, solid waste disposal facilities	Tremblay et al. (2019b)
forest fungal pathogens	active spore trap samples	urban areas with known forest disease presence	Aguayo et al. (2020)
fungi, including phytopathogens	water samples	secondary growth forest	Matsuoka et al. (2021)
oomycetes	planting soil	points of entry	Rossmann et al. (2021)
Bretziella fagacearum (Bretz) Z.W. de Beer, Marincowitz, T.A. Duong & M.J. Wingfield	insect traps	woodland areas (identified in risk analysis)	Gauthier et al. (2023)
Opiostomatales Benny & Kimbr	bulk insect specimens and insect trap conservation fluid	points of entry and *Pinus* plantations	Trollip et al. (2023)
fungi, including phytopathogens	water samples	areas with different land uses within a river basin	Pham et al. (2024)
bacteria and fungi, including phytopathogens	soil samples	moist and dry tropical forests	Saltonstall et al. (2025)
Arthropods, including plant pests			
non-native forest insects	insect traps	points of entry	Bowser et al. (2019)
arthropods (including pests and invasive species)	insect traps	forest national park	Hardulak et al. (2020)
Multiple target taxa			
viruses, bacteria, plants, fungi and arthropods	honey	eucalyptus tree and orange tree plantations	Bovo et al. (2018)

causal agents of disease (Piombo et al., 2021; EPPO, 2022). It can also be employed to determine the geographical origins of an already established outbreak (Hubbard et al., 2015; Piombo et al., 2021).

In the case of a non-pathogenic organism transitioning into a pathogenic one, if HTS data (especially whole-genome data from shotgun sequencing) are available from before and after the transition, these can be compared and the process studied from a genomic and genetic perspective (Piombo et al., 2021).

HTS enables the detection of fungi without the limitations of traditional isolation on growth media (Aguayo et al., 2018; Banchi et al., 2020; Piombo et al., 2021; Tremblay and Bilodeau, 2022). This means that problems with fungi that exert antagonistic effects on other fungi during culturing can be avoided (Brglez et al., 2020), and even obligate biotrophic fungi that cannot be cultured can be detected (Fierer et al., 2008; Hubbard et al., 2015; FAO, 2019; Tedersoo et al., 2019; Banchi et al., 2020; Piombo et al., 2021). Additionally, it enables the detection of slow-growing fungi, which would otherwise be overgrown by faster growing species during isolation on growth media and are therefore difficult to isolate (Bridge and Spooner, 2001; Piombo

et al., 2021). HTS can also overcome another issue that can arise when dealing with fungal pathogens, namely the fact that affected plants can remain asymptomatic for extended periods before developing symptoms that can be detected during visual surveys (Tremblay and Bilodeau, 2022; Zajc et al., 2023), as demonstrated by Cross et al. (2016) when monitoring the accumulation of *Hymenoscyphus fraxineus*, the pathogen responsible for ash dieback, in plant material during both asymptomatic and symptomatic stages of the disease.

Additionally, the methodology enables the sequencing of organisms whose DNA has not yet been sequenced or included in reference databases (Piombo et al., 2021). Unfortunately, the reason for an operational taxonomic unit (OTU) having low similarity (<97%) to sequences already present in, for example, the National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/) cannot be determined, as this may be due to a lack of sequences in the database, DNA degradation prior to sampling, sequencing errors or erroneous database entries (Sinha and Häder, 2002; Després et al., 2007). Still, repeated detection of the same "unknown" OTU across samples suggests a real organism not yet catalogued (Fröhlich-Nowoisky et al., 2009).

For the detection of fungal pathogens and insects targeting woody plants, two basic types of HTS can be used for different outcomes:

a) Amplicon sequencing or metabarcoding: taxon-specific barcodes (standardised genetic markers) are amplified, sequenced and used to identify taxa present in the sample (Tremblay et al., 2019a; Piombo et al., 2021). Standard EPPO barcodes for fungi and arthropods, as proposed by the European and Mediterranean Plant Protection Organisation (EPPO), can be used (EPPO, 2021), as well as additional markers that have been developed for this purpose.
b) Shotgun or whole-genome sequencing: all extracted DNA from a sample is fragmented (either by enzymatic digestion or by sonication) into shorter strands that can then be sequenced and reassembled. In this way, whole genomes of organisms present in a sample can potentially be recovered, including any phytopathogens and pests (Piper et al., 2019; Piombo et al., 2021; EPPO, 2022).

For the detection of fungal pathogens and insect pests that target woody plants (either in forests or in urban settings), many possible sample types can be utilised, including:

- Individual organisms (e.g. fungal isolates obtained from samples and individual insect specimens)
- Soil (targeting fungi and insects) and frass samples (targeting insects and insect-associated fungi)
- Water (targeting fungi and insects)
- Entomological traps: preservation fluid and bulk specimens (targeting insects and fungi); individual specimens (as fungal vectors, targeting insect-associated fungi)
- Honeybees, their honey and pollen (targeting fungi)
- Air sampling – spore traps: active and passive (targeting fungi)
- Plant samples – symptomatic or asymptomatic tissue (targeting fungi) and woody tissues from bores/galleries (targeting insects and insect-associated fungi)

As environmental samples can contain a diverse range of (micro)organisms, often in the presence of organic inhibitors, DNA extraction methods should be chosen according to the sample type and target organism(s) (Tedersoo et al., 2019). Optimisation of extraction efficiency is of utmost importance, as target organisms in samples are often present in low quantities or present other challenges that lower the success rate of extraction, such as thick spore cell walls (Tremblay and Bilodeau, 2022). The ability to analyse environmental samples also enables the use of more unconventional samples, such as spider webs (Gregorič et al., 2022) or even teabags (Krehenwinkel et al., 2022), and the utilisation of samples from already established monitoring networks, such as aerobiological networks set up to monitor pollen dispersal (Aguayo et al., 2020).

2.1 Sampling of bees, honey and pollen
2.1 Vzorčenje čebel, medu in cvetnega prahu

As pollinators, European honeybees play an important role in agriculture and the environment in general (Tremblay et al., 2019a; Cunningham et al., 2022). Pollen is an essential part of their diet, providing most of the necessary minerals, lipids and proteins. In addition to pollen, honeybee workers have been observed actively collecting willow rust spores (*Melampsora* spp.) (Migdał et al., 2024) and are able to ingest spores of potential plant pathogens, such as *Botrytis cinerea* Persoon, *Cladosporium* sp. and *Colletotrichum acutatum* J.H. Simmonds, which can still be viable after passing through their digestive tract (Parish et al., 2019). Pollen grains are collected by foraging workers and packed as pellets into corbiculae on their rear legs, which can then be collected upon their return to the hive via pol-

len traps (Cornman et al., 2015; Tremblay et al., 2019a; Cunningham et al., 2022) or from cells within the hive (Cunningham et al., 2022; Roberts et al., 2023). Several genera of potentially phytopathogenic fungi have already been detected in pollen with the help of HTS, including *Fusarium*, *Ophiostoma* Sydow & P. Sydow, *Peronospora* Corda, *Phytophthora* and *Pythium* Pringsheim (Tremblay et al., 2019a).

Worker bees themselves can be sampled from the hive or while foraging on a symptomatic plant (Cunningham et al., 2022; Roberts et al., 2023). The number of foraging workers per colony can climb into the thousands and they can forage within a radius of several kilometres from the hive (Beekman and Ratnieks, 2000; Tremblay et al., 2019a). This abundance and foraging range make them, as has been demonstrated previously, useful as sentinels for detecting plant pathogens, including fungi (van der Steem et al., 2018; Tremblay et al., 2019a; Cunningham et al., 2022; Roberts et al., 2023).

Honey can also serve as a source of information on the local presence of phytopathogenic and saprotrophic fungi (Magyar et al., 2016; Bovo et al., 2018). Bovo et al. (2018) used a metagenomic approach to characterise DNA signatures in orange and eucalyptus tree blossom honeys, detecting several potential plant pathogens.

2.2 Air sampling
2.2 Vzorčenje zraka

Bioaerosols are composed of airborne microorganisms and other (micro)biological material, e.g. free DNA and small pieces of tissue (Angenent et al., 2005). Airborne transfer represents one of the key transmission pathways for many plant pathogens (Fierer et al., 2008; Aguayo et al., 2018), even though prolonged exposure in the atmosphere presents a unique set of challenges, including low humidity, UV radiation and a lack of nutrients (Fierer et al., 2008). Consequently, the likelihood of detecting fungal DNA in bioaerosols is greatest when it is present in the form of spores, as they are less susceptible to environmental degradation than free DNA or parts of fungal tissues (Després et al., 2007; Fröhlich-Nowoisky et al., 2009). While the detection of free DNA and DNA from fungal tissues is still possible, the effects of degradation are likely to be more pronounced.

The small particles that make up bioaerosols have different residence times in the air depending on their size: fine dust particles (≤ 2.5 µm) remain in the air for days or weeks, while coarse dust particles (> 1 µm) are removed sooner, either by precipitation, sedimentation or scavenging by insects or other animals (Fröhlich-Nowoisky et al., 2009).

Fungal spores can be carried by air currents over long distances, which increases their potential for spread to novel geographical areas where their taxa are not actively targeted by phytosanitary surveys (Piombo et al., 2021). Employing HTS as a non-specific method with a broad scope increases the likelihood of the early detection of such introductions (Piombo et al., 2021). Because successful trapping of spores in the air is partly dependent on spore dispersal efficiency, it is inherently biased towards fungi, such as many basidiomycetes and ascomycetes, that spread their spores actively through ejection via water jets or droplets (Fröhlich-Nowoisky et al., 2009; Aguayo et al., 2020). Additionally, seasonal variation in airborne spore presence should be considered, as different fungal species produce fruiting bodies at distinct times of the year (Marshall, 1997; Fröhlich-Nowoisky et al., 2009; Banchi et al., 2020).

Examples of collectors and traps successfully used in plant pathogen studies include:

- Passive surface collectors such as double-sided tape, coated microscope slides or Petri dishes, or disks made from either wood or filter paper (Aguayo et al., 2018)
- Passive rainfall collectors with a cellulose nitrate or filter membrane used to collect particles in the funnel (Bérubé et al., 2018b; Tremblay et al., 2018)
- Active rotary-arm spore samplers with two vertical rods dipped in silicone grease or covered in double-sided tape (Aguayo et al., 2018; Bérubé et al., 2018a; Bérubé et al., 2018b; Tremblay et al., 2018; Chandelier et al., 2021)
- Volumetric ("Hirst") traps with petroleum jelly-coated tape (Núñez et al., 2017; Aguayo et al., 2020; Banchi et al., 2020; Chandelier et al., 2021; Piombo et al., 2021; Zajc et al., 2023)

Aguayo et al. (2018) used mock communities in combination with passive spore trapping to test the use of metabarcoding for the detection of fungi, including plant pathogens, while Nicolaisen et al. (2017) focused on employing active volumetric samplers to assess the detection of plant pathogens in urban and rural settings. While passive samplers are generally less costly and easier to employ, active samplers are capable of processing greater air volumes (Evenhuis et al., 1997; Aguayo et al., 2018). Chandelier et al. (2021) employed volumetric and active rotary-arm samplers to evaluate the detection efficiency of qPCR and HTS for three forest fungal pathogens, *Heterobasidion an-*

nosum, *Hymenoscyphus fraxineus* and *Erysiphe alphitoides*. They found rotary-arm samplers to be more suitable for forest environments than volumetric samplers and reported that pathogen-specific qPCR assays exhibited greater sensitivity compared with metabarcoding approaches.

2.3 Entomological traps
2.3 Entomološke pasti

For use with HTS methods, several sample types can be obtained from entomological traps. When looking for species of concern or studying insect diversity by extracting DNA from bulk insect samples from entomological traps, visual pre-sorting by taxonomic order (Morinière et al., 2016) or size (Elbrecht et al., 2021) seems to increase taxon recovery. Additionally, the preservation fluids used in wet types of traps can serve as a source of DNA (Marquina et al., 2019; Young et al., 2021; Gauthier et al., 2023), including for the detection of fungal pathogens, which has already been tested in urban, industrial and forest settings (Tremblay et al., 2019b; Trollip et al., 2023). Bérubé et al. (2022) found, when searching for forest pathogens, that they were able to detect more species with metabarcoding in trap fluids compared with passive spore collectors, while Trollip et al. (2023) had greater success detecting *Ophiostomatales* taxa in insect trap preservation fluids than in the bulk insect samples from the same traps. For *Bretziella fagacearum*, the causal agent of oak wilt disease, direct detection with metabarcoding is possible, although detection on insects proved to be more successful than in the conservation fluid from the same traps (Gauthier et al., 2023).

Depending on the target taxa, several types of traps are available for use. Malaise traps and their more versatile version, Sea, Land and Air Malaise (SLAM) traps, are examples of wet traps that target insects that instinctively fly upwards when hitting a barrier (Ritter et al., 2019). Their tent-like design, with a large opening on the bottom and a vertical central wall, directs the flying insects upward towards the small opening at the top, leading into a container with the killing agent (Townes, 1962). Samples from Malaise traps have already been shown to be useful for screening invasive and pest species with HTS methodology in a national park (Hardulak et al., 2020). Sticky (glue) traps, comprising paper covered in glue, often in combination with a pheromone lure, are a simple and cost-effective way of trapping insects. Funnel traps, in combination with DNA metabarcoding techniques, have been employed to monitor the arrival of non-native insect species that pose potential threats to forest ecosystems in Alaska (Bowser et al., 2019). Additionally, these methods have facilitated the investigation of fungal associates of bark beetles, including the detection of potential tree pathogens (Miller et al., 2016; Malacrinò et al., 2017).

Due to frequent physical damage incurred during specimen collection, morphological identification is often impractical or even impossible. However, such degradation does not compromise the suitability of these samples for HTS analysis. Additionally, HTS analysis is independent of the life-cycle stage of the targeted organism, which is an obstacle often encountered when using morphological methods for entomological identification. On the other hand, one of the main drawbacks of using entomological trap samples for HTS is that the trapped insects are exposed to environmental effects until the trap is collected, which can impact DNA quality (Butterwort et al., 2022).

When extracting DNA from individual insects, non-destructive methods enable preservation of the specimens for optional further morphological analysis (Piper et al., 2019; Lebas et al., 2022). In addition to the insects themselves, samples from entomological traps can provide information on insect parasites or parasitoids and on fungi that the insects might be carrying (Ritter et al., 2019). As insects, especially those capable of flight, can come into contact with many different plants and fungi during their activities, they can be a valuable source of information on local biodiversity and for the early detection of specific pathogenic fungi.

It has also been suggested that HTS methodology has the potential to help estimate population size based on a mixed sample (Piper et al., 2019), which could be useful for estimating the severity of a possible outbreak.

2.4 Soil sampling
2.4 Vzorčenje zemlje

Soil sampling can target the insects and fungi contained within the samples, as well as insect faeces and frass, parts of decomposing tissue and free DNA (DNA present outside of cells) that might be present (Ritter et al., 2019). In comparison with sampling via entomological traps, this increases the chance of detecting insects that were not alive at the time of sampling (Carini et al., 2016; Ritter et al., 2019). That might be an advantage when searching for the first signs of presence in an area, or a disadvantage when looking for a snapshot of current biodiversity in a specific location. In the latter case, protocols have been developed that can render free DNA non-amplifiable by binding it with an intercalating dye (Carini et al., 2016). It has

also been suggested that, for metabarcoding of soil samples, pooling several technical replicates of DNA extractions from a sample might improve the accuracy of detected diversity within that sample (Aguayo et al., 2018). For example, metabarcoding has already been used to investigate *Phytophthora* presence in soil samples sourced from different forest types (Vannini et al., 2013; Català et al., 2015), to detect phytopathogenic oomycetes in potting soil from internationally shipped plants (Rossmann et al., 2021) and to compare the relative abundance and diversity of potential plant pathogens between different types of forests (Saltonstall et al., 2025). Unlike most fungal metabarcoding experiments that usually target the ITS region, Karlsson et al. (2016) developed a genus-specific primer set to characterise the frequently phytopathogenic *Fusarium* species in soil samples.

2.5 Water sampling
2.5 Vzorčenje vode

Analyses of (sea) water samples have shown that DNA fragments degrade within days, which makes taxa detected in water more likely to have been (recently) present as living organisms (Ruppert et al., 2019). Typically, water samples are collected at various depths into sterile bottles, then filtered, and DNA is extracted from the filter papers (Shaw et al., 2016) or membranes (Banerji et al., 2018). In addition to sampling the water itself, sediment samples from the bottom can be collected and DNA extracted using techniques similar to those used for soil samples (Shaw et al., 2016). In the study by Català et al. (2015), which investigated *Phytophthora* species in soil samples sourced from various forest types, water samples were also utilised as a DNA source and yielded even greater success in species detection. Pham et al. (2024) detected potential forest tree pathogens in agriculturally important freshwater reservoirs with the help of metabarcoding, while Matsuoka et al. (2021) used it to investigate fungal communities and detect plant pathogens by sampling a stream in a restored (secondary-growth) forest. Bačová et al. (2024) investigated irrigation water used in forest and ornamental nurseries to detect *Phytophthora* species.

3 SYNERGY OF HTS METHODOLOGY WITH OTHER DETECTION METHODS
3 SINERGIJA METODOLOGIJE VISOKOZMOGLJIVEGA SEKVENCIRANJA Z DRUGIMI NAČINI ZAZNAVE

In comparison with traditional morphological identification, HTS methodology demands less taxonomic expertise and time per analysis and has a much higher throughput level (Piper et al., 2019; Banchi et al., 2020). While a lack of advanced taxonomic expertise is also not a problem for traditional molecular methods such as end-point PCR, real-time PCR (qPCR), digital droplet PCR (ddPCR) and loop-mediated isothermal amplification (LAMP), their throughput level is substantially lower than that of HTS. Whereas traditional DNA barcoding generally allows for resolution to the rank of species, end-point PCR, qPCR, ddPCR and LAMP require time-consuming development of specific protocols in order to allow for such resolution (FAO, 2019). Still, previous studies have found that species-specific qPCR assays may have a higher rate of detection compared with HTS (Aguayo et al., 2018). Additionally, ddPCR enables absolute quantification of the target pathogen and may be more robust when dealing with inhibitor-heavy matrices, as the method requires dilution of the sample to a single detectable molecule, which in turn also dilutes the inhibitors and lowers their effect on the polymerase used in the reaction (Rački et al., 2014; Mehle et al., 2018). Despite the high throughput and breadth of scope of HTS, it does not make more traditional morphological and molecular methods obsolete; rather, it is optimal to use them as complementary methodologies, both for confirmation of HTS results and for building and improving the databases that HTS relies on. In recent years, a new type of protocol that combines amplification with specific qPCR primers and high-throughput sequencing for targeted detection of invasive species in eDNA samples has emerged (Westfall et al., 2022). Successfully used to detect invasive crab species, the methodology could potentially be adapted for use in forest protection as a means of detecting specific taxa of high importance.

4 TECHNICAL CONSIDERATIONS AND ANALYSIS
4 TEHNIČNI VIDIK IN ANALIZA

At each consecutive step of the HTS process, biases that impact the final outcome of the analysis can be introduced (Cristescu, 2014; Aguayo et al., 2018; Piombo et al., 2021). For each application, these biases must be considered and efforts made to minimise their impact on the final result (Aguayo et al., 2018).

Implementing high-throughput sequencing in forest health surveillance requires careful attention to technical aspects that influence data quality and diagnostic reliability. While the methodology is robust, its sensitivity and broad target range make it susceptible to contamination and bias (Piper et al., 2019; Tedersoo et al., 2019; Piombo et al., 2021; EPPO, 2022; Lebas et al., 2022). Therefore, appropriate controls, validated

reference materials and reliable bioinformatics pipelines are essential to ensure accurate interpretation, especially when detecting low-abundance or novel pathogens (Budowle et al., 2014; Olmos et al., 2018; EPPO, 2022; Massart et al., 2022).

For practical integration into forest monitoring systems, laboratories should consider scalable IT infrastructure, curated databases relevant to forest pests and pathogens, and sequencing platforms suited to environmental samples. The choice of primers and target regions should prioritise taxa of concern in the studied ecosystems, while minimising host DNA amplification (Tedersoo et al., 2019; Lebas et al., 2022). Ultimately, HTS should be viewed as a complementary screening tool that enhances—but does not replace—traditional morphological and molecular diagnostics.

When interpreting the results, each step should be clearly documented (Budowle et al., 2014). Bioinformatics workflows typically begin with quality filtering of raw reads, followed by trimming of adapters and low-quality regions. Artefacts and background sequences (e.g. host DNA or contaminants) can be removed, and reads are either clustered into operational taxonomic units (OTUs), denoised into amplicon sequence variants (ASVs) or, if using shotgun sequencing, assembled into contiguous sequences (contigs) based on sequence similarity and overlap (Budowle et al., 2014; Edgar, 2017; Caruso et al., 2019; Piper et al., 2019; Ruppert et al., 2019; Tedersoo et al., 2019; Piombo et al., 2021; Lebas et al., 2022; Runnel et al., 2022; Tremblay and Bilodeau, 2022). Taxonomic assignment should be performed using curated databases, and results should be evaluated through ecological and geographical plausibility (Piper et al., 2019). Validated, automated and reproducible pipelines are recommended to ensure accuracy and consistency, especially when processing large volumes of environmental samples in forest health applications.

5 CONFIRMATION OF DETECTION
5 POTRDITEV ZAZNAVE

HTS is a very useful tool, especially when employed as a broad-spectrum screening method. Any positive results should, once analysis of controls has excluded a false positive, be confirmed by a previously established morphological or specific molecular test (e.g. real-time PCR or conventional PCR in combination with Sanger sequencing), ideally targeting different regions (Olmos et al., 2018; Piper et al., 2019; Aguayo et al., 2020; Massart et al., 2022; Tremblay and Bilodeau, 2022). Confirmation of plant health test results is crucial and is particularly recommended in the following situations: when a pest/pathogen is detected in a new area; when a pest/pathogen is detected for the first time by a specific laboratory and in cases in which the sample originates from an area where the pest/pathogen has been declared eradicated; or when a pest/pathogen is detected in a consignment that has been subjected to phytosanitary treatment (EPPO, 2018). For official plant health laboratories in the European Union, these confirmatory methods should be chosen in accordance with the EU Regulation on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products (Regulation 2017/625). In other cases, the need for additional confirmation can be determined on a case-by-case basis, depending on the organism and its distribution and impact, the presence of unusual symptoms and other contextual factors (Massart et al., 2022). Additionally, HTS methods (as well as other molecular methods) detect only the presence of genetic material, irrespective of whether it was part of a viable organism or spore, or present only in trace amounts (FAO, 2019; Ritter et al., 2019; Massart et al., 2022). This is even more relevant if a plant pest/pathogen is detected without evidence of an associated host (Massart et al., 2022). Thus, if a novel fungal pathogen is detected, it should still be confirmed with further targeted sampling and, where possible, Koch's postulates (which may not be feasible in the case of disease complexes involving several biotic and/or abiotic components) (Olmos et al., 2018; Piombo et al., 2021; Massart et al., 2022).

6 CONCLUSIONS
6 ZAKLJUČKI

HTS is a relatively young methodology and has only recently started to be incorporated into the international plant protection framework of standards and guidelines. Used alongside traditional methods, both molecular and morphological, it is a powerful tool that enables the processing of large numbers of samples. Still, the importance of traditional methods and tools for confirmation and for further specific investigation of HTS findings—whether as stand-alone tests or as tools for improving and maintaining the databases and voucher collections on which HTS relies—should not be underestimated or neglected. Protection of forest health is a very dynamic field that will, given current global and environmental trends, continue to change rapidly. As HTS continues to evolve, its role in proactive forest protection strategies will likely become increasingly central. Future developments may focus on improving detection sensitivity for low-abundance

pathogens, streamlining workflows for routine diagnostics and integrating HTS data into national surveillance systems. These advancements could enable faster response times, better risk assessment and more targeted management of forest health threats.

7 SUMMARY

7 POVZETEK

Globalizacija in rast mednarodne trgovine večata verjetnost za vnos novih rastlinskih škodljivcev in gliv, povzročiteljic rastlinskih bolezni. Če k tem okoliščinam prištejemo še spremembe v okoljskih razmerah, lahko pričakujemo zvišano verjetnost za stik škodljivcev in gliv z novimi potencialnimi gostiteljskimi drevesnimi vrstami. Za to, da zmanjšamo potencialno okoljsko in ekonomsko-socialno škodo, ki bi nastala, je bistveno zgodnje odkrivanje vnosa takih organizmov. Za ta namen je uporabna tudi razmeroma nova tehnologija visokozmogljivega sekvenciranja, ki teoretično omogoča sekvenciranja vsega genetskega materiala v vzorcu. Uporabi se lahko za iskanje večjega števila tarčnih taksonomskih skupin v velikem številu vzorcev hkrati. Dodatna prednost je tudi, da tip vhodnega vzorca ni omejen. Tako potencialno omogoča razširitev nabora tarčnih organizmov v okviru različnih monitoringov, katerih število je bilo s tradicionalnimi metodami omejeno zaradi finančnih in časovnih vložkov ter zahtevnosti morfoloških analiz. Pomembna prednost je tudi to, da nekateri načini vzorčenja omogočajo pokrivanje večjega tarčnega območja. V okviru zdravja gozda to pomeni, da lahko teoretično spremljamo tudi težje dostopna in manj obljudena gozdna območja, kjer običajno vizualne znake bolezni ali škodljivcev težko zaznamo, ali pa jih zaznamo z zamikom. Prav tako omogoča detekcijo asimptomatskih okužb, ki bi jih med vizualnimi pregledi lahko spregledali. Za organizme, ki jih na preiskovanem območju še ni, bi detekcija s pomočjo visokozmogljivega sekvenciranja lahko bila prvi znak njihovega pojavljanja ter bi bila v oporo pri načrtovanju nadaljnjega vzorčenja in analiz. Pri tem velja poudariti, da je zaznava simptomov v gozdnih predelih, tako zaradi težje dostopnosti kot manjše obljudenosti, pogosto počasnejša kot na kmetijskih in urbanih območjih. Način vzorčenja, ki ga uporaba visokozmogljivega sekvenciranja omogoča, in pri katerem z enim vzorcem pokrijemo širše geografsko območje, je tako uporabna rešitev za spremljanje stanja in poveča verjetnost zaznave škodljivih organizmov v gozdovih predvsem na račun usmerjenega spremljanja na taka območja. Visokozmogljivo sekvenciranje bi se prav tako lahko uporabilo za vzpostavitev osnovne linije - nabora organizmov, običajno razširjenih v preiskovanem okolju. Pridobljene sekvence bi lahko ob potencialnem izbruhu pomagale pri določanju geografskega izvora tarčnega organizma. Prednost metode je tudi njena neodvisnost od uspešnosti gojenja tarčnih gliv na gojiščih, s čimer zaobidemo težave z detekcijo obligatnih biotrofov ter počasi rastočih gliv, ki jih je težko izolirati, ne da bi jih hitrejše rastoče glive prerastle. Postopno naraščanje letnih objav na temo visokozmogljivega sekvenciranja s področja varovanja gozdov nakazuje, da se metodologija vedno bolj pogosto uporablja tudi za ta namen.

Za detekcijo žuželk in gliv, povzročiteljic bolezni lesnatih rastlin, metode visokozmogljivega sekvenciranja v grobem delimo na meta analizo črtnih kod, kjer pomnožimo in sekvenciramo genetske markerje, ter sekvenciranje celotnega genoma, kjer fragmentirano DNA iz vzorca sekvenciramo in dobljena zaporedja ponovno sestavimo. Uporabimo lahko posamezne organizme (npr. glivne izolate, pridobljene iz vzorcev, ali posamezne osebke žuželk), tla in vzorce črvine (detekcija žuželk in gliv, povezanih z žuželkami), vodo, konzervacijsko tekočino in množične vzorce žuželk iz entomoloških pasti, vzorce zraka (pasti za glivne trose). Za detekcijo gliv lahko uporabimo tudi rastlinsko tkivo in potencialne prenašalce (npr. čebele, njihov med in pelod), les iz rovov žuželk pa lahko služi kot vzorec za detekcijo žuželk ali gliv, povezanih z njimi. Lov žuželk je lahko učinkovitejši z uporabo atraktantov in sortiranjem ulova, prednost uporabe visokozmogljivega sekvenciranja za identifikacijo žuželk pa je tudi neodvisnost od razvojnega stadija in morfološke celostnosti osebkov. Ker žuželke prihajajo v stik z mnogimi organizmi, so koristen vir informacij o biodiverziteti in patogenih glivah. Prednost sekvenciranja je neodvisnost od razvojnega stadija in morfološke celostnosti žuželk.

Vzorčenje zemlje omogoča analizo prisotnih organizmov, iztrebkov, črvin, razkrajajočega tkiva in zunajcelične DNA. V raziskavah o zdravju gozda običajno vzorčimo gozdna tla ali tla iz gozdnih drevesnic. Metode za pridobivanje DNA iz tal so uporabne tudi za analizo vodnih sedimentov, medtem ko se sama voda lahko vzorči na različnih globinah. V kontekstu zdravja gozda se kot vir DNA uporablja voda iz vodotokov, zalivalnih sistemov in izcedna voda iz loncev v drevesnicah.

Visokozmogljivo sekvenciranje zahteva manj taksonomskega znanja, časa in ima večjo zmogljivost kot tradicionalne morfološke metode. Sicer druge specifične molekularne metode (npr. PCR, qPCR, ddPCR, LAMP) prav tako ne zahtevajo poglobljenega taksonomskega znanja, a imajo nižjo zmogljivost. Tradicionalne molekularne metode so tako lahko med

drugim odlična komplementarna metoda za potrditev izsledkov analize s pomočjo visokozmogljivega sekvenciranja.

Pri uporabi visokozmogljivega sekvenciranja za spremljanje zdravja gozdov je ključen tehnični vidik analize, saj občutljiva metodologija in širok nabor tarč povečujeta tveganje za kontaminacijo in posledično napačne rezultate. Potrebna je uporaba procesnih kontrol, validiranih referenčnih materialov ter baz in zanesljivih bioinformatičnih metod, prilagojenih okoljskim vzorcem in gozdnim škodljivcem. Prav tako je pomembna izbira ustreznih začetnih oligonukleotidov in tarčnih regij, ki zmanjšajo pomnoževanje gostiteljeve DNA. Bioinformatična analiza, ki sledi sekvenciranju, mora biti prilagojena glede na uporabljeno metodo in tarčne organizme. Za zagotavljanje točnih in konsistentnih rezultatov je priporočena uporaba preverjenega, avtomatiziranega in ponovljivega nabora bioinformatičnih metod. Analiza je procesno in podatkovno zahteven postopek, tako da je temu treba prilagoditi tudi strojno in procesno opremo laboratorija. Vsak korak, vključno z interpretacijo rezultatov, mora biti jasno zabeležen.

Visokozmogljivo sekvenciranje je učinkovito presejalno orodje, a pozitivne zaznave je kljub temu treba potrditi z uveljavljenimi morfološkimi ali molekularnimi metodami, zlasti pri fitosanitarni kontroli, zaznavi karantenskih organizmov ali prvem pojavu škodljivega organizma na novem območju.

Nujno pa je tudi upoštevati dejstvo, da metode visokozmogljivega sekvenciranja, tako kot druge molekularne metode, zaznajo genetski material, ne glede na to, ali ta obstaja le v sledeh ali dejansko pripada živemu organizmu ali viabilnemu trosu. Ta aspekt je še posebej pomemben v primeru detekcije novega škodljivca/patogena brez jasne povezave z gostiteljsko rastlino. V tem primeru je priporočljivo nadaljnjo tarčno vzorčenje in, v primeru gliv, če je možno, potrditev Kochovih postulatov.

Varovanje zdravja gozda je dinamično področje, ki se bo, glede na trenutne svetovne in okoljske trende, še naprej hitro spreminjalo. Visokozmogljivo sekvenciranje je relativno nova metodologija na področju zdravja rastlin, ki je v navezavi s tradicionalnimi morfološkimi in molekularnimi metodami močno orodje za procesiranje velikega števila različnih vzorcev. Vpeljava visokozmogljivega sekvenciranja kot komplementarne metode že obstoječim uveljavljenim metodam je tako izrednega pomena, saj bo omogočala boljšo zaščito naših gozdov pred obstoječimi in prihajajočimi grožnjami njihovemu zdravju.

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