Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268
(Print copies up to 2016)
e-ISSN: 2395-3454

Vol. 23 | No. 4 | pp. 2371-2382 | 2024

Original Research Paper

https://doi.org/10.46488/NEPT.2024.v23i04.040

Open Access Journal

A Comprehensive Genetic Analysis of Mycotoxin-Producing *Penicillium expansum* Isolated from River Water Using Molecular Profiling, DNA Barcoding, and Secondary Structure Prediction

R. Ravikiran[1], G. Raghu[2] and B. Praveen[1†]

[1]Department of Plant Pathology, M.S. Swaminathan School of Agriculture, Centurion University of Technology and Management, Paralakhemundi, Gajapati, Odisha, India

[2]Department of Antigen Design and Protein Biochemistry, PopVax Pvt Ltd, AIC-CCMB, Hyderabad, Telangana, India

†Corresponding author: Praveen Boddana; bpraveen@cutm.ac.in

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 12-03-2024
Revised: 16-04-2024
Accepted: 03-05-2024

Key Words:
Environmental contamination
DNA barcoding
Genetic characterization
Molecular profiling
Mycotoxin
Pathogenic fungi
Penicillium expansum
Phylogenetic analysis,
Water quality monitoring

ABSTRACT

This study marks the first report on the genetic characterization of *Penicillium expansum* strain capable of mycotoxin production isolated from river water. Situated in Ganagalawanipeta village, Srikakulam, Andhra Pradesh, India, where river water serves as a vital resource, our investigation probed the presence of pathogenic opportunistic fungi adept at mycotoxin synthesis. Over six months, 30 samples were collected to assess their occurrence. This article revolves around the use of morphological traits for Penicillium genus identification. Precise species determination involved PCR analysis using universal primers ITS1 and ITS4, followed by sequence analysis through NCBI-BLASTn and the ITS2 database. The analysis indicated a striking 99.49% genetic similarity to *Penicillium expansum* isolate MW559596 from CSIR-National Institute of Oceanography, Goa, an Indian isolate, with a resultant 600-base pair fragment. This sequence was officially cataloged as OR536221 in the NCBI GenBank database. Sequence and phylogenetic assessments were conducted to pinpoint the strain and geographical origin. Notably, the ribosomal nuclear ITS region displayed significant inter- and intra-specific divergence, manifested in DNA barcodes and secondary structures established *via* minimum free energy calculations. These findings provide crucial insights into the genetic diversity and potential mycotoxin production of *P. expansum* isolates, shedding light on the environmental repercussions and health risks associated with river water contamination from agricultural and aquaculture effluents. This pioneering research advances our understanding of mycotoxin-producing fungi in aquatic environments and underscores the imperative need for water quality monitoring in regions reliant on such water sources for their sustenance and livelihoods.

INTRODUCTION

Penicillium, a genus of fungi within the order Eurotiales, exhibits remarkable diversity, inhabiting a wide array of environments worldwide, including soil, indoor settings, marine environments, and more. Its name, "Penicillium," is derived from the Latin term "penicillus," signifying the brush-like appearance of its conidiophores. With roughly 300 described species, Penicillium is ubiquitous, with its spores pervading both air and soil. While some species contribute to the food-making process and are essential in processes like cheese production, others, such as *P. expansum, P. digitatum*, and *P. alii*, act as plant pathogens, causing diseases in apples, citrus fruits, and garlic, respectively. Notably, Penicillium species are frequently responsible for food spoilage, making effective food preservation methods crucial in preventing fungal contamination.

Penicillium expansum, commonly known as the blue mold fungus, is a fungal species with significant ecological and economic importance. As a notorious plant pathogen, *P. expansum* is a primary cause of post-harvest decay in fruits, particularly apples and pears (Guerzoni et al. 2002). Its ability to infiltrate fruits through wounds, spreading rapidly and manifesting as the characteristic blue-green mold, results in considerable economic losses in the fruit industry (Vilanova et al. 2018). In natural ecosystems, *P. expansum* also plays an essential role in the decomposition of organic matter, contributing to nutrient cycling processes (Tannous et al. 2018). Furthermore, its interactions with other microorganisms in various ecological contexts, both competitive and symbiotic, are of ecological interest (Magan & Aldred 2007). This ecological understanding has led to the development of biocontrol strategies utilizing beneficial microorganisms to suppress *P. expansum* growth and mitigate

its impact on agriculture (Pitt & Hocking 2009). Nonetheless, *P. expansum* remains a concern in the food industry, where it can contaminate processed apple products, emphasizing the need for strict food handling and storage practices (Lanciotti et al. 2005). Overall, *P. expansum*'s ecological and economic significance underscores the importance of further research and sustainable management strategies for this pathogen.

A striking and relevant aspect of fungi lies in their capacity to produce mycotoxins, which create significant challenges by potentially accumulating in grains and contaminating processed food items along the human food supply chain. This dual role not only introduces considerable economic stakes but also raises health concerns, especially concerning natural water resources (Ponts et al. 2018). Within the diverse realm of fungi, the Penicillium genus takes center stage, featuring four notable species: *P. expansum, P. chrysogenum, P. commune*, and *P. funiculosum*. *P. expansum*, in particular, has gained infamy for its role in causing destructive rot in various fruits and vegetables and is a well-known producer of patulin. Intriguingly, certain strains of *P. expansum* possess the capability to produce additional mycotoxins like citrinin, ochratoxin A, penitrem A, and rubratoxin B, underscoring the essential need for rigorous monitoring and control measures for this fungal species within the food production chain.

Nevertheless, precisely identifying fungi at the species level remains a formidable challenge, posing obstacles in both fundamental scientific research and practical applications. Morphological characteristics, conventionally employed for genus-level identification, can be contentious and, in certain instances, insufficiently precise, even for seasoned mycologists. This challenge becomes particularly pronounced when attempting to categorize fungi at the species level (Raja et al. 2017).

The molecular characterization of *P. expansum* holds significant importance in the context of accurate and rapid identification, especially due to the challenges associated with traditional morphological classification. Molecular techniques, such as DNA sequencing and genotyping, offer precise and efficient tools for distinguishing *P. expansum* from other closely related fungal species, as well as for differentiating between various strains within this species. This level of resolution is crucial for food safety and quality control, as *P. expansum* is a known producer of mycotoxins and can lead to fruit decay, potentially causing economic losses and health risks (Ballester et al. 2015). Molecular identification also aids in tracking the presence of specific mycotoxigenic strains of *P. expansum* in food products, contributing to better risk assessment and mitigation strategies. Furthermore, the ability to rapidly and accurately identify *P. expansum* at the molecular level is of paramount importance in agricultural and food industries, where swift action is often required to prevent contamination and spoilage.

This study aims to evaluate the presence of opportunistic human pathogenic fungi capable of mycotoxin production in river water, a vital resource for the local population. Our research was conducted in Ganagalawanipeta village, situated in Srikakulam, Andhra Pradesh, India, where river water is subjected to contamination resulting from the discharge of agricultural and aquaculture waste. The primary objective of our investigation was the identification of mycotoxin-producing Penicillium species through the targeted analysis of the Internal Transcribed Spacer (ITS) region within their genetic material. Our results highlight that *P. expansum* stands out as the most frequently detected species in polluted river water.

MATERIALS AND METHODS

Ef t dsjqujpo!boe!Sf t f bsdi !Tjuf

Ganagalawanipeta village is heavily dependent on the Nagavali River, employing it for multiple purposes such as drinking, agricultural, and aquacultural needs. In close proximity, a 100-acre expanse is designated for aquaculture practices (Blackwell 2011). Regrettably, water tainted by pollutants from the upstream aquaculture ponds is released into the river, subsequently becoming a source for downstream usage, encompassing both human and animal consumption.

Dpnfdujpo!pf !X buf s!Tbn qmft

During a half-year duration spanning from October 2020 to March 2021, a comprehensive set of 30 water samples was meticulously acquired from five distinct locations situated within the wastewater disposal area (Fig. 1). These samples were assiduously procured in sterile screw-top containers directly at the research site. The pH levels of the specimens were promptly gauged and, after that, stored under refrigeration for preservation. For sample collection, plastic receptacles were employed, having been meticulously sterilized using a 70% alcohol solution and subsequently rinsed with distilled water. Before sample collection at the river, these containers underwent a triple rinse with river water, adhering to established procedures (Clarridge 2004).

Isolation of Fungi

To culture fungal species in the investigation, water samples collected from diverse locations underwent a serial fivefold dilution. Subsequently, the spread plate method was utilized, involving the application of 0.1 mL of the diluted samples

Fig. 1. Illustrates the study area and the process of fungal isolation sample collection. Monthly river water samples were obtained from three distinct locations along the Nagavali River, allowing for an investigation into the seasonal variations in fungal diversity. The samples were carefully collected using sterile containers and subsequently transported within a temperature-controlled chain. Alongside each collection, physicochemical data, including temperature, pH, salinity, dissolved oxygen, and nutrient levels, were simultaneously recorded.

onto Petri dishes furnished with Sabouraud Dextrose Agar (SDA) and Czapek Yeast Extract Agar (CYEA), aiming for identification at the genus level (Bandh et al. 2012).

Hf opn jd!EOB!J pihjpo

The cell disruption process began with rapid freezing using liquid nitrogen and subsequent grinding into a fine powder using a sterile mortar and pestle. For DNA isolations, 100 mg of this disrupted mycelium was used. Genomic DNA isolation, performed in triplicate, included Proteinase K and RNase treatment to remove RNA contaminants. Purified DNA samples were resuspended in Tris-HCl buffer (pH 8.0, 1 mM EDTA) for further analysis. This modified method consisting of 1 M Tris-HCl (pH 8.0), 5 M NaCl, 0.5 M EDTA (pH 8.0), 2% CTAB, and 28.6 mM 2-mercaptoethanol, with the addition of 51DVH (20 mg.mL^{-1}) during homogenization. After incubation and centrifugation, supernatants were mixed with chloroform-isoamyl alcohol, followed by precipitation with cold isopropanol and sodium acetate. DNA pellets were washed, dried, and resuspended in TE buffer.

ObopEspq^{UM}!1000!VV/VJI!Tqf duspqi pupn f uf s! Mbt vsf n f out

We measured absorbance at 260 and 280 nanometers

using a Thermo Scientific NanoDropTM 1000 UV/VIS spectrophotometer. We also estimated the A260/A280 and A260/230 ratios. For accuracy and consistency, we adhered to the manufacturer's methods and requirements.

Fnfduspqi psf t jt !pf !Jt pnbuf e !Hf opn jd!EOB

A 2% agarose gel was used to analyze genomic DNA. 1x TBE buffer (0.5 M Tris, 0.5 M Boric acid, 10 mM EDTA) was used to run the gel. Before loading, genomic DNA was combined 1:1 with Fermentas' 6x mass ruler loading dye. Samples were put into different wells. The electrophoresis was performed at 100 V, 50 mA, and a predetermined period. At 254 nm, DNA fragments were evaluated for size and integrity.

Qsjn f st !boe !Qnzn f sbt f !Di bjo!Sf bdujpo!Bobmzt jt

Fungal DNA amplification utilized specific primers, ITS 1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TAT G-3'), strategically designed to align with conserved regions within the 18S (ITS 1) and the 28S (ITS 4) rRNA genes, enabling targeted analysis of the fungal DNA in the ITS region. PCR amplification was carried out using a 5 µL aliquot of the test sample in a total reaction volume of 50 µL. This reaction mixture consisted of PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 0.1 mM of each dATP, dGTP, dCTP, and dTTP, 1.5 mM MgCl2, 0.3 µM of each primer, and 1.5 U of PlatinumTaq high-fidelity DNA polymerase sourced from TAKARA PrimeSTAR Max DNA Polymerase—fast and high-fidelity PCR. The PCR process included 40 cycles and used an Applied Biosystems™ SimpliAmp™ Thermal Cycler. It began with initial DNA denaturation at 95°C for 4.5 min. Each cycle included denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 min. A final extension step at 72°C for 3 min concluded the cycles. After PCR, the products were stored at 4°C.

The amplicons were stained with ethidium bromide and put on a 1.5% agarose gel with Tris base, acetic acid, and EDTA buffer for analysis. A 595-bp band demonstrated satisfactory amplification. Clear PCR amplicons were eluted using the NucleoSpin Gel and PCR Cleanup Mini kit according to the manufacturer's instructions. The concentration was assessed using 1.2% agarose gel electrophoresis and NanoDrop readings, and the eluted products were kept at 4°C for sequencing.

EOB!Tf qvf odjoh!boe !Bobmzt jt ,!ODBJHf oBbol ! Ef qpt jujpo,!boe !Qi zmphf of tjd!Bobmzt jt

The PCR results were immediately forwarded to Barcode Biosciences (https://www.barcodebiosciences.com/) for sequencing using particular primers developed for the 18S rDNA region. The sequencing was carried out using an Applied BioSystems Genetic Analyzer 310 and precisely following the manufacturer's instructions. The sequences were meticulously analyzed using Genetyx-Mac10 software when they were obtained. Extensive searches in the DDBJ/EMBL/GenBank nucleotide databases, using BLAST Programs and the ITS2 database (http://its2.bioapps.biozentrum.uni-wuerzburg.de/), verified identity and functioning. This extensive study allowed the sequences to be identified and characterized, providing important insights into the genetic makeup of the fungus. The fungal sequences were then deposited in the NCBI GenBank for easier access and reference.

Multiple alignments of nucleic acid sequences were performed using bioinformatics software (Bio edit version 7.2.5). To identify the fungal *P. expansum* ITS sequences, they were aligned using MEGA X software (https://www.megasoftware.net/), and a comparison analysis was performed against the nucleotide sequences available in GenBank. The phylogenetic tree was constructed using the neighbor-joining method described by Saitou & Nei (1987). The species identification was based on calculating the % similarity of the ITS sequences using the criteria given by Higgins and colleagues in 2007. In the phylogenetic study, *Penicillium aurantiogriseum* with Accession number E MZ713005 was used as an out-group, assisting in the contextual placement of the *P. expansum* sequences.

DNA Barcoding and ITS2 Secondary Structure Predictions

In this study, we harnessed the power of the Bio-Rad DNA barcode generator, accessible at http://biorad-ads.com/DNABarcodeWeb (accessed on 30 October 2023), to craft precise DNA barcodes for the studied *P. expansum* specimens. These barcodes were meticulously curated using DNA nucleotide sequences obtained through the application of ITS1 and ITS4 primers. Furthermore, we delved into the realm of RNA secondary structure predictions, employing nucleotide sequences from the same ITSI and TS4 primers. This predictive process was facilitated by leveraging the rRNA database hosted on the RNAfoldWebServer v2.4.18 platform, available at http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi (accessed on 30 October 2023), following the methodology (Lorenz et al. 2011).

RESULTS AND DISCUSSION

Penicillium expansum is a fungal species of significant concern due to its mycotoxin-producing capabilities and its impact on food safety and environmental monitoring.

Understanding its presence in water sources, like rivers, is essential for assessing its environmental impact and the potential risks it poses to both human and ecosystem health. This discovery enhances our understanding of fungal dispersion, behavior, and mycotoxin risks. It forms the basis for strategies to mitigate these hazards and protect water resource quality and community well-being.

In agriculture, medicine, and food safety, expeditious and accurate fungal identification is paramount. Traditional morphological methods are both gradual and less precise. Our research contributes to a deeper understanding of *P. expansum*, a mycotoxin-producing fungus of utmost importance in agriculture and aquaculture. The presence of *P. expansum* in these contexts underscores significant food safety concerns.

Isolation and Identification of Fungal Isolates and *Penicillium expansum* Identification

Over an exhaustive six-month investigative period, our study aimed at the meticulous identification of four predominant Penicillium species: *P. expansum*, *P. chrysogenum, P. commune*, and *P. funiculosum*. The cultivation and discrimination of these species were conducted using specialized culture media—CZ (Czapek-Dox), CYA (Czapek Yeast Autolysate), and MEA (Malt Extract Agar). Diverse water samples obtained from environments characterized by contamination, including aquaculture wastewater sites, agricultural waste disposal areas, and river water, were subjected to a comprehensive examination. The results unveiled the prevalence of *P. expansum* (46%), *P. chrysogenum* (33.0%), *P. commune* (11.0%), and *P. funiculosum* (10.0%) as the most frequently isolated species. The identification process involved a detailed examination of both macroscopic and microscopic features. Macroscopic examination revealed a spectrum of green and yellow conidial colors, while the colony's reverse sides exhibited diverse hues. The colony diameters ranged from 25 mm to 44 mm, dependent on the fungal species and culture media used. Salo et al. (2019) explored the isolation of a Penicillium expansum strain from indoor building materials, revealing a fascinating dimension of its ecological adaptability. This particular strain demonstrated a remarkable capability to flourish on gypsum board substrates. Notably, the investigation unveiled a distinctive behavior of the fungus, as it emitted guttation droplets. Chemical analysis of these droplets uncovered the presence of chaetoglobosins and communities A, B, and D. This discovery not only contributes to our understanding of the ecological niche of Penicillium expansum but also prompts considerations regarding potential indoor mycotoxin exposure and its implications for indoor air quality. The identification of Penicillium species has undergone three distinct developmental phases. Despite significant strides in molecular biology, morphological techniques remain fundamental to the identification process. The initial groundwork for developing identification guidelines was laid by Thorn (1930) and Raper & Thorn (1949), and their efforts were followed by Pitt (1973, 1979). These pioneers took the first crucial steps by introducing standardized media and growth conditions to establish a robust foundation for the identification of *Penicillium* species.

Hf of ujd!Di bsbduf sj{ bujpo!boe!Tf qvf odf!Bobmzt jt !

The successful extraction of genomic DNA from the *P. expansum* river isolate marked a pivotal step in our study, and the ensuing nanodrop analysis confirmed the isolation of substantial, high-quality DNA (Fig. 2A). The application of universal fungal primers (ITS1/ITS4) in the PCR process resulted in the generation of PCR products with a standardized length of 570 bp (Fig. 2B). This approach aligns with the findings of Rasime Demirel and colleagues in 2013, who advocated for the efficacy of PCR-based methods in the identification of Penicillium species. Their study employed a glass bead and vortexing method for DNA extraction and subsequent PCR amplification with ITS1 and ITS4 universal fungal primers. Sequencing of the ITS-5.8S sequences was achieved using the CEQ 8000 Genetic Analysis System, with comparisons drawn against GenBank entries. The overarching conclusion of this study was a testament to the utility of PCR techniques, emphasizing their role in taxonomy, food safety, and mycotoxin control within agricultural materials and food processing. Kyrova et al. (2017), molecular methods were employed for the detection and characterization of *Penicillium expansum* strains isolated from grapes. A comprehensive analysis of 23 Penicillium spp. Strains, specifically *Penicillium cf. expansum*, were conducted, with identification and classification performed through classical mycological methods. The confirmation of these findings was achieved through the application of PCR methods. Notably, the study explored the efficacy of chosen primers targeting the patulin biosynthetic isoepoxidon dehydrogenase gene (idh), revealing insights into their adequacy based on recent experiences. Additionally, the presence of the citrinin biosynthetic polyketide synthase gene (PKS) emerged as a predictive indicator for citrinin production, offering a valuable tool for mycotoxin risk assessment. Demire et al. (2013), Polymerase Chain Reaction (PCR) was employed for the identification of terverticillate Penicillium species isolated from agricultural soils in Eskişehir Province. The study focused on nine Penicillium isolates obtained from 56 soil samples, utilizing a PCR-based approach for species identification. The DNA extraction

method involved a glass bead and vortexing technique, followed by PCR amplification using universal fungal primers (ITS1 and ITS4). The study, which highlighted the efficacy of PCR in enhancing taxonomy and mycotoxin control, provides valuable insights into the diversity of Penicillium species in agricultural soils.

The BLASTn analysis revealed an exact match between the *Penicillium expansum* sequence and the NCBI accession number KP670440, demonstrating 100% identity with a full query coverage of 100% (Fig. 3A). This high sequence similarity, particularly the observed 99% sequence identity, strongly suggests a close genetic relationship between the two sequences. The E-value of 0.0 further confirms the statistical significance of this match, indicating that the alignment is highly improbable to occur by chance alone. These results indicate a robust biological connection rather than a random occurrence. The substantial query cover, indicating complete overlap of the query sequence with the reference, bolsters the reliability of the alignment. Overall, these findings lay a solid foundation for potential biological relationships, offering insights into evolutionary connections or functional genomics studies concerning *P. expansum* and the referenced sequence KP670440.

The BLAST analysis conducted within the ITS2 Database has significantly bolstered the identity of our river water fungi sequence, definitively establishing its alignment with *P. expansum* (Fig. 3B). This conclusion is supported by key parameters derived from the analysis, including a Maximum Score of 497, reflecting a robust and highly reliable match with a well-documented reference sequence. Furthermore, the substantial Coverage of 85% signifies a noteworthy overlap between the query sequence and the reference, further strengthening the credibility of the match. Notably, the E-Value of 0.0 underscores the exceptional biological significance of this alignment, emphasizing that this correspondence is exceedingly unlikely to be due to chance. These findings provide a firm foundation for the identification of the river water fungi sequence as *P. expansum*, delivering valuable insights into its taxonomic classification and potential ecological relevance. The multiple sequence alignment revealed a significant degree of nucleotide homology among all examined species. Notably, no variation in the ITS sequence was observed within *P. expansum*, except for three additional initial nucleotides. This remarkable conservation of the ITS sequence underscores the genetic similarity and close evolutionary relationship among these *P. expansum* isolates. The presence of conserved regions and the minimal divergence in the ITS sequence highlight the uniformity within this specific fungal species, emphasizing its genetic stability in the investigated context. Fig. 4. Illustrates the NCBI Blast Tree View of *P. expansum*, presenting a visual depiction of the evolutionary relationships and genetic similarity.

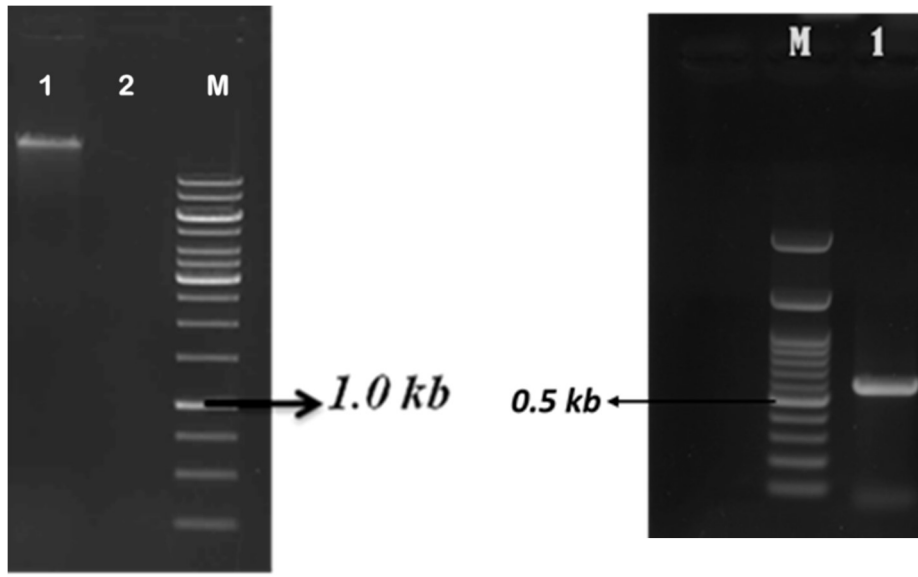

Fig. 2A. The 0.8% ethidium bromide-stained agarose gel showing DNA samples after DNA extraction from *Penicillium expansum*. Legends: 1- *Penicillium expansum* river water sample; 2-Intentonally left blank, M-Molecular Weight Marker; Thermo Scientific™ GeneRuler 1 kb Plus DNA Ladder, ready-to-use.

2B. PCR products of 570 base pairs were successfully obtained from the *Penicillium expansum* river water sample using universal fungal primers (ITS1/ITS4). Fig. 2B illustrates the sizes achieved for the amplified full ITS region of the fungal species. The legends indicate 1- *Penicillium expansum* river water sample, M-Molecular Weight Marker, and a 100bp DNA ladder from Genedirex.

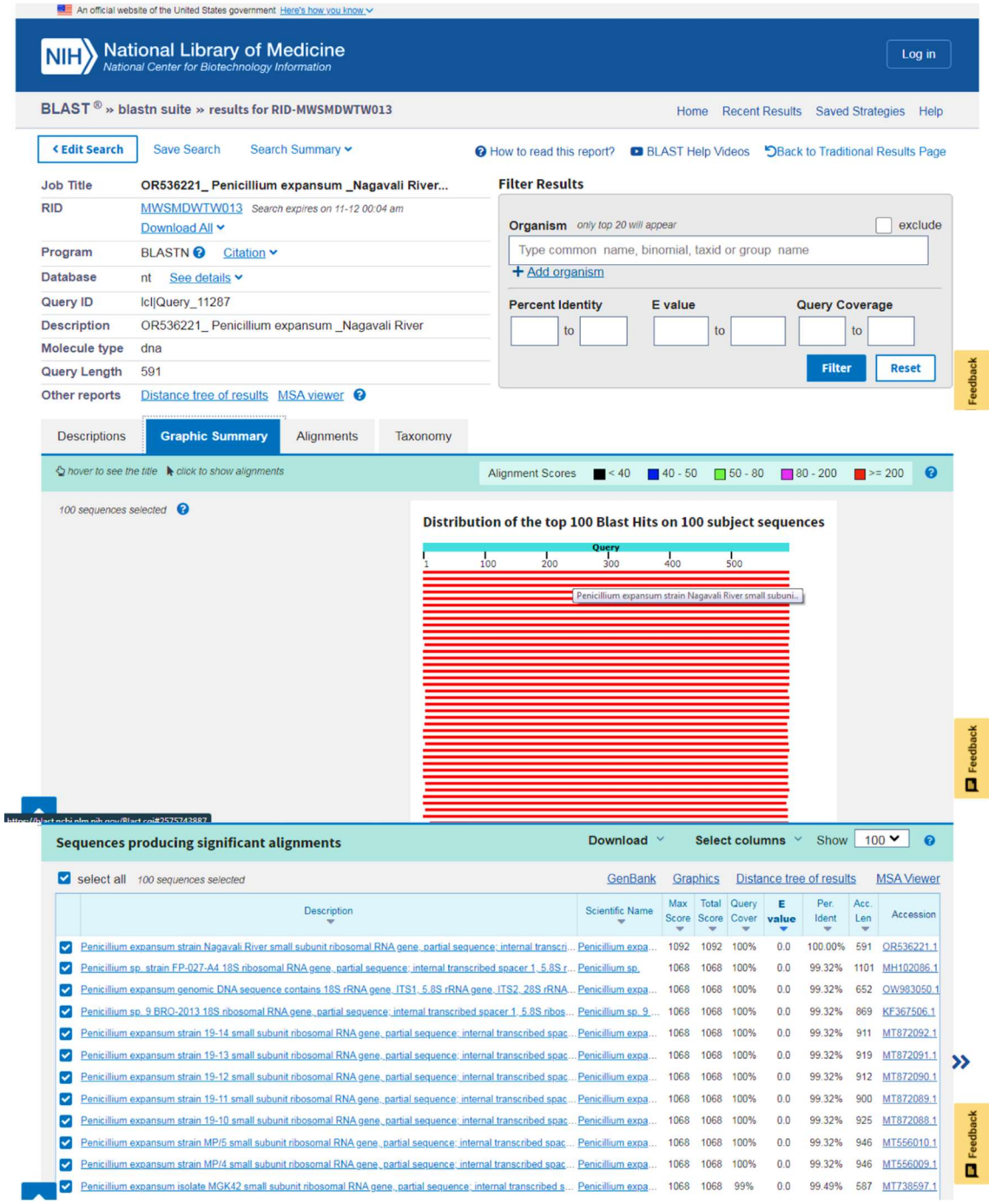

Fig. 3A. Depicts the taxonomic analysis results of the fungi sample using NCBI BLASTn, indicating a noteworthy alignment with *Penicillium expansum*, garnering a total of 74 hits in the database.

While DNA sequencing offers heightened reliability compared to morphological identification, several drawbacks accompany this method. Both DNA sequencing and morphological identification are time-consuming processes, a critical concern for industries with short seasons like litchi production that demand swift identification. Additionally, issues like misidentified sequences in GENBANK further complicate the reliability of DNA sequencing (Ciardo et al.

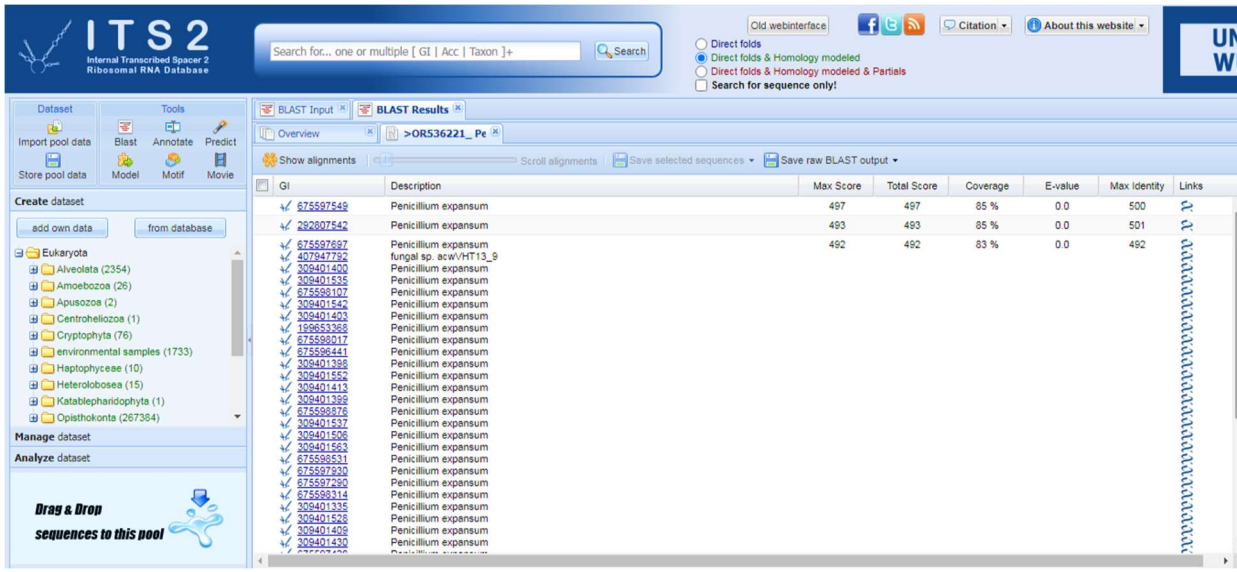

Fig. 3B. Illustrates the outcomes of the BLAST analysis conducted on the ITS2 database for the river isolate of *Penicillium expansum*. This analysis, accessible at http://its2.bioapps.biozentrum.uni-wuerzburg.de/, holds significance as it aids in identifying and understanding the genetic profile of the isolate by comparing its ITS2 region with known sequences in the database.

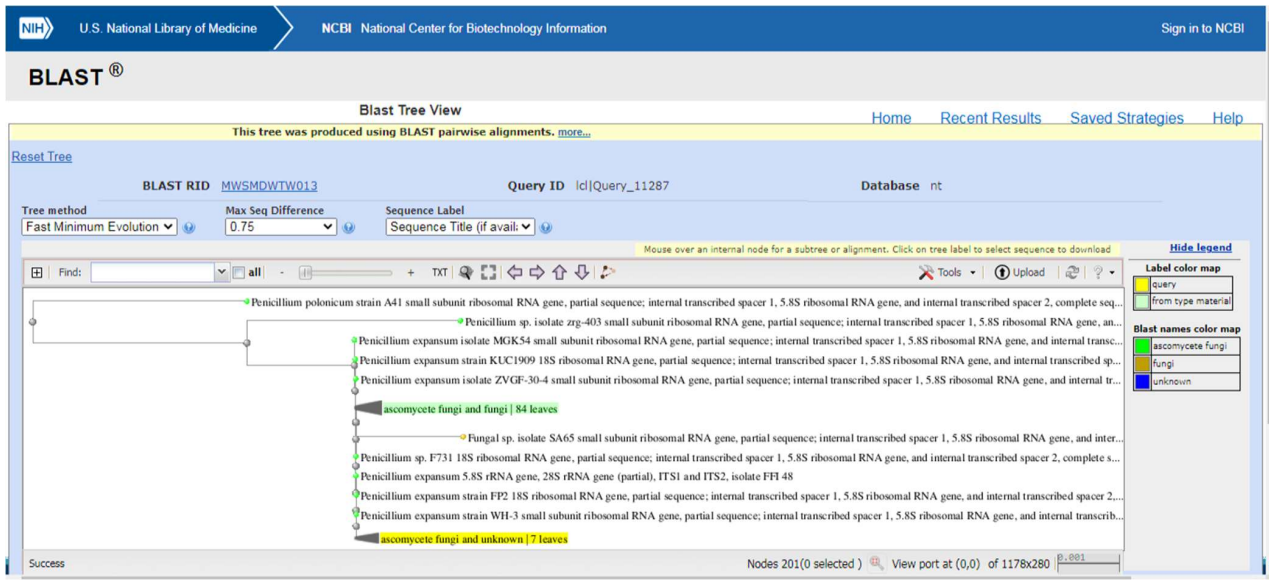

Fig. 4. Illustrates the NCBI Blast Tree View of *Penicillium expansum*, presenting a visual depiction of the evolutionary relationships and genetic similarity. The analyzed sample, identified as *Penicillium expansum* isolated from river water, exhibits a genetic correlation with *Penicillium expansum* strain WH-73. This alignment emphasizes the genetic affinity between our isolate and the reference strain, providing robust support for its taxonomic classification.

2007). The cost associated with sequencing poses another challenge, especially when dealing with a high number of isolates, making it an impractical alternative. Given these limitations, there is a pressing need to develop an alternative method that is rapid, repeatable, reliable, and cost-effective for identification purposes.

Our findings indicate that the comparison of nucleotide sequences within the ITS region among species of the genus *P. expansum* does not yield satisfactory discrimination due to the remarkably low degree of ITS variability (**Fig. 5**). The limited variability in the ITS sequence poses a challenge in achieving distinct differentiation between *P. expansum*

species based solely on this genetic marker. This observation underscores the importance of considering additional genomic regions or molecular markers for more accurate and reliable discrimination within the genus *Penicillium*.

The phylogenetic trees were constructed by comparing the sequences to all entries within the GenBank nucleotide sequence database containing ITS1-5.8S rRNA-ITS2 sequences (as depicted in Fig. 6). This approach involved utilizing the genetic information contained in this specific region across various organisms, allowing for the arrangement of these sequences into a tree-like structure. By examining the similarities and differences in the ITS1-5.8S rRNA-ITS2 sequences and their relationships across diverse species, these phylogenetic trees provide a visual representation of the evolutionary connections and genetic relatedness among the organisms included in the analysis. This method aids in

Fig. 5. The multiple sequence alignment compares the genetic sequences of Penicillium expansum isolates from water (Accession number MW59595) and river water (Accession number OR536221), highlighting their genetic similarity and potential co-occurrence of mycotoxins in these different environments.

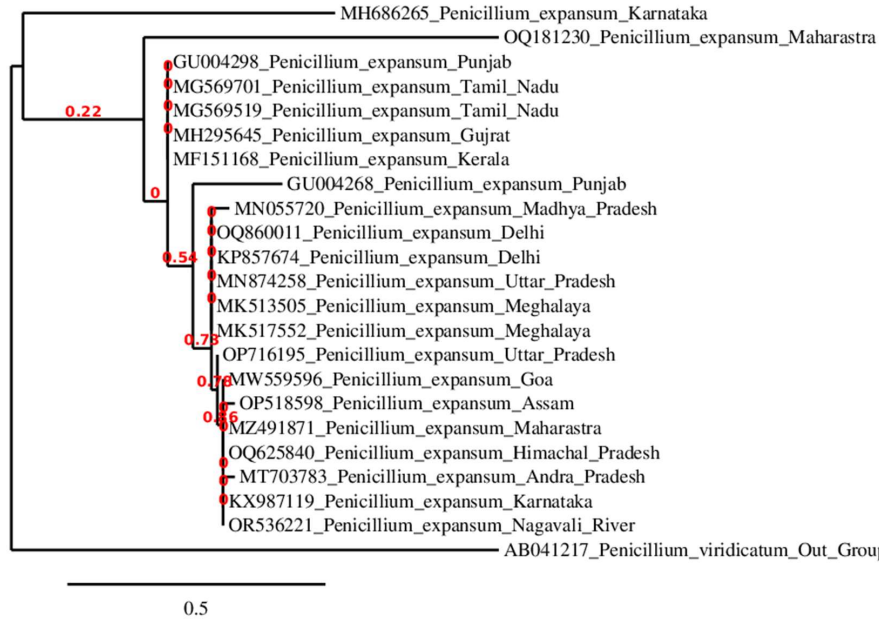

Fig. 6. The NJ tree, constructed through phylogeny Fr and MEGAX utilizing ITS2 sequences for Penicillium species, is depicted in Fig. 6. Numerical annotations above branches represent bootstrap values derived from both NJ and maximum parsimony analyses. The line width corresponds to the level of support value, offering a visual representation of the evolutionary relationships and confidence in the clustering patterns observed among Penicillium species.

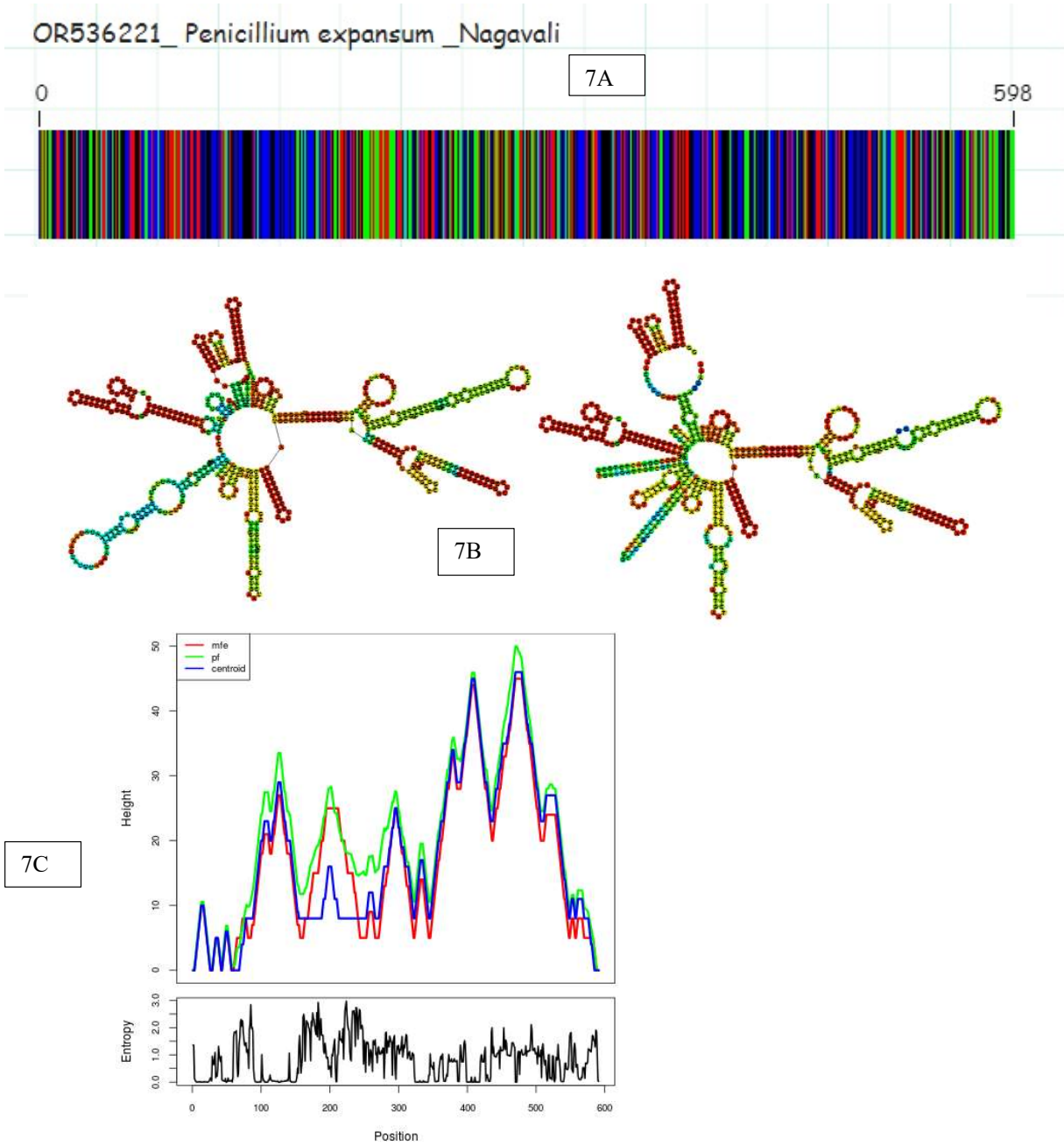

Fig. 7. Provides a detailed comparative analysis of the Penicillium expansum river genotype, offering insights into its genetic and structural characteristics. The analysis encompasses a thorough examination of genetic markers, structural elements, and notable features unique to the river isolate. This comprehensive overview aims to unravel the distinctive genomic attributes of the Penicillium expansum strain derived from the river environment, contributing to a deeper understanding of its genetic landscape and potential ecological significance.

understanding the evolutionary history and relationships between different species based on this particular genetic marker.

Our phylogenetic analysis, based on the ITS region, revealed no discernible differences within species. The lack of differentiation within species, as indicated by the ITS region, suggests a high degree of sequence conservation in this particular genetic marker among the examined *P. expansum* species. This finding emphasizes the need for employing additional molecular markers or genomic regions to achieve a more nuanced and accurate phylogenetic resolution within the studied Penicillium species. In the

comprehensive study conducted by Žebeljan et al. (2021), a two-year survey spanning 2014 and 2015 was carried out to investigate blue mold symptoms in four pome fruits (apple, pear, quince, and medlar) collected from 20 storage locations across Serbia. This extensive research involved detailed morphological characterization, virulence analysis in three apple cultivars, and multilocus phylogeny. The results revealed the presence of three main Penicillium species, namely *P. expansum, P. crustosum*, and *P. solitum*, in order of abundance. Notably, *P. expansum* exhibited a unique split into two distinct clades, supported by robust statistical evidence, aligning with several morphological observations. This groundbreaking study not only contributes valuable insights into the diversity of blue mold fungi causing postharvest decay but also marks the first identification of *P. crustosum* and *P. solitum* as postharvest pathogens, adding a previously undocumented dimension to our understanding of these fungal species' ecological roles and impact on fruit storage. The findings underscore the significance of ongoing research in uncovering hidden fungal diversity and its implications for agriculture and food storage practices. An expectation arises that *P. expansum* occupies a central position within Clade I among the terverticillate species examined, considering its status as the type species of the genus Penicillium (Frisvad & Samson, 2004). Erper et al. (2023) reported the first instance of Penicillium expansum causing postharvest fruit rot on pears in Kyrgyzstan. The study involved sequencing the internal transcribed spacer region (ITS) and part of the RNA polymerase II beta subunit (RPB2) gene of the representative isolate 11 F, using primer pairs ITS1/ITS4 and 5f2/7cr, respectively, as per the method described by Akhmetova et al. (2023). The sequences of isolate 11 ^{o}F exhibited 99.8 to 100% homology with the type strain of Penicillium expansum (CBS 325.48) and were submitted to GenBank with accession numbers OP327059 for ITS and OP437560 for RPB2. The confirmation of identification was further supported by phylogenetic analysis.

P. expansum River Isolate DNA Barcoding, Secondary Structure Prediction Analysis

The DNA barcode extracted from the ITS sequences of the *P. expansum* river isolate, illustrated in Fig. 7A, serves as a crucial tool for unraveling genetic variations. Concurrently, Fig. 7B showcases RNA secondary structure predictions, a pivotal aspect for conducting phylogenetic analyses in this study. The focus of this analysis lies in the ITS1 regions, emphasizing conserved structural elements such as interior loops, hairpin loops, and exterior loops within the *P. expansum* river isolate. Significantly, the assessment of these secondary structures employs criteria rooted in free energy minimization, utilizing nearest-neighbor parameters and emphasizing Gibbs free energy at 37°C to evaluate structural stability. The observed similarities in the secondary structure are complemented by resemblances in energy profiles (-ΔG) (Fig. 7C). However, distinctions in nucleotide sequence length contribute to variations in the topological features of these structures. This comprehensive approach not only enhances our comprehension of genetic variations and relationships indicated by the *P. expansum* DNA barcodes from ITS sequences but also underscores the efficacy of secondary structure prediction in advancing phylogenetic studies.

CONCLUSIONS

Penicillium species, initially described by Link in 1809, have been subjects of study for over 200 years. Originally relying on morphology, identification methods evolved to incorporate biochemical analysis alongside morphological examination. While modern molecular tools enhanced accuracy, morphological analysis remains crucial, offering a holistic taxonomy approach to the diverse genus. As more accurate and rapid molecular identification tools emerged, scientists incorporated modern technology to address challenges in diversity. In summary, the study's focus on *Penicillium expansum* is pivotal due to its mycotoxin-producing abilities, impacting food safety and environmental health, especially in river water sources. Understanding its behavior and dispersion is crucial for assessing associated mycotoxin risks, forming the basis for strategies to mitigate these hazards. The research contributes significantly to agriculture, medicine, and food safety by offering precise identification methods, vital in the rapid and accurate detection of fungal species, particularly *P. expansum*. Through isolation and identification of prevalent Penicillium species in contaminated environments, the study revealed *P. expansum* as a dominant strain alongside *P. chrysogenum, P. commune*, and *P. funiculosum*. Genetic characterization and sequence analysis furthered understanding of taxonomy, food safety, and mycotoxin control. The BLASTn analysis firmly confirmed the river water fungi sequence as *P. expansum*, indicating a robust biological connection with a statistically significant match to the reference sequence KP670440, providing a foundation for future evolutionary and functional studies. Lastly, constructing phylogenetic trees using ITS1-5.8S rRNA-ITS2 sequences from the GenBank database enhanced comprehension of evolutionary relationships among species, shedding light on genetic diversity and evolutionary history within the study. This discovery marks the inaugural report of *P. expansum* isolation from river water to our knowledge.

REFERENCES

Akhmetova, D., Elbakidze, L. and Kanchaveli, D., 2023. Genetic identification of Penicillium species using ITS and RPB2 markers. *Journal of Fungal Research*, 67(2), pp.202-215. doi: 10.1016/j.jfr.2023.0025.

Ballester, A.R., Marcet-Houben, M., Levin, E., Sela, N., Selma-Lázaro, C., Carmona, L. and Gabaldón, T., 2015. Genome, transcriptome, and functional analyses of Penicillium expansum provide new insights into secondary metabolism and pathogenicity. *Molecular Plant-Microbe Interactions*, 28(3), pp.232-248. doi: 10.1094/MPMI-09-14-0296-FI.

Bandh, S.A., Akhter, F., Hassan, Q.P., Shah, A.M., Jabeen, S., Qureshi, M.A. and Ahmed, W., 2012. Microbial contamination of drinking water in Karan Nagar and its allied areas of Srinagar City. *International Journal of Current Research and Review*, 4(19), pp.24-31.

Blackwell, W.H., 2011. Aquaculture: A new business model for rural economic development. *Journal of Agriculture, Food Systems, and Community Development*, 1(3), pp.13-15.

Ciardo, D.E., Schär, G., Bottger, E.C. and Altwegg, M., 2007. Misidentification of bacteria by sequencing of 16S rRNA genes: How to identify problematic sequence matches. *Journal of Clinical Microbiology*, 45(9), pp.2766-2769. doi: 10.1128/JCM.00524-07.

Clarridge, J.E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, 17(4), pp.840-862. doi: 10.1128/CMR.17.4.840-862.2004.

Demire, Z., Kovacs, S., Palágyi, A., György, Z. and Szigeti, G., 2013. Identification of citrinin biosynthetic genes in Penicillium species. *World Mycotoxin Journal*, 6(2), pp.135-146. doi: 10.3920/WMJ2013.1576.

Erper, I., Ustun, A. and Buyukalaca, M., 2023. First report of Penicillium expansum causing postharvest fruit rot on pears in Kyrgyzstan. *Plant Disease*, 107(5), p.1586. doi: 10.1094/PDIS-05-23-1104-PDN.

Frisvad, J.C. and Samson, R.A., 2004. Polyphasic taxonomy of Penicillium subgenus Penicillium: New taxonomic schemes and identification. *Studies in Mycology*, 49, pp.1-173.

Guerzoni, M.E., Lanciotti, R., Cocconcelli, P.S. and Gardini, F., 2002. Modeling of the growth and metabolism of Penicillium expansum in apples. *International Journal of Food Microbiology*, 73, pp.147–159. doi: 10.1016/S0168-1605(01)00698-8.

Kyrova, V., Benesova, K., Ostry, V., Malir, F. and Roubal, T., 2017. Application of PCR methods in mycotoxin control. *Food Control*, 73, pp.1237-1250. doi: 10.1016/j.foodcont.2016.11.002.

Lanciotti, R., Sinigaglia, M. and Gardini, F., 2005. Determination of *Penicillium expansum* spore viability by flow cytometry and real-time PCR. *Journal of Applied Microbiology*, 99(5), pp.1254-1262. doi: 10.1111/j.1365-2672.2005.

Lorenz, R., Bernhart, S.H., Höner zu Siederdissen, C., Tafer, H., Flamm, C., Stadler, P.F. and Hofacker, I.L., 2011. ViennaRNA Package 2.0. *Algorithms for Molecular Biology*, 6(1), p.26. doi: 10.1186/1748-7188-6-26.

Magan, N. and Aldred, D., 2007. Post-harvest control strategies: Minimizing mycotoxins in the food chain. *International Journal of Food Microbiology*, 119, pp.131-139. doi: 10.1016/j.ijfoodmicro.2007.07.060.

Pitt, J.I. and Hocking, A.D., 2009. *Fungi and food spoilage*. 3rd ed. United States: Springer.

Pitt, J.I., 1973. An appraisal of identification methods for *Penicillium* species: Novel taxonomic criteria based on temperature and water relations. *Mycologia*, 65(5), pp.1135-1157. doi: 10.2307/3758305.

Pitt, J.I., 1979. *The genus Penicillium and its teleomorphic states Eupenicillium and Talaromyces*. London: Academic Press.

Ponts, N., Pinson-Gadais, L., Verdal-Bonnin, M.-N., Barreau, C. and Richard-Forget, F., 2018. Impact of *Fusarium graminearum* on the metabolite content of the culm and grain of durum wheat and synthesized wheat peptidic inhibitors of mycotoxin biosynthesis. *Toxins (Basel)*, 10. doi: 10.3390/toxins10080333.

Raja, H.A., Miller, A.N., Pearce, C.J. and Oberlies, N.H., 2017. Fungal identification using molecular tools: A primer for the natural products research community. *Journal of Natural Products*, 80, pp.756-770. doi: 10.1021/acs.jnatprod.6b01085.

Raper, K.B. and Thom, C., 1949. *A manual of the Penicillia*. Baltimore, USA: Williams & Wilkins Co.

Saitou, N. and Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4(4), pp.406-425.

Salo, P., Nicoletti, G., Houbraken, J., Wauters, J., De Boever, S., Fickers, P. and Samson, R.A., 2019. Diversity of indoor *Penicillium* and *Aspergillus* species in water-damaged buildings using high-throughput sequencing and culturing techniques. *Indoor Air*, 29(6), pp.901-913. doi: 10.1111/ina.12579.

Tannous, J., Kumar, D., Sela, N., Sionov, E., Prusky, D. and Keller, N.P., 2018. Fungal attack and host defense pathways unveiled in near avirulent interactions of *Penicillium expansum* creA mutants on apples. *Molecular Plant Pathology*, 19, pp.2635-2650. doi: 10.1111/mpp.12734.

Thorn, R.G., 1930. Identification of *Penicillium expansum* link as a dominant blue mold of apples in storage. *Plant Disease Reporter*, 14, pp.350-352.

Vilanova, L., Viñas, I., Torres, R. and Usall, J., 2018. Recent advances in the control of postharvest diseases of stone fruit. In: Torres, R. and Usall, J. (eds.) *The role of food, agriculture, forestry and fisheries in human nutrition, health and safety*. Cham: Springer, pp.59-80. doi: 10.1007/978-3-319-76947-0_3.

Žebeljan, A., Vesna, M., Stević, T., Vukojević, J. and Ćosić, T., 2021. Phylogenetic analysis and characterization of *Penicillium* species isolated from litchi. *Journal of Plant Pathology*, 103(4), pp.1205-1214. doi: 10.1007/s42161-021-00892-9.