Elucidating Mycotoxin-Producing *Aspergillus* Species in River Water: An Advanced Molecular Diagnostic Study for the Assessment of Ecological Health and Contamination Risk

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ABSTRACT

The primary goal of this research is to isolate mycotoxin-producing fungus from the Nagavali River. Examining isolated fungi involved analyzing their mycelium growth on culture media and detailed microscopic inspection. We employed PCR analysis utilizing universal primers ITS1 and ITS4 to accurately identify the species. Furthermore, we sequenced the amplified ITS region and rigorously analyzed the sequences using NCBI-BLASTn and the ITS2 database. The analysis found a high 96.38% genetic similarity to the *Aspergillus flavus* strain, resulting in a 600-base pair fragment size. The sequence was given the accession number OR536222 in the NCBI GenBank database. Phylogenetic analysis was performed to ascertain the particular strain of *A. flavus* and its source. Remarkably, this analysis led to the identification of a single new strain gene, which represents a novel discovery in the field of fungal research. These results underscore the vital significance of molecular techniques in promptly and precisely identifying organisms. This research enhances our understanding of mycotoxin contamination in water, providing valuable insights to improve detection and prevention strategies. It accentuates the overarching importance of conserving our water resources and upholding ecological equilibrium, ultimately safeguarding the well-being of both humanity and the environment.

INTRODUCTION

The prevalence of mycotoxin-producing *Aspergillus* species in natural water sources has emerged as a significant ecological and public health issue (Houbraken & Samson 2011). These filamentous fungi, which are frequently connected with agricultural and aquacultural operations, can synthesize mycotoxins, which are a broad collection of secondary metabolites that have been linked to negative effects on human and animal health (Bennett & Klich 2003). Concerns about mycotoxin contamination in aquatic environments have grown in recent years as a result of their possible influence on ecological balance and the safety of water supplies (Brera et al. 2023).

Understanding the genetic variety of *Aspergillus* fungi is critical for understanding their critical ecological roles in the environment and agriculture. With their genetic variety, Aspergillus species perform critical roles in nutrient cycling, organic matter decomposition, and mycotoxin synthesis in a wide range of natural settings, including soil, water bodies, and plant environments. Their remarkable adaptability to diverse environmental conditions, viz., extreme pH ranges, temperature fluctuations, and variable substrate availability, enables them to thrive in disparate ecological niches and engage in intricate interactions with other microorganisms and host organisms (Smith et al. 2023). Additionally, the species genetic diversity among Aspergillus holds significant implications for agriculture. These fungi can function as beneficial biocontrol agents, plant partners, or notorious mycotoxin producers, affecting crop quality and food safety negatively. A comprehensive genetic analysis is imperative to harness their potential in sustainable agricultural practices while concurrently mitigating the associated risks of mycotoxin contamination.

Our project, based in Ganagalawanipeta village, Srikakulam, India, aims to thoroughly explore the prevalence of mycotoxin-producing *Aspergillus* species in river water—an ecologically critical reservoir (Gupta et al. 2023).

River water pollution with mycotoxins has been linked to agricultural runoff, aqua cultural waste, and other human activities (Hruska et al. 2019). These contaminants endanger not just aquatic life but also human populations that rely on these water supplies for a variety of functions, including drinking and agriculture. The major goal of this work is to discover and characterize mycotoxin-producing *Aspergillus* species using a molecular diagnostic method centered on the Internal Transcribed Spacer (ITS) region (Samson et al. 2014). In contrast to existing microbiological and chemical tests, which may lack specificity and sensitivity in detecting fungal pollutants, our study provides a PCR-based diagnostic method developed for rapid and accurate identification of toxin-producing fungi across genera (Klich & Pitt 1988).

This research not only raises awareness about the possible health hazards associated with mycotoxin pollution in aquatic environments but also gives critical insights for the development of efficient detection and prevention techniques (Battilani et al. 2016). Water resource conservation and ecological balance are critical for the well-being of both human populations and the environment (Viegas et al. 2021). Incorporating molecular characterization into our study not only improves the accuracy of our findings but also lays the groundwork for future studies and the development of tailored measures to decrease mycotoxin pollution in aquatic environments. This technique provides us with a thorough understanding of the genetic variety and behavior of mycotoxin-producing *Aspergillus* species, ultimately contributing to larger efforts to protect the environment.

MATERIALS AND METHODS

Study Area

Our investigation was conducted in Ganagalawanipeta village, situated within Andhra Pradesh, specifically in the Srikakulam district (Fig. 1). This area is renowned for its adjacency to the Nagavali River, which flows in a southwest to northeast direction. Characterized by a tropical monsoon

Fig. 1: Study Area and sample collection for fungal isolation.

climate, the region experiences distinct seasonal variations, marked by alternating rainy and dry periods (APHA 1998). The annual precipitation ranges between 900 to 1100 mm, with the highest levels observed typically from June to October (Sparrow 1960).

Sample Collection and Processing

We collected monthly river water samples from three places along the Nagavali River to study seasonal fungal diversity variations. The samples were collected in sterile containers and transferred in a temperature-controlled chain (Fig. 1). At each collection, physicochemical data (temperature, pH, salinity, dissolved oxygen, nutrients) were collected concurrently (Stevens 1974). This stringent technique ensured sample integrity while also providing critical data for the Nagavali River ecosystem fungus diversity and environmental study.

Isolation of Fungi

A serial dilution approach was employed to isolate fungi from water samples (Altschul et al. 1990). We created water dilutions by distributing 100 L of each dilution on Potato Dextrose Agar (PDA) plates using the spread plate method. Plates incubated for up to 7 days at 25°C 2°C. For pure cultures, colonies with specific physical characteristics were sub-cultured. The preliminary identification was based on colony features, which were validated by microscopic investigation (Abd-Elaslam et al. 2000). The DRBC (Dichloran Rose Bengal Chloramphenicol) medium, which included 2 micrograms of dichloran and 25 micrograms of rose bengal per mL, significantly limited spreading mold colony growth while recovering more fungal species in greater amounts than previous enumeration media (Manogaran & Lopez 2017).

The Growth of Fungi in Culture Media

For inoculation, we made a 10^{-6} CFU/mL conidia suspension. The fungal strain was grown in a GMS medium with a 2% sucrose solution and agitation at 150 rpm. After incubation, mycelium was removed using sterile filter paper, weighed, and kept at -80°C for DNA isolation.

Genomic DNA Isolation

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.

NanoDrop^{TM} 1000 UV/VIS Spectrophotometer Measurements

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.

Electrophoresis of Isolated Genomic DNA

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.

Primers and Polymerase Chain Reaction Analysis

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.

DNA Sequencing and Analysis, NCBI GenBank Deposition and Phylogenetic Analysis

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 Programmes 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 *A. flavus* ITS sequences, they were aligned using MEGA 11.0.10 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, *Aspergillus terrerus* with Accession number EU515150 was used as an out-group, assisting in the contextual placement of the *A. flavus* 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 16 October 2023), to craft precise DNA barcodes for the studied *Aspergillus flavus* 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 16 October 2023), following the methodology outlined by Lorenz et al. (2011).

RESULTS AND DISCUSSION

Detecting Mycotoxin-Producing *Aspergillus* species in river water extends beyond scientific inquiry; it represents a critical effort in protecting both the environment and public health. This discovery unveils insights into the distribution, behavior, and potential threats posed by these fungi, particularly their mycotoxin-producing capabilities. By delving into this research, we pave the way for the development of strategies aimed at mitigating these risks and preserving the integrity of water resources, along with the well-being of the communities reliant on them.

Rapid, accurate fungal identification is crucial across fields like agriculture, medicine, and food safety. Traditional methods like morphology are slow and less precise. Our study enhances knowledge of mycotoxin-producing *Aspergillus flavus*, which is vital in agriculture and aquaculture. *A. flavus* presence in these settings raises food safety concerns.

Isolation and Identification of Fungal Isolates and *Aspergillus flavus* Identification

During the six-month study, common *Aspergillus* species like *A. niger*, A. *flavus*, *A. fumigatus*, and *A. terreus* were identified. Differential culture media like CZ, CYA, and MEA were used. Sampling encompassed aquaculture wastewater, agricultural waste areas, and river water. Identification relied on macroscopic and microscopic traits. This comprehensive approach deepened our understanding of *Aspergillus* diversity and its implications for public and environmental health. For *A. flavus*, identification involved analyzing its morphology using specific culture media like AFPA and CDA. AFPA distinguishes *A. flavus* by its distinctive orange colony reverse. Morphological features, including conidia production, further differentiated *A. flavus* from other species. Intriguingly, variations in colony color, structure, and sclerotia formation were observed among *A. flavus* isolates. This highlights the need for molecular techniques to ensure precise identification.

Genetic Characterization

Our modified *A. flavus* genomic DNA extraction method yielded substantial, high-quality DNA confirmed by

nanodrop analysis (Fig. 2). Similar to the Aamir et al. (2015) approach, our method disrupts fungal cells, undergoes RNase treatment and employs phenol: chloroform: Isoamyl alcohol extraction and isopropanol precipitation. It delivers DNA in 1 h, with yields ranging from 60 µg to 230 µg per 200 mg wet fungal mass. This closed system minimizes contamination risks and supports diverse molecular methods, including PCR and RAPD analysis. Compared to Sarkanj et al. (2018) study, our method balances efficiency, cost-effectiveness, and time. It produces pure DNA with favorable ratios and suits downstream applications. Time ranges from 1 hour 15 minutes to 7 h 5 min, with reasonable per-sample costs, ensuring practicality for *A. flavus* genomic DNA isolation and PCR reactions. Our study aligns with Jin et al. (2000) findings, demonstrating the effectiveness and versatility of DNA extraction techniques.

Polymerase Chain Reaction Analysis

Following genomic DNA extraction from *A. flavus* isolates, PCR using ITS1/ITS4 primers produced consistent 600 bp fragments, consistent with Dıba et al. (2014) (Fig. 3). This size uniformity suggested genetic similarity among isolates. Electrophoresis and sequencing further confirmed *A. flavus* strain identification. Krulj et al. (2020) study on *A. flavus* from wheat grains also employed PCR amplification and PCR-RFLP with fragment length analysis, yielding similar results. Both studies identified all strains as *A. flavus* through ITS region and β-tubulin gene sequencing. Combining PCR-RFLP with Lab-on-a-chip electrophoresis, as shown by Kurlj et al. (2020), offers rapid *A. flavus* identification. Consistency with Stoll et al. (2003, Mohankumar et al. (2010), and Alrawi and Hussein (2017) highlights PCR's reliability

Sample collection from fish ponds

sample collection from river water

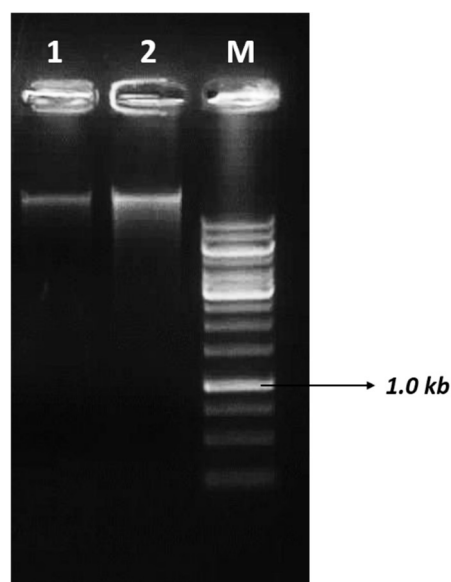sample collection from Fish catching area

Fig. 2: The 0.8% ethidium bromide-stained agarose gel showing DNA samples after DNA extraction from *Aspergillus* species. Legends: **1**- *Aspergillus* species sample as reference (Obtained and stored from previous studies); **2**- *Aspergillus flavus* river water sample; **M**-Molecular Weight Marker; Thermo Scientific™ GeneRuler 1 kb Plus DNA Ladder, ready-to-use.

for *A. flavus* detection using ITS1 and ITS4 primers. Gel electrophoresis, alongside a DNA ladder, confirms the technique's accuracy. These results underscore PCR's value in *A. flavus* identification across various applications.

NCBI BLAST Analysis

The NCBI BLAST analysis of the newly generated sequence revealed compelling results. It exhibited a 100% graphical similarity to *A. flavus*, with 404 BLAST hits on 100 subject sequences, indicating a precise alignment with no gaps and a plus/plus orientation. Taxonomically, 53 out of 103 hits were identified as *A. flavus*, affirming the sequence's affiliation with this species (Fig. 4). Furthermore, the Blast tree view confirmed a match with the *A. flavus* S7l6A strain, narrowing down the identification to a specific strain within the species. NCBI BLAST tree analysis is vital for the accurate identification and classification of genetic sequences, providing insights into genetic relationships, taxonomy, and potential functions, as demonstrated in this study (Fig. 5).

ITS2 Database Analysis

The BLAST analysis using the ITS2 Database has solidified the identity of our river water fungi sequence, confirming its alignment with *A.flavus*. Key parameters from the analysis include a Maximum Score of 488, indicating a robust match with a known reference sequence; a Coverage of 81%, signifying significant overlap between the sequences; and an E-Value of 0.0, denoting high biological significance. The ITS2 Database, housing comprehensive data on the ITS2 region of ribosomal RNA genes, enabled this comparison. This result is highly reliable, indicating a close genetic relationship between fungal isolate and *A. flavus*. Moreover, the presence of NCBI GenBank submitted sequences with the accession number OR53622 suggests a novel discovery, indicating that this *A. flavus* species from river water has not been previously reported in India or elsewhere, highlighting the significance of this finding for microbial diversity and ecological studies in water sources.

Sequence Data Analysis

In our study, we successfully isolated Mycotoxin-Producing *Aspergillus* species from river water, and the generated sequence was deposited in the NCBI database with the accession number OR536222. Subsequent NCBI BLAST search analysis revealed a highly significant match with
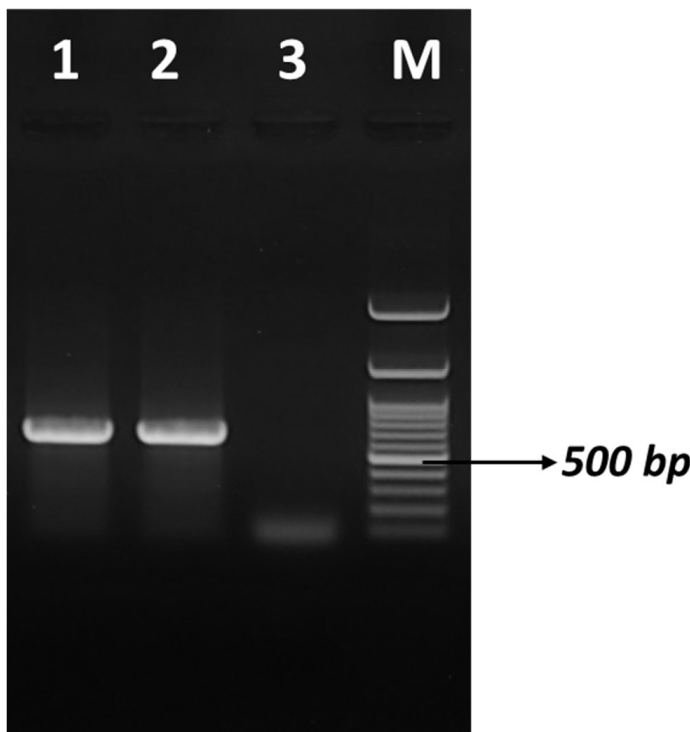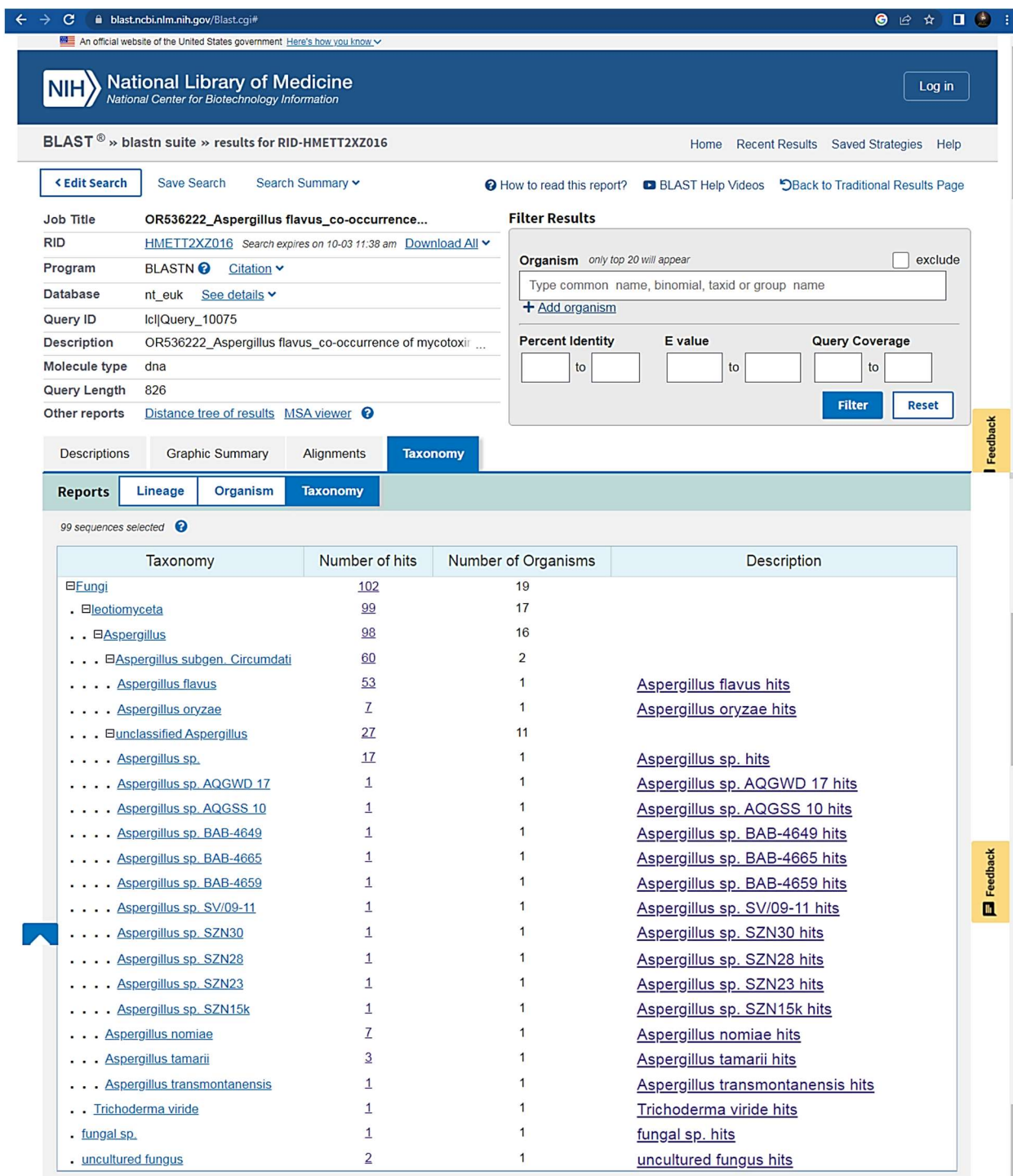
Fig. 3: **The PCR of screened 15 different fungal milk samples. The highly conserved region of (ITS) regions of the ribosomal DNA, primers pair ITS1and ITS4 produced the target band of 600bp,** Legends: **1**-*Aspergillus flavus* river water sample, **2**-Genomic DNA positive control; **3**-Genomic DNA negative control; **M**-Molecular Weight Marker; 100bp DNA ladder (Genedirex).

Fig. 4: NCBI BLASTn taxonomic analysis of the river fungi isolate sample reveals a significant match with *Aspergillus flavus*, with a total of 53 hits in the database.

NCBI nucleotide accession number KY234271, which corresponds to *A. flavus*. The sequence identity was exceptionally high at 96.38%, with an E-value of 0, indicating a robust and reliable match. Furthermore, the query coverage reached 100%, reinforcing the accuracy of our identification. These findings emphasize the presence of *A. flavus* in the aquatic environment, highlighting potential concerns related to mycotoxin contamination and underscoring the importance

Fig. 5: The NCBI Blast Tree View of *Aspergillus flavus* provides a visual representation of the evolutionary relationships and genetic similarity between our analyzed sample and this particular fungal species.

Fig. 6: The multiple sequence alignment compares the genetic sequences of *Aspergillus flavus* isolates from both fish feed (Accession number KY234271) and river water (Accession number OR536222), highlighting their genetic similarity and potential co-occurrence of mycotoxins in these different environments.

of monitoring and addressing such issues in the context of food safety and public health, particularly for smallholder farmers.

The alignment of KY234271_*Aspergillus flavus*_co-occurrence of mycotoxin in fish feed with OR536222_*Aspergillus flavus*_co-occurrence of mycotoxins in river water revealed a total of 34 nucleotide variations at specific positions within the sequences (Fig. 6). These variations were observed at positions 9 (C-G), 28 (G-C), 36 (-C), 41 (G-A), 53 (-A), 65 (-G), 69 (-A), 86 (-G), 91 (C-G), 92 (T-

OR237786.1_Aspergillus_flavus_isolate_AF2_small_subunit_ribosoma
KP686460.1_Aspergillus_sp._BAB-4659_18S_ribosomal_RNA_gene_parti
MG807064.1_Aspergillus_sp._clone_M-LHB03_small_subunit_ribosomal
KP686456.1_Aspergillus_sp._BAB-4649_18S_ribosomal_RNA_gene_parti
MF767617.1_Aspergillus_flavus_clone_EF12_internal_transcribed_sp
LC779009.1_Aspergillus_flavus_x88_genes_for_small_subunit_riboso
OL655391.1_Aspergillus_sp._isolate_SR1792-LTR2-ITS_internal_tran
KX067849.1_Aspergillus_flavus_isolate_Anhui-Rfsb15_18S_ribosomal
ON063449.1_Aspergillus_flavus_strain_PvarS-52_small_subunit_ribo
ON408308.1_Trichoderma_viride_strain_KUVKU-TV01_small_subunit_ri
KY234273.1_Aspergillus_flavus_strain_Beca_67_small_subunit_ribos
KP721599.1_Aspergillus_sp._AQGSS_10_internal_transcribed_spacer
KY234271.1_Aspergillus_flavus_strain_Beca_53_small_subunit_ribos
KY234272.1_Aspergillus_flavus_strain_Beca_60_small_subunit_ribos
KY234280.1_Aspergillus_oryzae_strain_Beca_95_internal_transcribe
KY234275.1_Aspergillus_flavus_strain_Beca_79_small_subunit_ribos
KY234263.1_Aspergillus_flavus_strain_Beca_5_internal_transcribed
KY234267.1_Aspergillus_flavus_strain_Beca_35_internal_transcribe
KY234265.1_Aspergillus_flavus_strain_Beca_20_small_subunit_ribos
OR192858.1_Aspergillus_flavus_strain_s88_internal_transcribed_sp
KP686465.1_Aspergillus_sp._BAB-4665_internal_transcribed_spacer
OQ723451.1_Aspergillus_sp._isolate_OTB1_internal_transcribed_spa
OQ723454.1_Aspergillus_sp._isolate_YCD3_internal_transcribed_spa
KF221065.1_Aspergillus_flavus_strain_Bp5_18S_ribosomal_RNA_gene
MH758698.1_Aspergillus_flavus_strain_CCTU145_small_subunit_ribos
OP526902.1_Aspergillus_flavus_isolate_Afla0001_small_subunit_rib
MW290493.1_Aspergillus_sp._isolate_Asp_13_small_subunit_ribosoma
MN537794.1_Aspergillus_flavus_isolate_2101434_small_subunit_ribo
FJ654485.1_Aspergillus_sp._SV/09-11_18S_ribosomal_RNA_gene_parti
OQ832049.1_Aspergillus_sp._isolate_OTB1_small_subunit_ribosomal
ON819577.1_Aspergillus_tamarii_strain_M24_small_subunit_ribosoma
KY234268.1_Aspergillus_flavus_strain_Beca_38_internal_transcribe
KY234266.1_Aspergillus_flavus_strain_Beca_32_internal_transcribe
OP596490.1_Aspergillus_flavus_isolate_SH3_small_subunit_ribosoma
KX067884.1_Aspergillus_sp._isolate_Sichuan-Rfsb01_internal_trans
KJ150716.1_Aspergillus_oryzae_strain_FNBR_L35_18S_ribosomal_RNA
JQ763433.1_Aspergillus_flavus_internal_transcribed_spacer_1_part
OK393670.1_Aspergillus_flavus_strain_Sh2_internal_transcribed_sp
MW929198.1_Fungal_sp._strain_F1_internal_transcribed_spacer_1_pa
KT221877.1_Aspergillus_sp._SZN28_internal_transcribed_spacer_1_p
MH716402.1_Aspergillus_flavus_strain_TM-3_internal_transcribed_s
KR905618.1_Aspergillus_flavus_isolate_SF45_internal_transcribed
KT221878.1_Aspergillus_sp._SZN30_internal_transcribed_spacer_1_p
KY978360.1_Uncultured_fungus_clone_S128_internal_transcribed_spa
KT221886.1_Aspergillus_sp._SZN15k_internal_transcribed_spacer_1
OM677817.1_Aspergillus_nomiae_strain_AS16_small_subunit_ribosoma
KT221874.1_Aspergillus_sp._SZN23_internal_transcribed_spacer_1_p
MF359724.1_Aspergillus_tamarii_strain_BPMF17_small_subunit_ribos
MF359732.1_Aspergillus_tamarii_strain_MF24_small_subunit_ribosom
OM677819.1_Aspergillus_nomiae_strain_AS27_internal_transcribed_s
MH664050.1_Aspergillus_oryzae_isolate_SZ1_internal_transcribed_s
KX067852.1_Aspergillus_flavus_isolate_Anhui-Rfsb27_18S_ribosomal
MZ322951.1_Aspergillus_sp._isolate_MSB1/SRLAAH/2019_small_subuni
MN031597.1_Aspergillus_flavus_isolate_AM-05_internal_transcribed
MN031598.1_Aspergillus_flavus_isolate_AM-08_internal_transcribed
MN161143.1_Aspergillus_flavus_isolate_Meg_S08b_small_subunit_rib
KP721583.1_Aspergillus_sp._AQGWD_17_internal_transcribed_spacer
ON171640.1_Aspergillus_flavus_isolate_S7L6A_small_subunit_riboso
MT645617.1_Aspergillus_sp._isolate_MEBP0048_small_subunit_riboso
ON063497.1_Aspergillus_flavus_cultivar_KRH-2_small_subunit_ribos
ON063528.1_Aspergillus_flavus_cultivar_MTU-7029_small_subunit_ri
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MN031596.1_Aspergillus_flavus_isolate_AM-04_internal_transcribed
OM677818.1_Aspergillus_nomiae_strain_AS33_internal_transcribed_s
KX431672.1_Aspergillus_nomius_internal_transcribed_spacer_1_part
OR100392.1_Aspergillus_transmontanensis_isolate_TP-RF11_small_su
MT497446.1_Aspergillus_sp._isolate_SS_12_small_subunit_ribosomal
MZ322955.1_Aspergillus_sp._isolate_MSB3/SRLAAH/2019_small_subuni
MN031599.1_Aspergillus_flavus_isolate_AM-11_internal_transcribed
OQ732719.1_Aspergillus_flavus_strain_CTd-R-EPI-9C_small_subunit
MN597042.1_Aspergillus_sp._isolate_ACSIKS_2100168_small_subunit
MW882244.1_Aspergillus_sp._strain_CBPFEAs4_small_subunit_ribosom
OQ784291.1_Aspergillus_sp._isolate_VMF-21_small_subunit_ribosoma
MN031601.1_Aspergillus_flavus_isolate_AM-16_internal_transcribed
MH664051.1_Aspergillus_flavus_isolate_SZ2_internal_transcribed_s
MF806072.1_Aspergillus_nomius_strain_DO2_internal_transcribed_sp
MW762713.1_Aspergillus_oryzae_isolate_SML5_internal_transcribed
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KY234277.1_Aspergillus_oryzae_strain_Beca_85_internal_transcribe
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MK120614.1_Aspergillus_flavus_isolate_20_small_subunit_ribosomal
MW741887.1_Aspergillus_flavus_isolate_PA37_internal_transcribed
ON063492.1_Aspergillus_flavus_cultivar_UPRI-133R_small_subunit_r
KX898361.1_Aspergillus_flavus_strain_RG01_internal_transcribed_s
OR536222.1_Aspergillus_flavus_strain_Nagavali_River_small_subuni
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KC907367.1_Aspergillus_flavus_isolate_GGV_BT03_internal_transcri
GU172440.1_Aspergillus_flavus_isolate_UPM_A8_internal_transcribe
KR905619.1_Aspergillus_nomius_isolate_SF46_internal_transcribed
KX067886.1_Aspergillus_flavus_isolate_Sichuan-Rfsb10_18S_ribosom
OQ723470.1_Aspergillus_sp._isolate_DDG1_small_subunit_ribosomal
MH553483_Aspergillus_terreus_isolate_S4P7-25_Out_Group

Fig. 7: The phylogenetic tree of specific *Aspergillus flavus* isolates, constructed using Neighbor-Joining methodology with the Tamura-Nei model.

C), 93 (C-T), 95 (C-G), 112 (C-G), 119 (C-A), 121 (-C), 130 (G-T), 192 (T-G), 193 (-T), 195 (-G), 656 (-T), 713 (G-A), 714 (-G), 715 (-C), 721 (-A), 722 (-A), 755 (A-C), 757 (-A), 758 (-C), 759 (-C), 760 (C-T), 761 (T-C), 791 (-C), 801 (-C), and 829 (G-C). The overall nucleotide variation observed in this comparison accounts for approximately 4.062% of the sequences.

It's noteworthy that these variations are primarily distributed in both the 5' and 3' regions of the sequences, while the core sequence remains unchanged. This pattern of variations is a common occurrence in genetic sequences, where certain regions exhibit more variability than others, often due to factors like mutations, insertions, or deletions.

Despite the observed nucleotide variations, the core genetic sequence maintains a consistent and highly similar 96% identity, affirming the fungal isolate's classification as *A. flavus* and reinforcing the accuracy of our identification process. A ClustalW multiple alignment analysis, comparing our river water Aspergillus flavus sequence with sequences from diverse sources, including mycotoxin in fish feed, textile industrial effluent, Russia spring water, Egypt canal water, and Turkey magnesite quarries, revealed significant homology within *A. flavus* species. This observation holds ecological and public health significance, as the mycotoxin-producing *Aspergillus* species' presence in natural water sources raises contamination and health concerns. It confirms the strong genetic similarity between the fungal isolate and *A. flavus*, suggesting its taxonomic classification as a strain or variant of this species. These findings enhance our understanding of genetic relatedness, aiding taxonomic classification and emphasizing potential ecological roles in water ecosystems or contamination scenarios.

Phylogenetic Tree Analysis

The clustering pattern in the trees was robustly confirmed through 2,000-replication bootstrap tests using MEGAX and Phylogeny Fr tools during Neighbor-Joining methodology (NL) tree analysis (Fig. 7). This demonstrated the consistent and reliable clustering of individuals of the same species, primarily guided by the similarity of the ITS sequence *of A. flavus* and *Aspergillus* species. The molecular marker's efficacy in distinguishing closely related fungal species remained uniform regardless of collection site or geographic location, emphasizing the universal applicability of ITS sequencing for species-level identification and phylogenetic analysis, further enriching our comprehension of fungal diversity and evolution.

Antonios Krimitzas et al. (2013) extensive phylogenetic tree work contributed significantly to understanding *Aspergillus* genus relationships, delineating seven well-supported clades using various methods. This research illuminated the evolutionary connections among different *Aspergillus* species.

A related study by Mays et al. (2019) in Diyala Province employed molecular techniques to detect *A.flavus* in milk samples. Using a conventional PCR assay with published primers (ITS1 and ITS4), they confirmed the presence of *A. flavus*, identifying toxigenic isolates through a distinct 600 bp sequence. Phylogenetic analysis pinpointed the specific *A. flavus* strain and its origin, uncovering a novel strain gene added to the Gene Bank database under the accession number MH213344, underscoring its significance in fungal research.

A. flavus River Isolate DNA Barcoding, Secondary Structure Prediction Analysis

The DNA barcode for the *A. flavus* river isolate, derived from the ITS sequences, has provided valuable insights into the genetic variations present, as depicted in Fig. 8A. In this study, the prediction of RNA secondary structures, as depicted in Fig. 8B, was employed to gather crucial information for conducting phylogenetic analyses. The analysis primarily centered on the ITS1 regions, emphasizing the examination of conserved structural elements, including interior loops, hairpin loops, and exterior loops within the *A. Flavus* River isolate. Notably, the assessment of these secondary structures incorporated criteria rooted in free energy minimization, utilizing nearest-neighbor parameters and focusing on Gibbs free energy at 37°C to gauge structural stability. The observed similarities in secondary structure were complemented by resemblances in energy profiles (-ΔG). Nevertheless, variations in nucleotide sequence length contributed to distinctions in the topological features of these structures. This comprehensive approach served to enhance our understanding of the genetic variations and relationships elucidated by the *A. flavus* DNA barcodes derived from the ITS sequences, and it further emphasized the utility of secondary structure prediction in phylogenetic studies.

Thangaraj et al. (2011) study highlighted the significance of RNA transcript secondary structures in systematic fungal studies, as these structures provide valuable information not discernible in primary sequences. The research involved predicting and comparing the secondary structures of the internal transcribed spacer region 1 (ITS1) in four Aspergillus species. The examination of these structures revealed a mix of conserved and varied topologies among the species. These
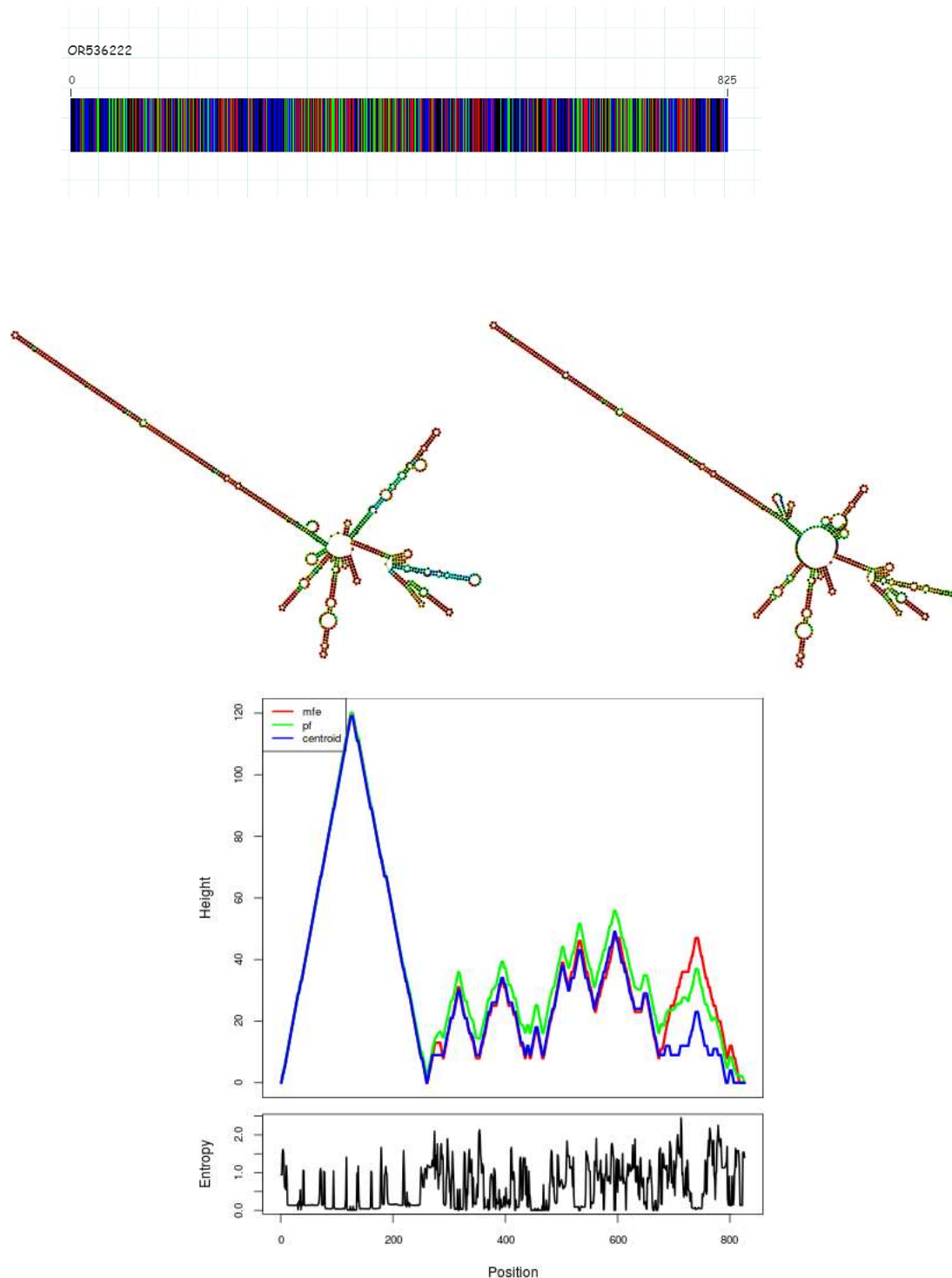
Fig. 8: A comprehensive comparative analysis of the *Aspergillus flavus* river genotype, delving into its genetic and structural characteristics.

distinctive structural characteristics can be employed in generic-level fungal identification. Interestingly, the study found variations in secondary structure topologies, particularly in features like the first stalk and junctions, across different species. Notably, the phylogenetic trees constructed based on both sequence and secondary structure data exhibited similar cladistics among the four *Aspergillus* species, underscoring the relevance of these RNA structural insights in fungal taxonomy.

CONCLUSION

In conclusion, mycotoxin-producing *Aspergillus* species in natural water sources have significant ecological and public health implications. Molecular methods, including PCR assays, provide rapid and precise species identification. Common *Aspergillus* species were identified using culture media and morphology over six months, with specific *A. flavus* identification based on colony characteristics and specialized media. Genomic DNA extraction from *A. flavus* was efficient. PCR analysis with ITS1/ITS4 primers consistently produced a 600-base pair fragment, confirming genetic uniformity. NCBI BLAST analysis and the ITS2 Database supported *A. flavus* identification. Our study uncovered novel *A. flavus* isolates, underscoring the need for ongoing microbial diversity research. Phylogenetic analysis using ITS sequencing demonstrated its robustness. This approach enhances our understanding of Aspergillus diversity and ecological roles, with future research focused on broader diversity exploration and ecological investigations.

ACKNOWLEDGMENTS

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