MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF A SOIL-DERIVED *Aspergillus niger* STRAIN

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ABSTRACT

Aspergillus niger is a filamentous fungus of great importance to the industry and biotechnology. A detailed strain-level characterization of *A. niger* isolated from soil was carried out in this study, which entailed the integration of morphological, biochemical, molecular and *in silico* analyses. Morphology showed black conidial heads together with biseriate phialides. Supportive evidence is given by enzymatic assays and carbohydrate fermentation tests. The molecular identification was performed by PCR amplification of the Internal Transcribed Spacer (ITS) region, with primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' which amplify a region of about 570 bp. Sanger sequencing generated a high-quality consensus sequence (Phred score > 40) which was deposited in NCBI GenBank database (Accession No. PV056011 and PV056012 forward and reverse respectively). Comparisons made by BLASTn with the reference *A. niger* showed homology of 99-100%. Phylogenetic analysis was done in MEGA12 using the neighbor-joining method with 1000 bootstrap replications. *In silico* PCR AmplifX confirmed the specificity of the Primer, producing one unique primer pair that amplified a 570 bp-length product with 57% GC content. To characterize genomic features of the strain, RNAfold was used to predict stable secondary structure with a minimum free energy of -213.10 kcal/mol, EMBOSS to determine the GC content, MEME Suite to determine identified three conserved motifs and DnaSP analysis revealed minimal genetic differentiation (Fst = -0.00338) and high gene flow (Nm = 40.67) among populations. Haplotype network analysis with PopART revealed seven different haplotypes, the most prevalent being Hap_3 located in the center of the network. It is a combination of the processes that provides a useful base for the precise identification, characterization of fungus and the biotechnological potential of isolates for enzyme production and organic acid synthesis.

Keywords: Molecular identification, Phylogenetic analysis, *In silico* PCR, RNAfold, Motif discovery, Haplotype

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INTRODUCTION

Aspergillus niger is a filamentous fungus that is a member of the phylum *Ascomycota*, *Eurotiomycetes* class, and genus *Aspergillus*. It is among the most industrially significant fungal species, as it has the outstanding ability to produce large quantities of extracellular enzymes and organic acids. As a result, *A. niger* has been broadly used in the production of pectinases, xylanases, and other hydrolytic enzymes commercially. Consequently, *A. niger* has been extensively studied among filamentous fungi because it is diverse and holds great importance to the food, pharmaceutical and biotechnological industries (Samson *et al.*, 2014).

Morphological characteristics, such as the structure of the conidial head, color of *A. niger*, and nature of hyphae have been major contributors to the traditional identification of *A. niger*. Despite their utility, they are prone to be ambiguous because of phenotypic plasticity and overlap with other black-spored members of *A. niger* (Banos *et al.*, 2019). In order to deal with the constraints outlined above, the biochemical profiling and molecular approaches have become potent methods to complement the classical identification (Rong *et al.*, 2023).

The high inter-species variability and intra-species conservation of the internal transcribed spacer region of ribosomal DNA make it a suitable molecular marker of molecular identification, which has become a standard method of fungal taxonomy. Amplification of this region is usually performed using ITS1 and ITS4 primers, and subsequent sequencing and search against curated databases such as NCBI GenBank via BLAST (Aamir, 2015). Phylogenetic analysis provides an additional level of confirmation by utilizing software such as MEGA, which gives understanding of the evolutionary relationships between closely similar fungal strains (Kumar *et al.*, 2018; Cairns *et al.*, 2021). The current

investigation in bioinformatics means that primers and sequence alignment can now be validated *in silico*, providing yet more assurance at species level identification (Bantihun and Kebede, 2021).

Investigations in bioinformatics have made it possible to validate primers and sequence alignment *in silico* so that molecular identification can be improved more accurately. These techniques are useful not only in enhancing molecular diagnostics but also in developing special diagnostic means (Bonin *et al.*, 2021). RNA fold is used to predict the secondary structures of ribosomal RNA, which is useful in refining molecular markers. EMBOSS GC is used to determine the GC content, that provide information on the total genomic composition and its possible effects on metabolic pathways. The MEME Suite was employed to determine conserved motifs in the genome, that point out functional regions that play an important role in the production of enzymes or tolerance of the environment (Bradshaw *et al.*, 2020). In addition, DnaSP was used to examine the genetic diversity and haplotype structure that might have been present in the *A. niger* strain to provide information supporting the population genetics and evolution of the fungus. Hence, Tandem Repeat analysis is used to identify repetitive sequences that could contribute to genomic stability and to the response of environmental stresses. These *in silico* studies reinforce taxonomic characterization and assist in developing a broad genetic description of the *A. niger* strain (Cairns *et al.*, 2021; Choudhary *et al.*, 2025). Despite these advances, most *A. niger* identification studies rely on one or two approaches, and an integrated framework that simultaneously applies morphological, biochemical, ITS-based molecular phylogeny but still a panel of *in silico* genomic characterization tools remains absent. This gap is particularly pronounced for soil-derived isolates, which are often underexplored compared to clinical or industrial strains, yet may harbour unique enzymatic and metabolic traits relevant to biotechnology. This gap limits the robustness of strain-level identification and the extraction of genome-level insights relevant to industrial application. No previous investigation has assembled such a multi-layer profile for a single soil-borne *A. niger* strain. In this study, a soil-derived *A. niger* isolate was comprehensively characterized using morphological, biochemical, molecular phylogenetic and *in silico* genomic approaches. This integrated strategy enabled unambiguous species identification and provided a genomic foundation for future biotechnological applications.

MATERIALS AND METHODS

Collection, isolation and purification of fungal isolates: Soil samples (50 g) were aseptically collected using sterile spatulas from the top 10 cm of compost heaps and decaying leaf litter in an agricultural field located at University of Agriculture, Faisalabad, Pakistan. This site is rich in organic matter, such as compost and decomposing plants, and transferred into pre-labeled sterile polyethylene bags. Samples were placed in sterile media to preserve viability and were processed within 2 hr. Until processing, samples were temporarily stored at 4°C in the Enzyme Biotechnology Lab., Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan (Rong *et al.*, 2023). Fungal isolation was performed through serial dilution of soil samples in sterile distilled water and inoculation of 100 µL aliquots were inoculated onto Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) using the spread plate method. For isolation of pure colonies, the streak plate method was subsequently employed. The media was autoclaved for sterilization to prevent contamination. The plates were incubated at 28 ℃ for 5-7 days and pure isolates of fungal colonies were sub-cultured on new PDA (Romsdahl *et al.*, 2018). PDA was used for *Aspergillus* sporulation and colony morphology, SDA with chloramphenicol for bacterial suppression in soil samples, while enzyme assays (amylase, pectinase and xylanase) and antifungals (amphotericin B and fluconazole) were selected based on *A. niger's* known industrial enzyme profile and standard antifungal classes for susceptibility testing.

Macroscopic and microscopic observation: Colony features including color, texture, margin, topography, pigmentation and reverse coloration were observed after 5 days of incubation at 28 ℃ (Mandal *et al.*, 2021). Lactophenol Cotton Blue (LPCB) preparations were prepared based on microscopic features. Fungal mycelium was gently lifted from the colony using a sterile inoculating needle, placed on a glass slide with a drop of LPCB stain, covered with a coverslip, observed under a compound microscope (40X and 100X) and the characteristics of conidiophore structure, vesicle shape, metulae, phialides and arrangement of conidia were noted (Choudhary *et al.*, 2025).

Physiological and biochemical tests

Gram staining, catalase, oxidase, urease, citrate utilization and gelatin hydrolysis tests: Gram staining was performed to confirm culture purity and rule out bacterial contamination. Although fungal hyphae may retain crystal violet due to their cell wall composition, the absence of bacterial cells confirmed culture purity. A set of biochemical tests was performed for characterization of the isolate. A small quantity of fresh fungal mycelium was placed on a clean glass slide and 1 drop of 3% hydrogen peroxide was added, a positive result was indicated by immediate effervescence (bubble formation) (Mbareche *et al*., 2021). A sterile filter paper was moistened with 1% tetramethyl-p-phenylenediamine and rubbed a portion of fungal mycelium on the paper, appearance of dark purple color within 10s was considered as positive.

The urease activity was assessed by growing *A. niger* for 48 hr at 30 ℃ on Christensen's urea agar slants (Pérez Rodríguez *et al*., 2023). Citrate utilization was determined by incubation of Simmons citrate agar (pH 6.9) at 28-30 ℃ for 5 days and a color change from green to blue was considered positive (Paranos *et al*., 2024). Biochemical tests were carried out using the uninoculated media as negative controls. The oxidase and citrate test results were interpreted according to standard microbiological protocols. Each biochemical assay was repeated thrice and results were consistently identical for each replicate, indicating reproducibility. Fungal mycelium was inoculated in the nutrient gelatin tubes and incubated at 28 ℃ for 14 days; the tubes were then refrigerated at 4 ℃ for 30 min and liquefaction was considered as gelatinase activity. All biochemical tests were performed in triplicate to ensure reproducibility of observations. Since these assays were qualitative, the results were interpreted descriptively and no statistical analysis was performed.

Fermentation profile of carbohydrates and enzymatic screening: Carbohydrate fermentation was assessed based on phenol red broth with the addition of 10 sugars such as glucose, fructose, sucrose, maltose, lactose, mannitol, galactose, xylose, arabinose, and raffinose (1%) to the medium. Durham tubes were put in place to trap the formation of gases. The tubes were incubated at 28-30 ℃ for up to 7 days when the color changed (red to yellow), signifying acid production (Cairns *et al.*, 2018). The agar media were tested to determine extracellular enzyme production, and these were the following agar media: amylase: starch agar, to which iodine was added to be able to see clear zones. Pectinase: Pectin agar plates stained with Congo red (1%). Xylanase: Xylan agar flooded with Congo red (0.1%) and stained with 1 M NaCl. The plates were incubated at 28-30 ℃ for 48 h. The presence of clear zones around the colonies was a sign of enzymatic hydrolysis (Cairns *et al.*, 2021). A known enzyme producing *A. niger* strain (ATCC 16404) was inoculated on separate plates as a positive control and uninoculated agar plates served as negative controls. For enzyme assays, the absence of clearing in the uninoculated agar plate served as a negative control; the clear zone around the colony relative to the background provided an internal control for enzyme activity.

Antifungal susceptibility testing: Antifungal susceptibility was assessed by the agar well diffusion method. The conidial suspensions (1×10^6 conidia/mL) were added uniformly on PDA plates 50 µL per plate. Autoclaved PDA media was inoculated with 50 µL freshly prepared fungal suspension and poured into the culture plates for solidification. After solidification, wells (6 mm in diameter) were formed with sterilized cork-borer. These wells were filled with respective 100 µL of amphotericin B 10 µg, itraconazole 10 µg, fluconazole 25 µg and ketoconazole 10 µg under sterilized conditions in laminar air flow, incubated at 30 °C for 72 h (Paranos *et al.*, 2024). The antifungal susceptibility assay followed a Completely Randomized Design (CRD) consisting of four antifungal treatments with three independent replicates per treatment (Akram *et al*., 2025).

Inoculum preparation for molecular studies: Five days old pure cultures were harvested in 0.1% Tween 80 solution, filtered with sterile muslin cloth, and measured using a hemocytometer to a spore concentration of 1×10^6 spores/mL. DNA extraction and PCR amplification were performed using this standardized inoculum (Gupta *et al.*, 2024).

DNA extraction and amplification by PCR: Genomic DNA was prepared by the use of 5 days old *A. niger* cultures in PDA using SDS, EDTA and CTAB based protocols (Schenk *et al.*, 2023). The fungal mycelium (200 mg) was ground in liquid nitrogen and extracted with CTAB extraction buffer, 700 µL of pre-warmed CTAB extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA and 0.2% β-mercaptoethanol) was added and the mixture was incubated at 65 ℃ for 45 min. An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added, mixed gently and centrifuged at 12,000 rpm for 10 min. The aqueous phase was transferred to a fresh tube and DNA was precipitated with chilled isopropanol at -20 ℃ for 45 min. DNA pellet was washed with 70% ethanol and resuspended in TE buffer. The purity was determined by measuring the A260/A280 ratio of 1.8 to 2.0 and integrity by 1% agarose gel electrophoresis. The DNA sample (200 µL) was treated with RNase from a 10 mg/mL stock solution. The sample was incubated at 37 ℃ for 30 min and then analyzed by agarose gel electrophoresis. The DNA was subsequently purified by salt and cold ethanol precipitation by adding one-tenth volume of 3 M sodium acetate (pH 5.2), followed by ethanol addition and centrifugation. Finally, the DNA pellet was dried and stored for subsequent PCR amplification (Raja *et al.*, 2017). The universal primers of ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' were used to amplify the rDNA ITS region in a 25 uL PCR reaction mixture. The 25 µL reaction mixture contained: 12.5 µL of 2X PCR Master Mix (Thermo Fisher Scientific), 1 µL each of forward and reverse primers, 2 µL of template DNA (50 ng/µL) and 8.5 µL of nuclease-free water. PCR amplification was performed using a modified Touchdown PCR protocol (Nischala *et al*., 2022). The thermal cycling conditions were as follows: initial denaturation at 95 ℃ for 3 min; followed by 5 cycles of denaturation at 95 ℃ for 30 s, annealing at 45 ℃ for 30 second and extension at 72 ℃ for 1 min; then 30 cycles of denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 1 min; with a final extension at 72 ℃ for 5 min. The amplified products were separated on 1.2% ethidium

bromide-stained agarose gel and a single clear band was observed with the anticipated size of ITS of *A. niger* (Gupta *et al.*, 2024).

Sequencing, chromatogram analysis, BLAST, submission in NCBI and phylogenetic analysis: The purified PCR products were then subjected to bidirectional sequencing of the product using forward and reverse primers. Chromas v2.6 and BioEdit v7.2.5 were used to analyze sequence chromatograms. Phred scores below 30 were trimmed and high-quality overlapping regions were assembled into a 570 bp consensus sequence that represented the ITS region of the isolate (Choudhary *et al.*, 2025). BLASTn was performed on the NCBI GenBank database to analyze the consensus ITS sequence. Matches with 99 percent sequence identity and E-values less than 1e-100 were regarded as significant. The isolate was most similar to the reference *A. niger* strain, which identified the isolate as a species. The verified sequence was then uploaded to GenBank (Mbareche *et al.*, 2021). The ITS sequence of the isolate and 14 reference species of *Aspergillus* were aligned with CLUSTAL W in MEGA 12 to assess evolutionary relationships. To identify the reliability of the nodes, a neighbor-joining (NJ) tree was made using the Kimura 2-parameter model with 1,000 bootstrap replicates to evaluate the reliability of the nodes. The resultant topology placed the isolate squarely in the *A. niger* clade. The tree was rooted using *A. tubingensis* and *A. arizonicus* as outgroups. Evolutionary distances were calculated as substitutions per site (Kumar *et al.*, 2018).

***In silico* analysis of the ITS region:** Additional *in silico* analyses were performed to complement strain identification with genomic characteristics of potential industrial interest. Stability of RNA secondary structure (RNAfold) supports ITS-based identification by PCR; GC content (EMBOSS) and conserved motif detection (MEME) may indicate genomic adaptation to high-yield fermentation; genetic differentiation (DnaSP) and haplotype networking (PopART) provide an evolutionary perspective on the soil isolate relative to over-used laboratory strains; and scanning for tandem repeats indicates genomic stability, a desirable trait for industrial fermentation. *In silico* PCR was confirmed with AmplifX v2.x (Kalendar *et al.*, 2024). Motifs were discovered by MEME Suite v5.5.9 (three motifs, 6-20 bp wide) (Harrison *et al.*, 2022). GC content was calculated with EMBOSS Geecee. Secondary structure was calculated with RNAfold (ViennaRNA package) (Nystrom and McKay, 2021). Genetic differentiation and gene flow were measured with DnaSP v6.12 (populations classified by isolation source). Tandem repeats were analyzed using Tandem Repeats Finder (default parameters) (Dash *et al.*, 2025). Haplotype networks were generated with PopART (Minimum Spanning Network and TCS algorithms) (Liang *et al.*, 2023).

Statistical analysis: Qualitative assays, including Gram staining, biochemical characterization, and carbohydrate fermentation tests, were interpreted descriptively because they generated categorical outcomes rather than continuous numerical data. All quantitative experiments, including antifungal susceptibility testing, enzyme screening assays were subjected to statistical analysis under a Completely Randomized Design (CRD). Data are presented as mean ± standard deviation (SD). Statistical analyses were performed using Minitab Statistical Software v17.0. Where appropriate, inhibition zone diameters obtained from antifungal susceptibility testing were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's Honest Significant Difference (HSD) test at $p \leq 0.05$.

RESULTS

Isolation, colony morphology and microscopic examination: Among the fungal isolates obtained, one black-colored colony grew rapidly on PDA and SDA after 5 days of incubation. The colony was velvety, black pigmented, and pale yellow on the reverse. These characteristics are consistent with *A. niger*, as shown in Fig. 1a-b. Microscopic examination of LPCB-staining preparation revealed septate hyphae, erect conidiophores, and globose vesicles with biseriate phialides that formed chains of black conidia. These characteristics were similar to the morphological characteristics of *A. niger*, as shown in Fig. 1c-d.

Biochemical characterization; Biochemical profiling was used to support the identification. The isolate was positive for catalase, urease and citrate utilization (Table 1). The isolate exhibited positive reactions for catalase, urease, citrate utilization, and gelatin hydrolysis, whereas the oxidase test was negative. Gram staining confirmed the absence of bacterial contamination and the presence of filamentous fungal hyphae, indicating a pure fungal culture. All biochemical assays were repeated three times and yielded consistent observations across replicates, demonstrating the reproducibility of the results.

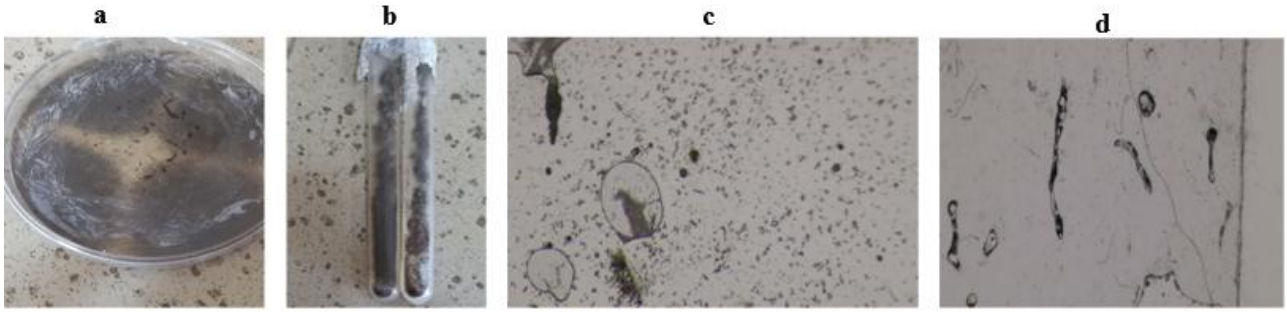

Fig. 1. Morphological and microscopic characterization of the soil-derived *A. niger* isolate. (a-b) Colony morphology on PDA after 5 days at 28 ℃ showing black conidial heads and velvety texture. (c) LPCB-stained preparation (40X) showing septate hyphae and conidiophores. (d) Higher magnification (100X) showing globose vesicle with biseriate phialides and chains of black conidia. Scale bars = 10 µm.

Table 1. Physiological and biochemical characterization of the isolated fungal strain.

Test	Observation	Result	Interpretation
Gram Staining	No bacterial cells observed; filamentous fungal hyphae present	ND	Pure fungal culture confirmed
Catalase	Bubble formation	+	Catalase positive
Oxidase	No color change	–	Oxidase negative
Urease	Bright pink slant	+	Urease positive
Citrate Utilization	Blue coloration	+	Citrate utilization
Gelatin Hydrolysis	Liquefaction	+	Gelatinase activity

ND means presence or absence of bacteria not detectable, "+" sign represents identification of bacteria and "–" sign denoted no identification of bacteria.

Carbohydrate fermentation tests and enzymatic activities: The carbohydrate fermentation profile of the isolate is presented in Table 2. The isolate fermented most monosaccharides and disaccharides, indicating an active carbohydrate metabolism consistent with the reported physiological characteristics of *A. niger*. Since carbohydrate fermentation tests are qualitative in nature, the results were interpreted descriptively as positive (+) or negative (−), and no statistical analysis was performed.

In contrast, enzyme activity was evaluated quantitatively by measuring the diameters of the hydrolysis (clearance) zones. The isolate produced distinct clearance zones for amylase (19.00 ± 1.50 mm), pectinase (22.00 ± 1.80 mm), and xylanase (24.00 ± 2.10 mm). The experiments were performed in triplicate under a Completely Randomized Design (CRD), and the results are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) demonstrated significant differences among the enzyme clearance zone diameters ($p \leq 0.05$).

Table 2. Carbohydrate fermentation profile of the isolate.

Sugar	Acid Production	Gas Formation	Result
Glucose	+	+	Yellow with gas
Fructose	+	+	Positive
Sucrose	+	–	Positive
Maltose	±	–	Variable
Lactose	–	–	Negative
Mannitol	+	–	Positive
Galactose	+	+	Positive
Xylose	+	–	Positive
Arabinose	+	–	Positive
Raffinose	–	–	Negative

Results are presented as qualitative observations based on carbohydrate fermentation reactions. (+) positive fermentation; (−) no fermentation. All assays were performed in triplicate under a Completely Randomized Design, and identical observations were obtained across all replicates.

Table 3. Quantitative assessment of extracellular enzyme activities of the isolated *A. niger* strain based on hydrolysis zone diameters.

Enzyme	Clearance zone (mm)	Enzyme production
Amylase	19.00 ± 1.50^{c}	Positive
Pectinase	22.00 ± 1.80^{b}	Positive
Xylanase	24.00 ± 2.10^{a}	Positive

Values are expressed as mean ± standard deviation (SD) of three independent replicates (n = 3). Means followed by different superscript letters differ significantly according to Tukey's Honest Significant Difference (HSD) test following one-way ANOVA ($p \leq 0.05$).

Antifungal susceptibility profile: The isolate displayed greater susceptibility to polyenes and triazoles. The antifungal susceptibility profile of the soil-derived *A. niger* isolate is presented in Table 4. One-Way ANOVA revealed significant differences among the antifungal agents tested ($F = 66.48$, $p \leq 0.001$). Amphotericin B exhibited the highest inhibitory activity, producing a mean inhibition zone of 21.00 ± 1.20 mm, which was significantly greater than those of the remaining antifungal agents ($p \leq 0.05$). Ketoconazole showed moderate inhibitory activity with a zone diameter of 18.33 ± 0.58 mm, followed by itraconazole (15.67 ± 0.58 mm). Fluconazole produced the smallest inhibition zone (12.00 ± 0.82 mm) and was classified as exhibiting intermediate susceptibility. Overall, the isolate demonstrated significantly greater susceptibility to amphotericin B than to the azole antifungal agents.

Table 4. Antifungal susceptibility profile of the soil-derived *A. niger* isolate against different antifungal agents.

Antifungal agent	Zone of inhibition (mm)	Susceptibility
Amphotericin B	21.00 ± 1.20^{a}	Sensitive
Ketoconazole	18.33 ± 0.58^{b}	Sensitive
Itraconazole	15.67 ± 0.58^{c}	Intermediate
Fluconazole	12.00 ± 0.82^{d}	Intermediate

Values are presented as mean ± standard deviation (SD) of three independent replicates (n = 3). Means followed by different superscript letters (a–d) differ significantly according to Tukey's Honest Significant Difference (HSD) test following one-way ANOVA ($P \leq 0.05$).

DNA extraction, PCR amplification and gel electrophoresis: The CTAB DNA extraction protocol resulted in good quality genomic DNA with A260/A280 ratio of 1.84 to 1.92, indicating high-quality DNA. Strong and sharp bands were observed on 1% agarose gel. The optimized PCR conditions produced a single bright amplicon of 570 bp. Amplification using the Touchdown PCR profile was successful and specific, suggesting that initial low-stringency cycles allowed the primers to bind to the template. The final high-stringency cycles enhanced amplification specificity, consistent with the Touchdown PCR strategy. DNA from *A. niger* ATCC 16404 was used as a positive control, and nuclease-free water served as the negative control. The negative control was not amplified, confirming that the reactions were not contaminated. The occurrence of a single discrete DNA band at 570 bp was verified by electrophoresis, which matched the ITS region length of *A. niger*. The sample loaded in lane 2 along with the 1kb ladder from Thermo Fisher Scientific in lane 1 in Fig. 2 shows the quantity of obtained DNA in (Supplementary Figure 1) as 1786.8ng/µL.

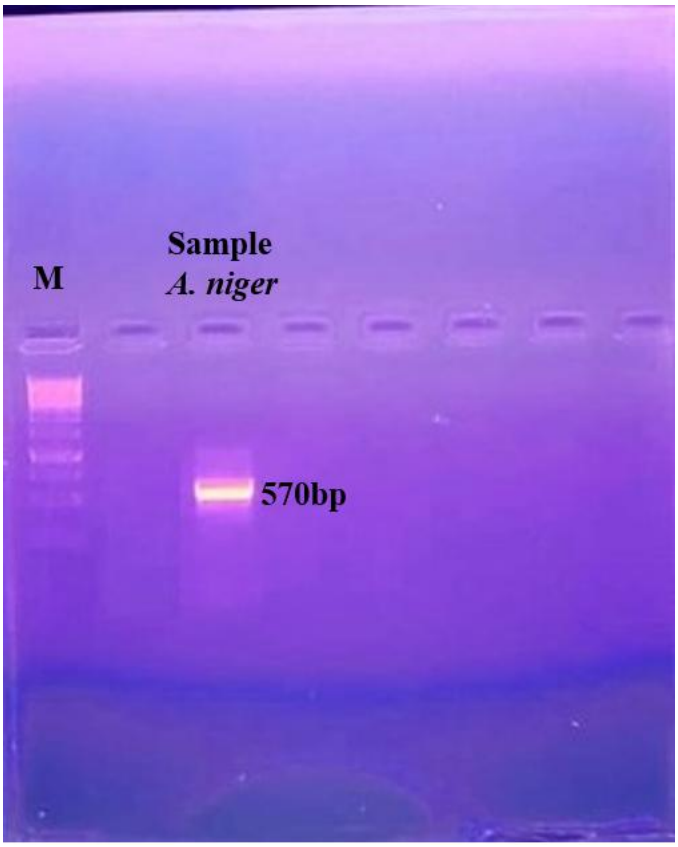

Fig. 2. The PCR gel electrophoresis image shows two lanes: Lane 1 contains a molecular weight ladder (M) with bands at 1000, 750, 500, 250, and 100 bp. Lane 2 shows a PCR product from *A. niger* with a band of 570 bp.

Sequencing and chromatogram analysis, BLAST and sequence identity: Sanger sequencing of the ITS region that was PCR-amplified produced good chromatograms (Supplementary Fig. 2) with sharp and well-resolved peaks in the central parts of the forward and reverse reads in *A. niger*. Poor quality bases and ambiguities at terminal ends were clipped off, and the good quality high confidence bases were stitched together to provide a consensus sequence of about 570 bp. BLASTn analysis of the 570-bp ITS sequence returned a top match of 99.65% identity to *A. niger* (MH064151.1) with 98% query coverage and an E-value of 0.0; several additional matches were also *A. niger* strains with 99.65% identity (accession numbers provided in Supplementary Table S1). Clear peak resolution in the central regions of the chromatogram was retained, while low-confidence ends were trimmed, to ensure accurate species identification, and the bidirectional sequences was verified via GenBank database of the NCBI under accession no. PV056011, PV056012 forward and reverse respectively.

Phylogenetic relationship: The resulting neighbor-joining phylogenetic tree placed the isolate in a well-supported *A. niger* clade (bootstrap 100%) and closely related to *A. niger* strains MH064151.1 and OP237079.1 (Fig. 3). The isolate was different from *A. tubingensis* (MW789023.1) and *A. arizonicus* (OP810889.1) supporting *A. niger* identification.

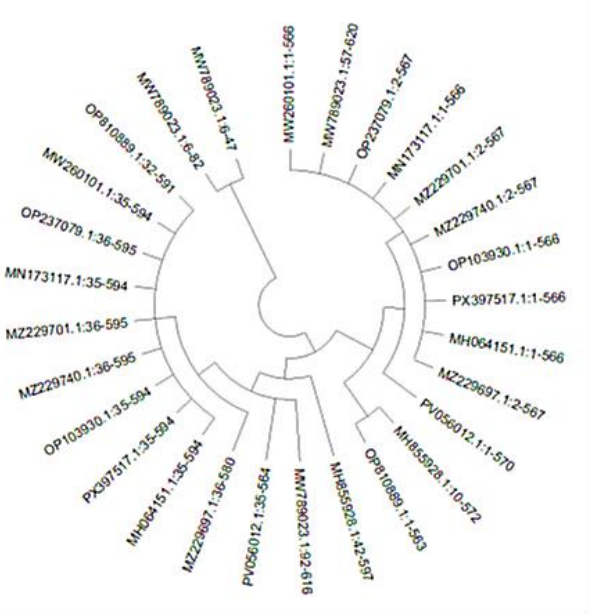

Fig. 3. Circular representation of the neighbor-joining phylogenetic tree showing the relationship of the isolate with related taxa.

***In silico* profiling supports strain identity and genomic stability:** *In silico* profiling also supported the strain identification. AmplifX showed that only one product of 570 bp was amplified with ITS1/ITS4 primers (Fig. 4A). The ITS sequence was 57% GC-rich (EMBOSS), a normal fungal ITS feature and likely to have ensured primer attachment during identification. The AT content was 43.0% (GC:AT ratio of 1.33:1), which is consistent with the slightly GC-biased *Aspergillus* ITS sequences (Supplementary Fig. 3-4). Three conserved motifs (MEME; E-values ≤ 6.9) (Fig. 4B) were observed, suggesting structural features that support the reliability of ITS-based phylogeny. RNA Secondary Structure Prediction showed a stable minimum free energy (MFE) of −213.10 kcal/mol (Supplementary Fig. 5S), and a clear centroid structure, indicating the sequence structural stability as a taxonomic marker. No tandem repeats were found, confirming that the ITS region is free from length variations and can be used for reliable PCR-based identification. Population differentiation analysis between the soil-isolate and populations from other environments revealed a lack of differentiation ($F_{st} = -0.00338$, $N_m = 40.67$, $P = 0.3518$) in Table S2, confirming that the isolate is part of a genetically unified *A. niger* population, further supporting species identification. Lastly, haplotype network analysis revealed the isolate in the central, most common haplotype (Hap3) in Fig. 4C, shared with other reference *A. niger* strains, further confirming its identity and evolutionary position within *A. niger*.

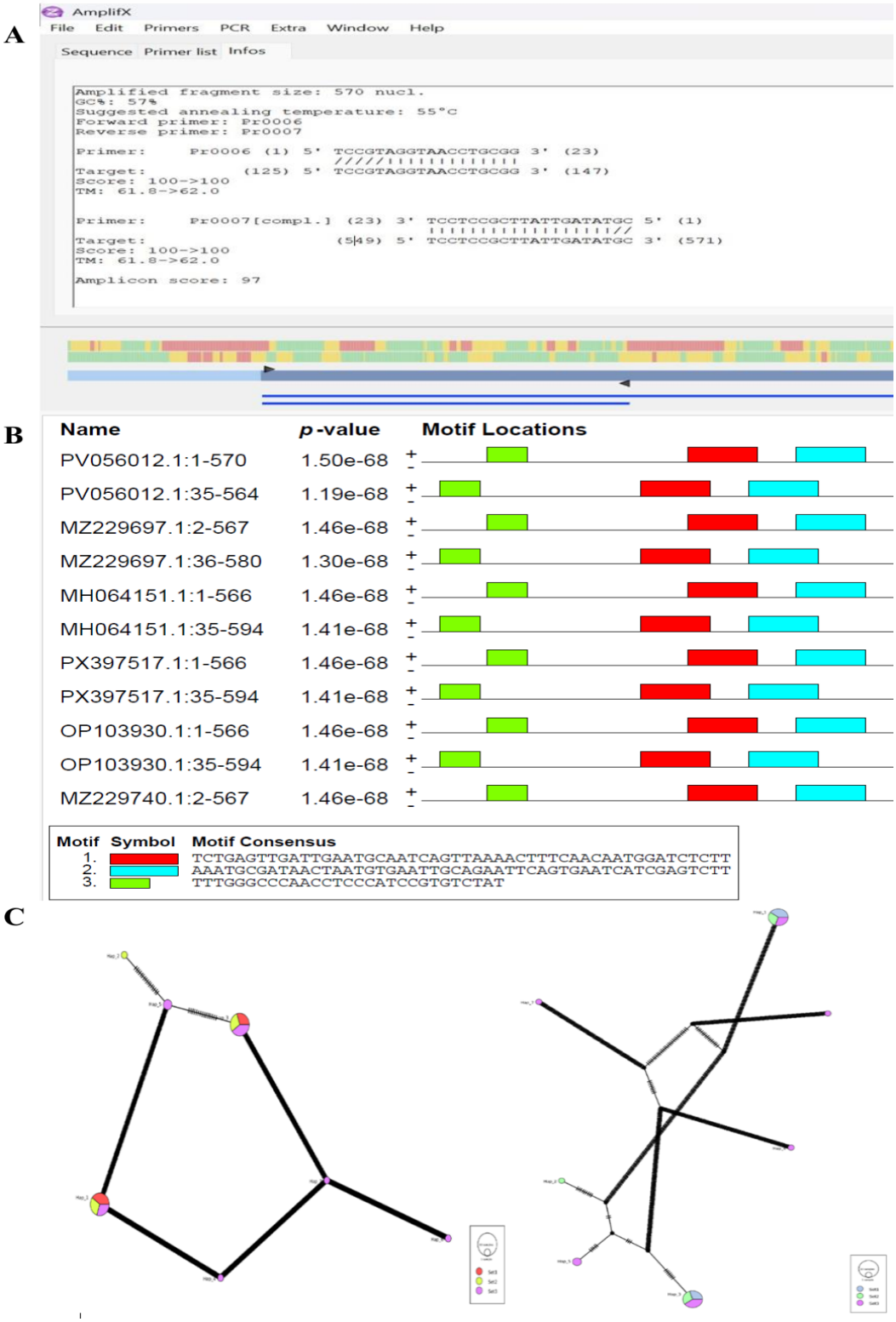

Fig. 4. (A) *In silico* PCR using AmplifX, showing a single ~570 bp amplicon. (B) Three conserved motifs identified by MEME Suite (E-values: Motif 1 = 2.2e-04, Motif 2 = 6.9e+00, Motif 3 = 6.9e+00). (C) TCS haplotype network constructed by PopART; each circle represents a haplotype, circle size proportional to frequency, colors denote sampling sets. The isolate belongs to the central haplotype (Hap3), shared with reference *A. niger* strains.

DISCUSSION

Strain-level identification of filamentous fungi is a critical yet often overlooked aspect of industrial biotechnology. Comprehensive characterization of *A. niger* is indispensable for maintaining industrial reliability, optimizing productivity, and preventing variability associated with strain misidentification. Most existing studies have combined morphological and ITS-based identification (Mandal *et al.*, 2021), but this work takes things a step further by incorporating molecular identification into a broader *in silico* genomic profiling that, in turn, establishes a quality assurance framework for both taxonomy and bioprocess development. This is the first report of its kind to integrate phylogenetics based on ITS, *in silico* primer testing, analysis of secondary structure of RNA, GC content, conserved motifs, tandem repeats, genetic differentiation indices and haplotype networking into a single, transferable workflow for the characterization of a soil-derived *A. niger* isolate. This multi-step approach not only validates species identity but also offers genomic insights that directly impact commercialization, a feature rarely reported in the literature.

The morphological and biochemical observations offered rapid presumptive identification but are known to be unreliable in *A. niger* (Banos *et al.*, 2019); therefore, molecular identification was needed to confirm the species. The PCR amplification of the ITS region with universal primers ITS1/ITS4 and its confirmation as a reliable marker are in line with the universal barcode concept (Schoch and Seifert, 2012). However, the *in-silico* primer validation step (100% query coverage and single amplicon) goes beyond species identification. It addresses an industrial need: single-direction sequencing qPCR reactions are often performed and sequencing reactions can yield false-negative results when there is a mismatch in the PCR primer sequence, which is not detected. Our study offers a readily available primer template pair which can be easily adopted in production (Choudhary *et al*., 2025).

The evolutionary position of the isolate, as well as the technical validation of the molecular marker, allows conclusion to be drawn about its metabolic capabilities. The phylogenetic position of the isolate in a strong branch of *A. niger*, distinct from *A. tubingensis* and *A. arizonicus*, excludes the possibility of cryptic species. This finding demonstrates a close evolutionary relationship with high performing industrial strains MH064151.1 and OP237079.1. The evolutionary proximity of the isolate to high-performing industrial strains suggests that it may possess metabolic traits relevant to enzyme and organic acid production. Therefore, the phylogenetic tree is not only a means to confirm species identity, it also predicts biotechnological potential.

The *in silico* analyses contribute to the identification process and reveal biological insights. The GC content 57% of the ITS amplicon is within the ideal range for primer annealing, enhancing PCR stability under varying laboratory conditions, a benefit when the identification system is translated to industrial quality-control laboratories. The stable RNA secondary structure (MFE = -213.10 kcal mol^{-1}) also positively impacts identification: a homogeneous fold prevents artefacts of size-heterogeneity in capillary electrophoresis, ensuring that the amplicon size matches the target sequence, a critical requirement for size-based identification (Kapoor *et al.*, 2018). This structural stability, combined with the three conserved MEME patterns, suggests intact rRNA processing motifs. The rRNA biogenesis is intimately coordinated with biomass production and protein export; these motifs also suggest the ability of isolate to produce high levels of enzyme. Likewise, the absence of tandem repeats is consistent with low intragenomic variation and removes a potential source of length polymorphism from the amplicon, further confirming the stable and distinctive nature of the ITS region (Bradshaw *et al.*, 2020), and suggests long-term genomic stability, a trait that helps prevent strain degeneration during continuous cultures. The absence of tandem repeats not only supports the identification but also explains the reliability, and at the same time identify genomic features that are compatible with industrial performance. While the above features are isolate specific, population genetic analyses broaden the perspective to the global *A. niger* population, and address the question of genetic differentiation between soil-derived and industrial populations (Bradshaw *et al*., 2020).

Population genetic analyses open the discussion to a broader perspective. The almost zero genetic differentiation between soil and other environmental populations (Fst = -0.00338, Nm = 40.67, P = 0.3518) suggests that *A. niger* is a globally panmictic population with strong gene flow. This is in line with other broadly distributed saprophytic fungi and has an important biotechnological implication: the genetic potential for desirable traits (such as high enzyme production) is likely to be distributed across the entire gene pool. Therefore, there is no need to fear bioprospecting, since it can safely focus on local soil isolates, which will presumably carry the same metabolic properties as the current commercial strains. Finally, the central placement of our isolate in the haplotype network also indicates its genetic typicality; i.e. that it is not a genetic outlier but a typical member of the *A. niger* population (Dumaidi *et al.*, 2020). In a world of industrial microbiology where genetic background information becomes an important selection criterion, this blueprint is a competitive edge. The novelty of this work thus relates not to the technologies themselves, but to the integrated use of different technologies to one environmental isolate to obtain a genomic and functional passport that can serve as a starting point for industrial strain selection and regulatory documentation (Mbareche *et al.*, 2021).

There are some limitations. First, only one soil isolate was investigated; population-level analyses with multiple isolates from diverse geographic locations are required to verify the presence of the genomic features. Second, only the ITS region was used for species identification. Although ITS is universally adopted as the fungal barcode, it does not always discriminate close relatives in section *niger* (Samson *et al.*, 2014). Finally, the population genetic analyses were based on a small number of ITS sequences from public databases; genome-wide data from a larger and better-sampled population would be more informative. Despite these limitations, the integrated approach described here represents a major improvement in the standard for industrial fungal strain characterization and shows the benefits of integrating traditional and *in silico* method.

Conclusion: The soil-derived isolate was definitively identified as *A. niger* using an integrative approach that combined morphological, biochemical, ITS-based phylogenetic and *in silico* genomic analyses. The integrated framework encompassing secondary structure prediction, GC content analysis, motif discovery and haplotype networking, validated species identity and generated a comprehensive genomic profile. The strong enzymatic activities of the isolate (amylase, pectinase, and xylanase) highlight its potential as a candidate for industrial bioprocessing and enzyme production.

Declarations: All authors read and approved the final manuscript.

Ethical approval: This study did not involve human participants or vertebrate animals. The fungal isolate was obtained from soil samples collected from an agricultural field at the University of Agriculture, Faisalabad, Pakistan. No specific permits were required for soil sampling at this location and the study did not involve endangered or protected species. Since this paper is part of a broader PhD research project approved by the Institutional Review Board (ORIC), University of Agriculture, Faisalabad (Certificate No. 2306/ORIC); the present study reports only the fungal isolation and identification component, which did not involve animal use.

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Conflict of Interest: The authors declare no conflict of interest.

Data Availability: The ITS sequences generated in this study have been deposited in the NCBI GenBank database under accession numbers PV056011 (forward) and PV056012 (reverse).

Authors Contribution: Nayya Waseem Dar prepared the manuscript. Muhammad Anjum Zia, Muhammad Shahid, and Bushra Akhtar supervised the study and critically reviewed the manuscript. All authors approved the final version of the manuscript.

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