IDENTIFICATION OF ENDOPHYTIC *CLADOSPORIUM* SPECIES USING MORPHOLOGICAL, PHYSIOLOGICAL, AND rDNA SEQUENCE ANALYSIS

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Abstract. Endophytic *Cladosporium* species are important for plant health, but their identification is difficult due to their diverse morphology and physiology. In this study, our objective is to identify endophytic Cladosporium species by integrating morphological characterization, physiological profiling, and rDNA sequence analysis. This combined approach was selected because morphological traits alone can be highly variable and often insufficient for distinguishing closely related species. The study identified endophytic Cladosporium species using an integrated approach that included morphological characterization, physiological profiling, molecular analysis, and rDNA sequence examination. Morphological traits, observed from isolates grown on Potato Dextrose Agar, showed structures resembling *C. cladosporioides* or *C. halotolerans*. Physiological tests, including various temperatures, pH levels, and media, provided insights into species-specific adaptations. Overall growth patterns revealed no significant differences between the two *Cladosporium* species. Molecular identification using a combination of universal and species-specific markers, confirmed the identity of the isolate as *C. halotolerans*. Our findings highlight the significance of integrating traditional and molecular techniques for precise fungal taxonomy, paving the way for future research on endophytic *Cladosporium* species and their applications in agriculture and biotechnology.
Keywords: *Cladosporium species, endophytic fungi, optimal growth, rDNA sequence, molecular identification, fungal taxonomy, ITS, ACT, TEF1 markers*

Introduction

Although endophytic *Cladosporium* species are widespread and functionally important, accurate identification remains challenging due to their overlapping morphological features and the limited use of integrative analytical approaches. This gap hinders consistent classification and comparison across studies. To address this, our work identifies endophytic *Cladosporium* species using a combined framework of morphological, physiological, and rDNA-based molecular analyses. This integrative approach represents the main novelty of the study and provides a more robust basis for distinguishing closely related species. The findings are expected to strengthen taxonomic clarity and support future applications of these fungi in agriculture and biotechnology.

Endophytic *Cladosporium* species play important ecological and functional roles in promoting plant growth and enhancing nutrient-related processes (Choudhary et al., 2023). hey also contribute to natural disease suppression through the production of bioactive antibacterial and antifungal compounds (Yehia et al., 2020). A variety of bioactive metabolites have been isolated from *Cladosporium* strains, including

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cladosporin, macrolides, sulfur-containing diketopiperazines, indole alkaloids, hybrid polyketides, and diterpenes with a 5-8-5 ring system (Zhang et al., 2019). By secreting beneficial secondary metabolites, these species enhance plants' ability to adapt to new habitats and maintain their health and performance. Among the secreted metabolites, gibberellins play a crucial role. These hormones are responsible for stimulating plant growth, particularly in seed germination, stem elongation, and leaf expansion (Răut et al., 2021). *Cladosporium* species have been demonstrated to promote the growth of various plants, including rice, cucumber, and soybeans (Halo and Al-Sadi, 2021).

Traditional methods of *Cladosporium* species identification, based on morphological and physiological characteristics, often fall short due to the high variability and subtle differences between species. *Cladosporium* is primarily characterized by its asexual morphological structures. Both the conidiogenous cells and conidia have distinctive conidiogenous loci with a unique coronate structure, comprising a central convex dome surrounded by a raised, thickened, refractive, and darkly pigmented. Microscopy is essential for obtaining morphological characters and cellular structures, except for macroscopic features visible to the naked eye. Various graphical approaches and software programs also provide additional instruments for providing precise descriptions. Conventional practices, errors, and ideas to avoid potential downsides are discussed here (Chinthani et al., 2020).

To overcome these limitations, the integration of molecular techniques, such as rDNA sequence analysis, has proven invaluable (González et al., 2021). Amplifying rDNA sequence analysis using a Thermocycler polymerase chain reaction (PCR) is a fundamental tool in molecular biology and the most common technique used in laboratory research and DNA studies (Khalil, 2020). To accurately identify and distinguish closely related species with similar morphological features, molecular sequences are used. However, ITS sequencing alone is often insufficient for differentiating these species within complexes. Therefore, some researchers opt for multilocus DNA sequencing, utilizing actin, ITS, and TEF1 genes, which provide high discrimination among closely related species (El-Dawy et al., 2021). This study employs a comprehensive approach combining morphological characterization, physiological profiling, and rDNA sequence analysis to accurately identify endophytic *Cladosporium* species. By doing so, it aims to enhance our understanding of the diversity within this genus and explore its potential benefits for sustainable agriculture and biotechnology.

Materials and methods

Morphological characterization

An endophytic *Cladosporium* species isolated from *Catharanthus roseus* was obtained from the culture collection of the Microbiological Laboratory at the Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia. To evaluate the morphological characterization, the purified fungal isolate was grown on a PDA medium. Colony characteristics such as growth rate, color and texture, and reverse pigmentation were considered. This isolate was stained using lactophenol and examined under a light microscope (Wang et al., 2023).

Physiological Characterization Physiological studies were undertaken to determine the growth parameters for *Cladosporium* species to discover potential for growth of these fungi under varying conditions including temperature, pH levels, and different types of media.

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Effect of temperatures: The impact of different temperatures (5°C, 15°C, 25°C, 35°C, and 45°C) on fungal growth was examined. The isolates were cultured on Potato Dextrose Agar (PDA) medium and incubated for 14 days.

Effect of pH values: To assess optimal fungal growth, eight pH values (ranging from 2 to 7) were tested. The isolates were cultured on PDA media and incubated for 14 days at a constant temperature of 25°C.

Effect type of media: Five different types of media (PDA, CZA, SDA, MEA, and YEA) were utilized to observe fungal growth. The isolates were cultured and incubated for 14 days at a constant temperature of 25°C.

These experiments aimed to understand how temperature, pH levels, and various media types influence fungal growth, providing valuable insights for further research and practical applications.

Molecular identification

For molecular identification, pure *Cladosporium* sp. culture was used for DNA extractions (Mukhtar et al., 2020).

The fungal single spore technique of *Cladosporium* sp. was conducted from pure culture in the laboratory as follows: Using 0.1 mL of sterilized distilled water (SDW) and a sterilized loop, one fungal inoculation was spread on PDA medium's surface. The plate was left in laminar flow for 10 min to dry. The inoculated medium was incubated at 25°C for 2-5 days. Thereafter, the germinated spores were selected and transformed into a new PDA medium. The fungal growth was collected after 14 days of incubation at 25°C (Moharram et al., 2022).

The isolate was identified through molecular techniques utilizing ITS, ACT, and TEF1 presented in *Table 1* (González et al., 2021). These sequences were subjected to BLAST analysis using default parameters, and the sequencing data were then analyzed against the nucleotide collection database.

Evolutionary analysis

The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions were reproduced in less than 50% of bootstrap collapsed replicates. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (1000 replicates). Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated by using the Tamura-Nei model (Bolivar et al., 2022). The isolate was identified through molecular techniques utilizing ITS, ACT, and TEF1 as shown in *Table 1* (González et al., 2021; Denis et al., 2015). These sequences were subjected to BLAST analysis using default parameters, and the sequencing data were then analyzed against the nucleotide collection database.

Results

Morphological identification

An endophytic *Cladosporium* species culture was purified, and its appearance in the PDA medium was characterized by velvety a dark grey colony with 4.6 cm in diameter after 14 days of a PDA medium. When young, the mycelium surface of the strain displays

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tiny dark dots, which darken to a dark olive as it matures. The hyphae of the mycelium measure 4–6 µm in width, are branched, and spaced apart. Conidiophores branch out, yielding abundant conidia in branched chains, with a dense concentration at the base and a sparser distribution towards the top of the chain *Figure 1*. Conidia of *Cladosporium* are typically produced in extended, branching chains. They are released either by fragmenting the chain or by rupturing the supporting cell. These conidia are usually darkly pigmented because of melanin in their cell walls, which protects against environmental stressors like UV radiation and desiccation. The thick cell walls of the conidia enhance their durability and ability to withstand harsh conditions. A key morphological feature of *Cladosporium* conidia is their lemon or ellipsoidal shape, which is elongated and slightly tapered at both ends, resembling a lemon. The conidia are relatively small, typically measuring between 3 to 7 µm in length. The morphological analysis of the *Cladosporium* isolate was similar to that of two species: *C. cladosporioides* and *C. halotolerans*. These morphological findings provided the initial basis for species differentiation and directly supported our objective of identifying the endophytic *Cladosporium* isolate through combined morphological, physiological, and molecular analyses. Physiological and molecular studies were carried out to identify the accurate species.

Table 1. *Molecular identification of endophytic Cladosporium species was conducted using three primers*

Locus	Domain amplified	Primer name	Primer sequence
ITS-rDNA	The internal transcribed spacer ribosomal DNA	ITS1 (universal)	TCCGTAGGTGAACCTGCGG
Actin	Partial translation elongation factor 1-a, actin	ACT-512F ACT-783R (Specific)	5'-ATGTGCAAGGCCGGTTTCGC-3' 5'-TACGAGTCCTTCTGGCCCAT-3'
TEF1	Translation elongation factor 1-a	EF1-728F EF1-986R (Specific)	5'-CATCGAGAAGTTCGAGAAGG-3′ 5'-TACTTGAAGGAACCCTTACC-3′

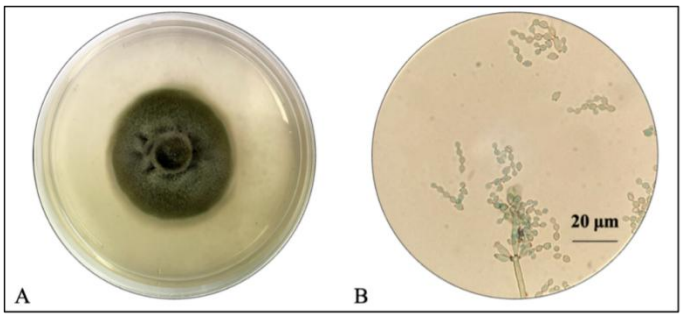

Figure 1. *Cladosporium sp. isolate (A) grey colony on PDA medium at 25℃ for 14 days of incubation, (B) microscopic appearance showing conidiophores and conidia by using a light microscope at 100X magnification*

Effect of cultural conditions

After 14 days of cultivation, the different temperatures, PH values, and media types significantly affected the growth of *Cladosporium* sp. isolate. The greatest fungal growth was at 25°C, 5 pH value, and on CZA medium as shown in *Figure 2*.

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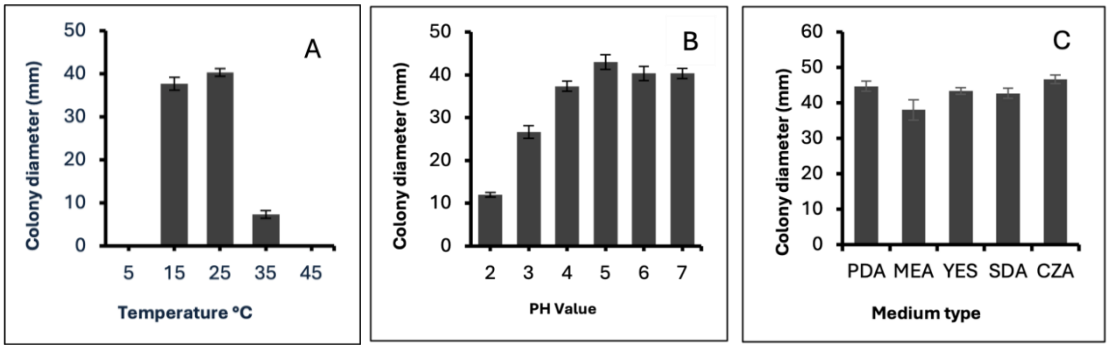

Figure 2. *Physiological studies indicate that various factors influence the colony diameter (mm) of an endophytic Cladosporium species. These factors include (A) different temperatures (5°C, 15°C, 25°C, 35°C, and 45°C), (B) pH levels (2, 3, 4, 5, 6, and 7), and (C) media types (PDA, MEA, YES, SDA, and CZA)*

Colony growth of *Cladosporium* species varies significantly with temperature at 5°C, 15°C, 25°C, 35°C, and 45°C. At 5°C, there is no observable growth, while substantial growth occurs at 15°C and 25°C, with colony diameters reaching around 40 mm and showing consistent results indicated by small error bars. At 35°C, growth reduces noticeably, with diameters averaging around 10 mm and larger error bars indicating greater variability. At 45°C, no growth is observed. The optimal growth temperature is between 25°C, where the largest colony diameters are seen. Higher temperatures, 35°C and above, significantly reduce growth, with no growth at 45°C. Similarly, low temperatures at 5°C inhibit growth completely. Thus, the colony thrives best at moderate temperatures (15°C to 25°C), while extreme temperatures, both low (5°C) and high (35°C and above), significantly inhibit colony growth, indicating the colony's sensitivity to temperature extremes.

The impact of varying pH levels on the colony diameter of an endophytic *Cladosporium* species was measured in millimeters (mm). The pH levels tested are 2, 3, 4, 5, 6, and 7. The colony diameter increases progressively from pH 2 to pH 5, where it reaches its maximum, indicating optimal growth conditions. Beyond pH 6, the colony diameter remains relatively stable, showing no significant further increase up to pH 7. The error bars are small across all pH levels, suggesting consistent and reliable results. This data indicates that while the *Cladosporium* species can grow across a range of pH levels, it thrives best in slightly acidic to neutral conditions, specifically around pH 7.

The effect of different medium types on the colony diameter of an endophytic *Cladosporium* species, measured in millimeters (mm). The media types tested are PDA, CZA, SDA, MEA, and YEA. The colony diameters vary slightly across the different media, with PDA, SDA, and CZA showing the highest growth, each around 50 mm. The medium MEA supports the least growth, with a colony diameter of approximately 40 mm, while YES also shows moderate growth, with a diameter close to 45 mm. The error bars are small, indicating consistent results across replicates. This data suggests that while the *Cladosporium* species can grow on all tested media, PDA, SDA, and CZA provide the most favorable conditions for colony expansion. These physiological results helped distinguish species-level growth patterns and supported our objective of identifying the endophytic *Cladosporium* isolate through an integrative analysis of temperature, pH, and media preferences.

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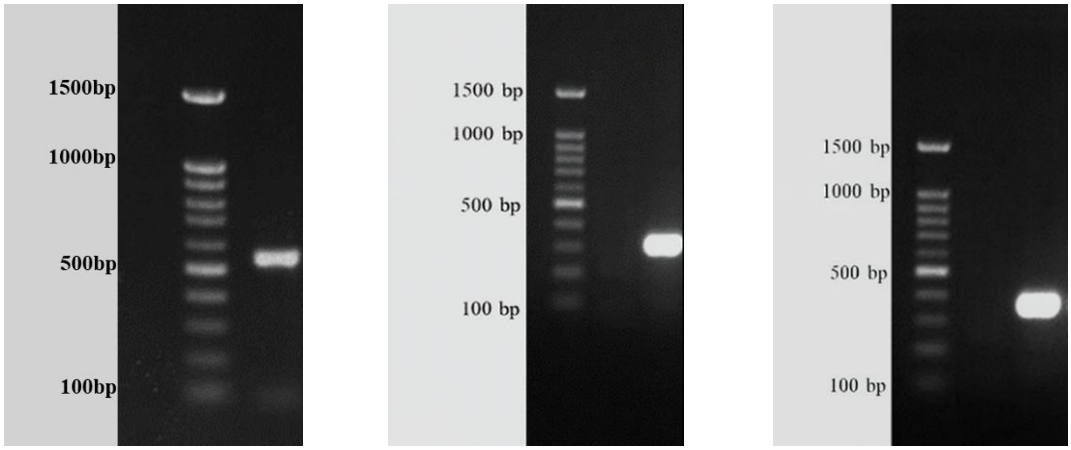
Molecular identification

An endophytic *Cladosporium* species were subjected to PCR amplification using three markers: ITS-rDNA, Actin, and TEF1. After agarose gel electrophoresis, distinct bands were observed. Primer-BLAST was employed to estimate the sizes of the PCR products. The predicted sizes were approximately 549 base pairs for the ITS region, 272 base pairs for the Actin gene, and 348 base pairs for the TEF1 gene as shown in *Figure 3.*



(A) (B) (C)

Figure 3. *Gel electrophoresis of PCR products. The data shown were obtained with (A) ITS1, (B) ACT, and (C) TEF1 primers. The range of DNA molecular size was about 549 bp with ITS, 272 with ACT, and 348 with TEF1, compared to M (100 bp marker on the left side of the image)*

Cladosporium cladosporioides was identified using the ITS-rDNA primers, showing a 100.00% identity with sequences registered under GenBank accession numbers MK265717.1. However, *C. halotolerans* was identified using the Actin primers, displaying a 99.43% identity with the GenBank accession number OQ185000.1. Additionally, *C. halotolerans* was also identified using the TEF1 primer, with a 98.51% identity corresponding to GenBank accession number LC426379.1 as shown in *Table 2*.

Table 2. *Molecular identification of the endophytic Cladosporium isolate, including GenBank accession numbers and percentage sequence identity for each marker used*

SI no.	Primer	Species identified	GenBank accession No.	Max. identity (%)
1	ITS	*Cladosporium cladosporioides*	MK265717.1	100.00
2	Actin	*Cladosporium halotolerans*	OQ185000.1	99.43
3	TEF1	*Cladosporium halotolerans*	LC426379.1	98.5

The phylogenetic tree was constructed using Maximum Likelihood, and the percentage in which the related taxa clustered together is indicated next to the branches in *Figure 4*. The dendrogram analysis depending on the ITS sequence elucidates the hierarchical relationships among various *Cladosporium* species including *C. cladosporioides*, *C. halotolerans*, *C. sphaerospermum,* and *C. parahalotolerans*. The phylogenetic tree was

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resolved into seven clades, with bootstrap support values ranging from 11 to 61%. The fungal *Cladosporium* species was positioned within the first sub-clade of Clade One and showed a close relationship to *C. cladosporioides*. The Actin and TEF1 gene tree analysis revealed five to six clades, with the homology level of the endophytic *Cladosporium* species to other isolates of *C. halotolerans* ranging from 7% to 74%. The endophytic *Cladosporium* species found within the initial and secondary clades displayed a strong association with *C. halotolerans*. Overall, the endophytic *Cladosporium* species identified using three markers in this study was confirmed to be the isolate *C. halotolerans*. These molecular and phylogenetic analyses provided definitive species-level resolution and fulfilled our objective of accurately identifying the endophytic *Cladosporium* isolate as *C. halotolerans* using an integrative approach.

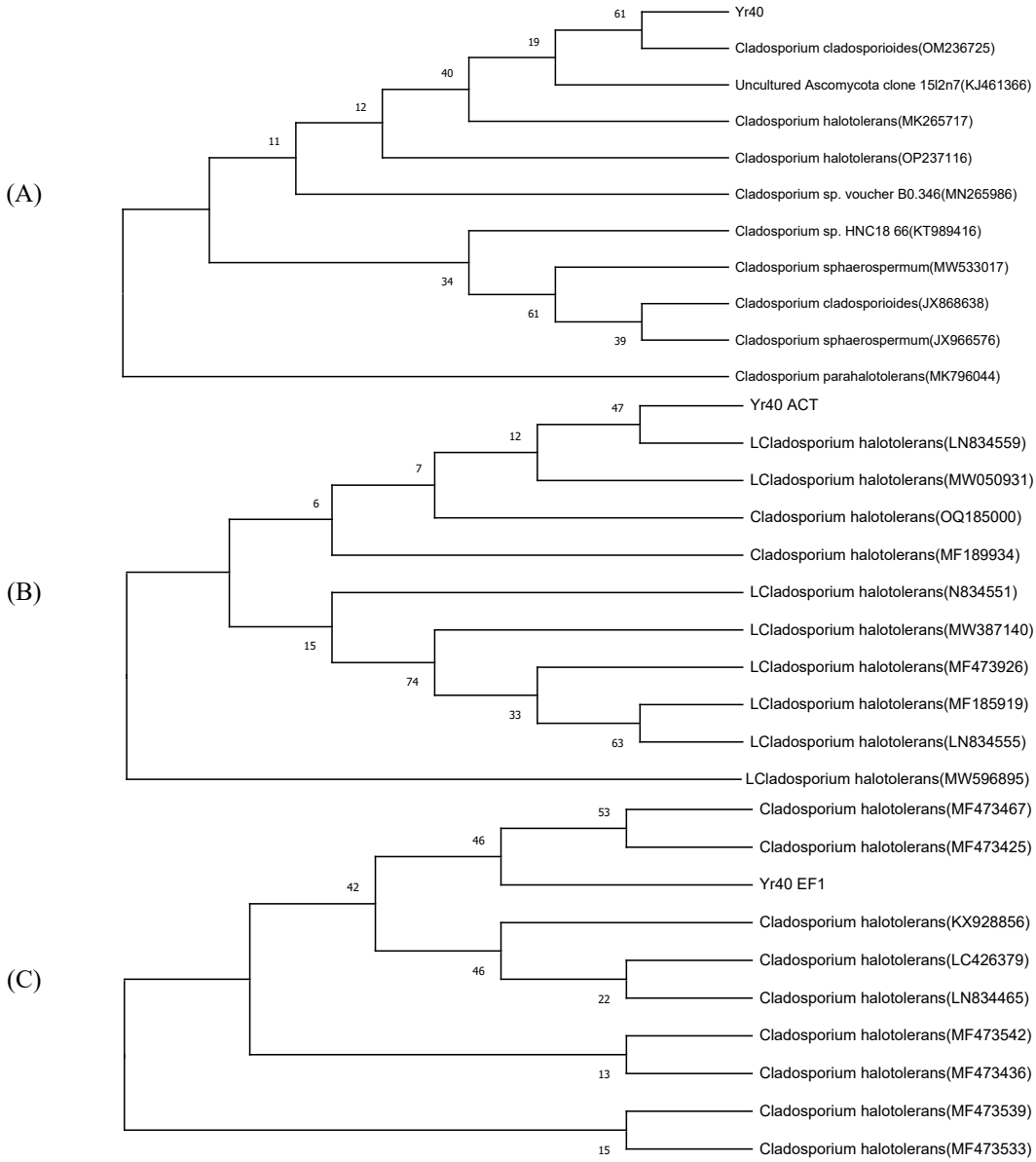

Figure 4. *Dendrogram showing phylogenetic analysis based on (A) ITS, (B) ACT and (C) TEF regions and NCBI GenBank database*

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Discussion

This study aimed to accurately identify an endophytic *Cladosporium* isolate through a combined morphological, physiological, and molecular approach. The discussion below interprets these results in relation to this objective. In this study, macro and micro morphological chaotizations were recorded to evaluate the selected endophytic *Cladosporium* species. Morphological identification of *Cladosporium* species is crucial for several reasons. Firstly, it aligns with traditional taxonomy, which has historically relied on morphological traits for classifying fungi within established frameworks. Additionally, morphological characteristics are indispensable for distinguishing closely related species, complementing the high-resolution data provided by molecular techniques. Traits such as colony color, conidial morphology, and conidiophore structure often exhibit species-specific features, aiding in accurate identification (Chinthani et al., 2020). Moreover, morphological traits are readily observable in the field or under a light microscope, enabling swift identification without the need for specialized equipment or molecular expertise. Furthermore, historical data from fungal collections and taxonomic literature are based on morphological characteristics, emphasizing the importance of maintaining consistency for the continuity of fungal taxonomy and facilitating comparisons across studies (Huang et al., 2018). Depending exclusively on morphology for species identification may lead to inaccuracies because of factors such as hybridization, cryptic speciation, convergent evolution, and phenotypic plasticity. The morphological traits of the isolate—particularly colony pigmentation, branched conidiophores, and lemon-shaped conidia—aligned closely with *C. cladosporioides* and *C. halotolerans*, indicating that morphology alone was insufficient for precise identification and required physiological and molecular confirmation.

Physiological studies of endophytic *Cladosporium* species including pH, temperature, and media composition are fundamental aspects related to species identification in fungi. By evaluating the growth response of fungi to these environmental variables, researchers can uncover species-specific traits and patterns that contribute to accurate identification and classification. Regarding its optimum growth conditions, it is important to note that different species of *Cladosporium* may have slightly different preferences. However, many strains of *Cladosporium* tend to thrive in conditions with moderate temperatures and slightly acidic pH levels. The optimum temperature for the growth of *Cladosporium* species, including *C. cladosporioides* and *C. halotolerans* is typically reported to be around 25°C (77°F) (Jiang et al., 2021). This temperature range is consistent with the mesophilic nature of many *Cladosporium* species, preferring moderate temperatures rather than extremes. In terms of pH, *Cladosporium* species generally prefer slightly acidic conditions. The optimum pH for growth varies among species but is often reported to be around pH 5 (Rajagopal et al., 2018). This preference for acidic conditions is common among many fungi and is likely related to the pH of their natural habitats, such as decaying organic matter or plant surfaces. Furthermore, it is noteworthy that *Cladosporium* species often exhibit robust growth on CZA and PDA media. Czapek Dox Agar and PDA are both commonly used media for culturing a wide range of fungi due to their nutrient-rich composition and ability to support vigorous fungal growth. In conclusion, *Cladosporium* species, including *C. cladosporioides* and *C. halotolerans*, generally exhibit optimal growth at a temperature around 25°C, a pH of approximately 5, and tend to thrive on CZA and PDA media. These conditions are reflective of their natural ecological niches and can vary slightly depending on the specific species and environmental factors. The identification of endophytic *Cladosporium* species can

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fluctuate due to factors like geographic location, host plant types, and environmental variables. In environments with elevated salt levels, such as coastal areas, saline soils, or regions affected by anthropogenic activities like irrigation with saline water like Jeddah city, xerotolerant *Cladosporium* species may be more prevalent. These species have adapted mechanisms to cope with osmotic stress caused by high salt levels, allowing them to colonize and thrive in such environments (Zalar et al., 2023). We believe that these physiological signatures helped differentiate the isolate from *C. cladosporioides*, supporting the integrative identification process.

The BLAST sequence analysis of *Cladosporium* species using ITS-rDNA indicated a 100% similarity match with *C. cladosporioides*. Also, the dendrogram analysis based on the ITS sequence clarifies the hierarchical relationships among several *Cladosporium* species. In previous studies, utilizing molecular universal markers like ITS is indeed broadly applicable for fungal identification and phylogenetic analysis across diverse taxa. These markers are commonly used due to their conserved nature across a wide range of fungal species. They are particularly valuable for assessing fungal community composition and diversity in environmental samples, providing insights into the richness and abundance of fungal taxa present. However, it is important to note that while universal markers like ITS are useful for broad-scale studies, they may lack the resolution needed for distinguishing between closely related species or identifying species-specific genetic traits (González et al., 2021). This limitation arises because universal markers target regions of DNA that are conserved across multiple species, leading to overlapping sequences among closely related taxa. As a result, these markers may not provide sufficient discriminatory power to differentiate between closely related species or identify genetic variations specific to particular taxa. Therefore, while universal markers like ITS are valuable tools for initial screening and broad-scale analysis of fungal diversity, researchers often complement their analyses with additional specific markers or sequencing techniques to achieve higher resolution and specificity, especially when studying closely related taxa or investigating species-specific traits (Yang et al., 2023; El-Dawy et al., 2021).

Molecular identification of endophytic *Cladosporium* species, utilizing ACT and TEF1 sequencing, revealed a similarity ranging from 98.51% to 99.43% with sequences of known species published in NCBI databases. The sequencing data provided confident confirmation that it belonged to *C. halotolerans.* Appling ACT and TEF markers for endophytic *Cladosporium* species identification provide numerous advantages. These markers offer enhanced resolution, enabling precise differentiation among closely related species. They are particularly valuable for discriminating between morphologically similar or cryptic species, overcoming challenges in traditional identification methods. Molecular techniques yield consistent and reproducible results, reducing subjectivity and ensuring reliability across different studies and laboratories. They also enable rapid identification, streamlining the process compared to labor-intensive traditional methods. Molecular markers facilitate the study of genetic diversity and population dynamics within *Cladosporium* species, offering insights into evolutionary relationships, population structures, and dispersal patterns. Additionally, they aid in identifying and describing new *Cladosporium* species by revealing unique genetic traits and their phylogenetic position within the genus (Yang et al., 2023). Although the ITS marker initially suggested similarity to *C. cladosporioides*, both ACT and TEF1 provided higher-resolution data that clearly grouped the isolate with *C. halotolerans*. This demonstrates the importance of combining universal and species-specific markers to resolve closely related *Cladosporium* taxa.

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For future research, the identification of the isolate as *C. halotolerans*, a species adapted to saline environments, is consistent with the environmental conditions of the collection site, supporting the ecological plausibility of the result. Indeed, *Cladosporium halotolerans* refers to the ability of certain species within the *Cladosporium* genus to withstand and thrive in high-salt environments. While *Cladosporium* species are commonly found in various habitats, including indoor and outdoor settings, not all of them possess the capability to tolerate high salt concentrations (Lee et al., 2023). We believe that understanding *C. halotolerans* is important for various reasons, including its potential applications in bioremediation of saline environments, agriculture in saline soils, and ecological studies focusing on habitats with high salt concentrations. Future studies should investigate the functional roles of *C. halotolerans* in planta, assess its distribution across diverse environments, and explore its biotechnological potential in saline agriculture.

Conclusion

This study successfully identified the endophytic isolate as *Cladosporium halotolerans* through an integrative combination of morphological, physiological, and multilocus molecular analyses, contributing to improved taxonomic resolution within the genus.". This multidisciplinary strategy is crucial for advancing our understanding of fungal diversity, ecological roles, and potential applications in various fields, including agriculture, medicine, and biotechnology. In conclusion, the confirmed identification of *C. halotolerans* enhances our understanding of salt-tolerant endophytes and highlights their potential applications in crop improvement, stress tolerance, and biotechnological processes.

Author contributions. All authors contributed equally to the writing of this paper. All authors have read and approved the final manuscript.

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Data availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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