

Research Article

Molecular identification and genetic variation of *Alternaria* species isolated from leaves of ornamental plants using Internal transcribed spacer (ITS) sequencing

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Abstract

Alternaria species are pathogens that cause significant economic losses in ornamental plants. The present study focused on the molecular identification and genetic diversity of *Alternaria* species, a common fungal genus that includes pathogens causing plant diseases. Accurate identification and understanding of the genetic relationships of *Alternaria* species are important for managing plant diseases. This study included 100 samples collected from several plant species: Ivy plant (*Convolvulus*), Chrysanthemum plant (*Chrysanthemum*), Infernal (*Bougainville*), Rubber plant (*Ficus*), Cardina flower (*Gardenia*) infected with fungi. All samples cultured on PDA medium and purified with the single-spore method, followed by Polymerase Chain Reactions PCR identification through internal transcribed spacer ITS1 and ITS4 gene and sanger or next-generation sequencing of positive samples to construct a phylogenetic tree. Preliminary results showed the presence of *Alternaria* in 23 of the 100 samples, which were confirmed by PCR. Genetic sequencing revealed 10 newly recorded isolates, which were registered with National Center for Biotechnology Information (NCBI) under the following names: *A. infectoria* strainWS1-iqOR053817.1, strainWS3-iqOR064285.1, strainWS5-iqOR064342.1, strainWS9-iqOR083596.1, strainWS10-iqOR083599.1, *A. angustivoidea* strainWS4-iqOR064286.1, *A. tenuissima* strainWS2-iqOR053945.1, strainWS7-iq OR083592.1, *Alternaria burnsii* strainWS8-iq, OR088248.1, *A. alternate* strain WS6-iqOR072637.1. These species were classified into groups based on their genetic patterns. This molecular study confirmed the association of various *Alternaria* species with ornamental plants and revealed the importance of ITS for diagnosing and identifying genetic diversity within the genus *Alternaria*.

Keywords: *Alternaria* species, Genetic diversity, Internal transcribed spacer (ITS) sequencing, Ornamental plant

INTRODUCTION

Fungi are highly diverse and ubiquitous organisms. It is estimated that the fungal kingdom comprises 2.2 to 3.8 million species, of which 2 to 3 million species remain unidentified. These organisms can live symbiotically or as saprophytes on organic materials (Jayawardena *et al.*, 2021; DeMers, 2022). Despite receiving relatively little attention, fungi play a crucial role in human life and have widespread applications in various fields. However, fungal diseases are considered a serious global health and economic threat, causing significant damage to agriculture and public health each year (Barzee *et al.*, 2021; Rokas, 2022; Qassim *et al.*, 2024b). In addition

to extensive economic losses, fungi pose a serious risk to human and animal health by producing mycotoxins (Altomare *et al.*, 2021; Qassim *et al.*, 2024a). One genus of fungi that is globally widespread and abundant in both natural and human-impacted ecosystems is *Alternaria* (Dang *et al.*, 2015). *Alternaria* species exhibit various modes of life. Most of these fungi thrive as saprophytes in soil and decaying plant materials. Certain species can cause human health issues such as respiratory diseases, skin conditions, and allergies. In plants, they act as necrotrophic pathogens, causing diseases that reduce product quality and lead to post-harvest decay (Schmey *et al.*, 2024). The most common *Alternaria* species include *A. alternata*, *A. tenuissima*

uissima, *A. arborescens*, *A. radicina*, *A. brassicae*, *A. brassicicola*, *A. malorum*, and *A. infectoria*, each of which poses significant challenges to humans and animals in various ways (Lee *et al.*, 2015; Al-Healy and Al-Tae, 2023; Salem-Bango *et al.*, 2023).

Accurately diagnosing fungal species, along with evaluating the diversity of their genes, is a fundamental step in understanding plant and human pathogens. This is essential to comprehend of the biological attributes of fungi and how they interact with their hosts (Leiminger *et al.*, 2013; Perez-Nadales *et al.*, 2014 ., Younis and Al-tae 2020). *Alternaria* is a genus comprising more than 50 species, both pathogenic and non-pathogenic, that share striking morphological characteristics. Diagnosing them with conventional morphology-based techniques is frequently laborious, time-consuming, and challenging, and often inadequate due to the high degree of variation in their physical attributes. Nowadays, after significant advances in molecular biology, researchers use various DNA-dependent techniques to determine the sequences of specific genetic regions for quick and precise identification of these fungi at the species level (; Somma *et al.*, 2019; Al-Nuaimy and Mulla Abed, 2021). Because of their wide compatibility and high degree of conservation, molecular-based methods, for example, ITS sequencing, provide a reliable means of recognising closely associated species and discovering phylogenetic relatedness (Rajammohan *et al.*, 2019; Saleem *et al.*, 2022; Almashhadani and Qassim, 2025a). Different plants are vulnerable to fungal infections. Ornamental plants are among the most susceptible plants to fungal contamination, particularly by various species of the genus *Alternaria* and their toxins, due to their fragile epidermis (Rocha *et al.*, 2022). This fruit, is considered as one has great importance to human wellbeing as it contain different biological compounds such as antioxidants, lycopene, and vitamin C. *A. infections* cause a significant financial damage to ornamental plants as a results of various diseases they are associated with which marked by black or brown spots on leaves, stems, and fruits, eventually result in reduced production and fruit quality (Bacha *et al.*, 2023; Haro *et al.*, 2023). At the molecular level, variability in pathogen populations is typically observed. The genetic makeup of many different organisms is currently possible to be described with the assistance of DNA markers. To rapidly examine genetic variation, the internal transcribed spacer (ITS) region and random amplified polymorphic DNA (RAPD) have been used to evaluate intra- and interspecific variation among various fungal species (Zelmat, L. *et al.*, 2021; Almashhadani and Qassim, 2025b). Considering the importance of identifying and differentiating *Alternaria* species, the present study aimed to conduct molecular recognition and genetic variability analyses of *Alternaria* species obtained

from infected ornamental plant leaves using Internal transcribed spacer (ITS) sequencing.

MATERIALS AND METHODS

Collection of samples, culture, and identification of fungi

The current study included collecting 100 samples of ornamental plants at a rate of 20 samples from each of: Ivy plant (*Convolvulus*), Chrysanthemum plant (*Chrysanthemum*), Infernal (*Bougainvillea*), Rubber plant (*Ficus*), and Cardina flower (*Gardenia*). In the City of Mosul/Iraq, for the period from November 2023 to February 2024. All samples were collected and transferred to the laboratory under sterilized conditions.

Morphological identification

Upon arrival at the laborator, the plants were washed with distilled water to remove surface contaminants. The infected areas were then excised and disinfected using a 1% sodium hypochlorite solution. The disinfected samples were directly cultured onto Potato Dextrose Agar (PDA) and incubated for 7 days at a temperature (2 ± 25) °C with daily observation during the incubation period (Al-Healy and Al-Tae, 2023). After fungal growth was observed, the single-spore method was used to purify the isolates, yielding pure cultures for further analysis. This identification process was carried out in accordance with the *Alternaria* Identification key (Ellis, 1971 ; Barnett and Hunter, 1972) .

Molecular identification

DNA extraction from the isolates

All previously identified *Alternaria* fungal isolates were subjected to DNA extraction. DNA was meticulously extracted from the mycelium of 10 distinct fungal samples using the Geneaid Genomic DNA Mini Kit (Cat. No. GT100;Kit provided by Geneaid/Taiwan).

Amplification process

The highly conserved ITS region in fungi was detected using PCR technique. The following universal primer (F: TGAATCATCGACTCTTTGAACGC, R: TTTCTT TTCCTCCGCTTATTGATAT) and thermal program shown in (Table 1) were used. The process was carried out by adding 4 microliters (100 nanograms) of template DNA and 1 microliter (10 pmol) of each gene-specific primer to the master mix. The amplification products then electrophoresised on a 2% agarose gel.

Sequencing

The sanger or next-generation sequencing (NGS) method was used to sequencing the amplified product. The obtained sequences were compared with the entire sequences available in the NCBI GenBank database to

identify homology. This is a reliable approach for biological identification and categorisation because of the ITS1 region's high species diversity, sensitivity, and the availability of large databases. (Bellemain *et al.*, 2010; Fathy *et al.*, 2023, Almashhadani, and Qassim, 2025b).

Genetic diversity analysis using ISSR markers

To assess the genetic diversity of *Alternaria* spp. isolates, ISSR markers were utilized. five primers: primer1 (OPP-02 5'TCGGCACGCA 3') , primer2(OPB-03 5'CATCCCCCTG 3') , primer3 (OPQ-05 5'CCGCGTCTTG 3') , primer4 (OPR-07 5'ACTGCCTGA 3') , primer5 (OPO-14 5'AG-CATGGCTC 3') , known for their high reproducibility and polymorphism , were selected for the analysis. The PCR process was carried out using the thermal program shown in (Table 2) The amplification products obtained were examined on a 1.6% agarose gel, and the genetic diversity among the isolates was assessed. For data analysis, a genetic similarity matrix was calculated using the Simple Matching (SM) coefficient, and a dendrogram was subsequently constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in NTSYSpc (version 2.02). Polymorphic Information Content (PIC) values were also calculated to assess primer efficiency.

Phylogenetic tree

Using the BLASTn The Nucleotide Basic Local Alignment Search Tool (BLASTn) program (Altschul *et al.*, 1990), the entered sequences were compared to the whole sequences which are available on the NCBI GenBank database to find homology. The phylogenetic tree was constructed by bootstrap (100X) analysis using MEGA-11 software, and genetic distances were calculated using the Kimura 2-parameter (K2P) model

(Tamura *et al.*, 2021).

RESULTS AND DISCUSSION

Isolation and morphological identification

Among a total of 100 samples initially collected from various ornamental plants, 23 isolates were morphologically identified as *Alternaria* spp. Based on their macroscopic and microscopic characteristics, such as the size, color, and arrangement of conidia, the presence or absence of surface ornamentation, the length and shape of conidiophores, and other morphological features, For molecular validation, 10 representative isolates were selected for DNA sequencing and further genetic diversity analysis using ISSR markers.

Molecular identification of *Alternaria* spp. fungi isolates

Molecular diagnosis was performed for all samples that, by phenotypic diagnosis, were identified as belonging to the genus *Alternaria* and confirmed, as the results were identical to the previous results. After examination under an ultraviolet light source, it was found that each of the isolates contained a single, undispersed band, which is evidence of presence of *Alternaria* in the sample. as can be seen in (Fig. 1)

DNA sequencing

Genetic sequencing revealed ten new fungal strains belonging to the genus *Alternaria*, as can be seen in (Table 3) These strains appear for the newly recorded isolates, and all have been registered in the NCBI strain bank. The results indicated that the fungi had the highest identity, consistent with the isolates matched in the Basic Local Alignment Search Tool (BLAST) database.

Table 1. Thermal program used for amplification of internal transcribed spacer (ITS) region

No.	Stage	Temperature	Time	Cycle number
1	Initial denaturation	95	5 min.	1
2	Denaturation	95	45 sec.	
3	Annealing	55	1 min.	35
4	Extension	72	1 min.	
5	Final extension	72	7 min.	1

Table 2. Thermal program used for amplification of ISSR markers

No.	Stage	Temperature	Time	Cycle number
1	Initial denaturation	94	3 min.	1
2	Denaturation	94	55 sec.	
3	Annealing	55	1 min.	35
4	Extension	72	1 min.	
5	Final extension	72	7 min.	1

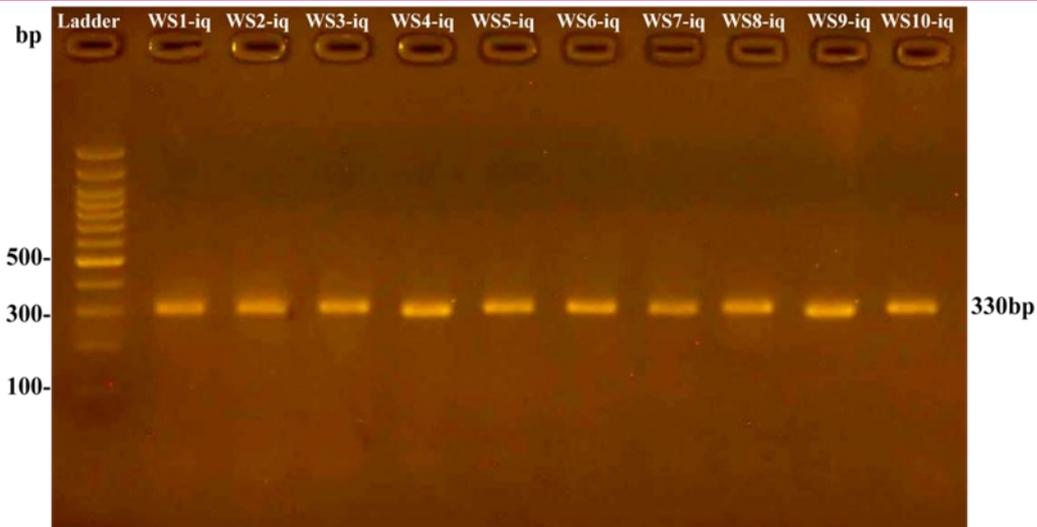


Fig. 1. Showing the PCR reaction result for the ITS region of fungal samples, with a reaction size of 330 pb, and carried over to a 2% agarose gel

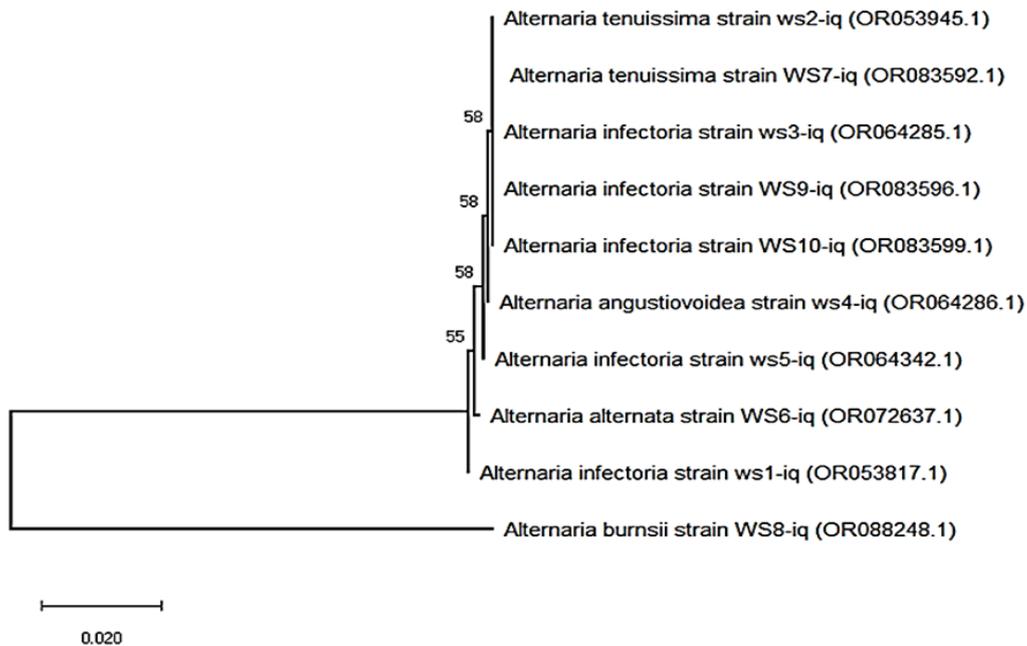


Fig. 2. Neighbor-joining phylogenetic tree showing the relationship among the strains of *Alternaria* species of included in this study based on ITS sequences using MEGA-11 software. The percentage of replicate trees in which the associated strains clustered together in the bootstrap test (100 replicates) are shown next to the branches

Representation of their phylogenetic relationships

Fungi with their vast diversity play crucial roles in ecosystems and human activities, both positively and negatively. *Alternaria* is a genus of fungi that has garnered notable attention because of its effects on food safety and human and plant health. Therefore, it is necessary to study the species of this genus at the molecular level and to recognise their genetic variability, particularly those that harm key ornamental plants, to comprehend their ecology, pathogenic abilities, and the possibility of mycotoxin production (Carvalho *et al.*, 2019; Lodovica and Wardlow, 2002). In this study, genetic diversity of several *Alternaria* species obtained from various infect-

ed ornamental plants leaves was investigated using ITS sequencing and ISSR markers. The results provided important new insights into the distribution, phylogenetic relationships, and genetic variability of species in this genus. This diversity could be linked to differences in host plants and environmental conditions among the nurseries where samples were collected. The fact that some isolates from different hosts clustered together suggests a potential gene flow or the transfer of plant materials between nurseries. On the other hand, the distinct grouping of other isolates might indicate specific adaptation to their respective host plants.

ITS sequencing technology can be highly reliable in

Table 3. Local Iraqi strains of *Alternaria* spp.

Name	Scientific name	Accession No.
WS1-iq	<i>Alternaria infectoria</i>	OR053817.1
WS2-iq	<i>Alternaria tenuissima</i>	OR053945.1
WS3-iq	<i>Alternaria infectoria</i>	OR064285.1
WS4-iq	<i>Alternaria angustivoidea</i>	OR064286.1
WS5-iq	<i>Alternaria infectoria</i>	OR064342.1
WS6-iq	<i>Alternaria alternate</i>	OR072637.1
WS7-iq	<i>Alternaria tenuissima</i>	OR083592.1
WS8-iq	<i>Alternaria burnsii</i>	OR088248.1
WS9-iq	<i>Alternaria infectoria</i>	OR083596.1
WS10-iq	<i>Alternaria infectoria</i>	OR083599.1

identifying *Alternaria* and distinguishing evolutionarily similar species. Traditional recognition methods often fail to account for species with high morphological variation, and errors can occur (Adeyemo and Schmidt-Heydt., 2024; Saeed *et al.*, 2024). This study confirmed the ability to distinguish *Alternaria* species using ITS sequencing, even when morphological characters are inadequate. The ITS region is considered a perfect

marker for fungal diagnosis due to its preservation across taxa and its high variability. All of the studied species in this study (*A. alternata*, *A. tenuissima*, *A. infectoria*) and other species possess noteworthy significance due to their ability to cause crop damage and pose public health risks through their potential to excrete mycotoxins (Escrivá *et al.*, 2017; Ismail *et al.*, 2023).

The groups are classified according to the similarity (Fig. 2) into the following:

Group A: includes *A. tenuissima* and *A.infectoria*. These two species share a high level of similarity, indicating that could potentially share similar pathogenic mechanisms or ecological sources (Jayawardena *et al.*, 2021).

Group B: constitutes *A. infectoria* which forming a separate group, as it poses a distinguish genetic composition, which might suggests the possibility of specific adaptations that distinguish it from the other species within the same genus (Rokas., 2022).

Group C: due to the high degree of similarity the species *A. angustivoidea* and *A.infectoria* are lay in a single group. Such similarity highlights the hereditary homogeneity among these two species, which may be related to their identification in various plant hosts.

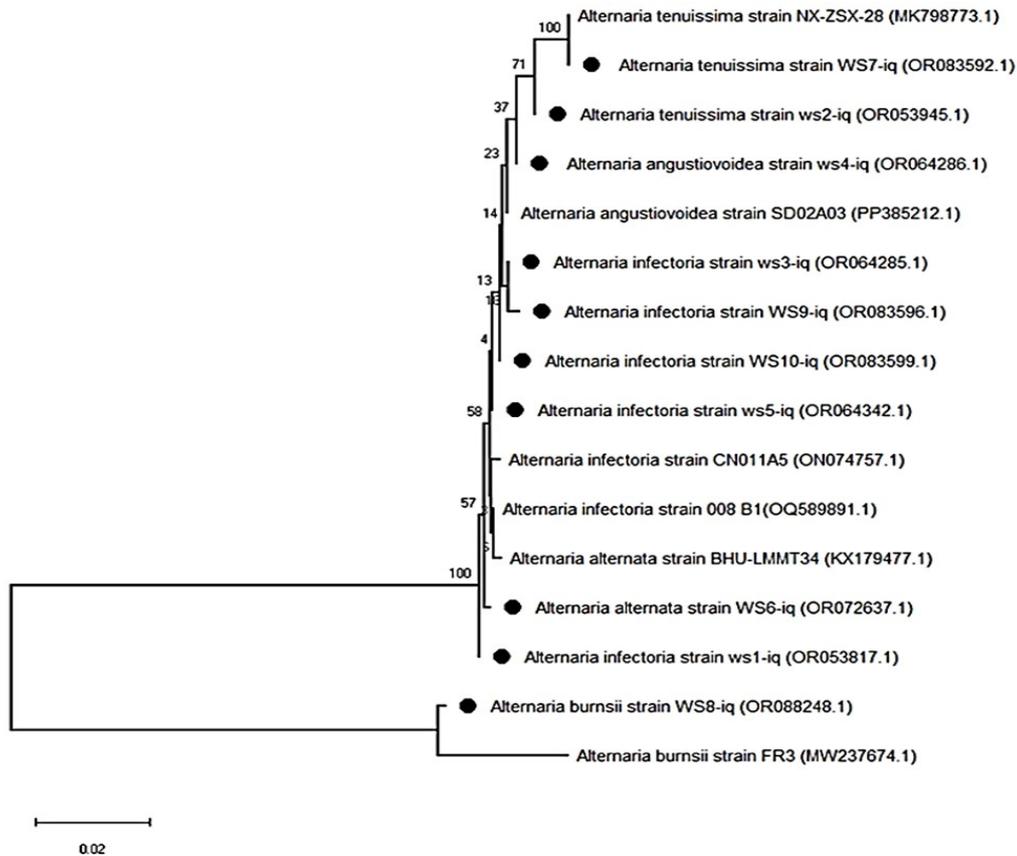


Fig. 3. Neighbor-joining phylogenetic tree showing the relationship between the strains of *Alternaria* species included in this study (indicated in black circles) with the closely related strains of *Alternaria* species that retrieved from NCBI based on ITS sequences using MEGA-11 software. The percentage of replicate trees in which the associated strains clustered together in the bootstrap test (100 replicates) are shown next to the branches

Table 4. Most closely related strains of local *Alternaria* spp. with *Alternaria* spp. recorded in the National center for biotechnology information (NCBI)

strains	Most closely strain	Accession No.	Similarity (%)
WS1-iq	<i>Alternaria infectoria</i> strain CN011A5	ON074757.1	99.2
WS2-iq	<i>Alternaria tenuissima</i> strain NX-ZSX-28	MK798773.1	99.57
WS3-iq	<i>Alternaria infectoria</i> strain 008_B1	OQ589891.1	99.52
WS4-iq	<i>Alternaria angustivoidea</i> strain SD02A03	PP385212.1	99.57
WS5-iq	<i>Alternaria infectoria</i> strain CN011A5	ON074757.1	99.53
WS6-iq	<i>Alternaria alternata</i> strain BHU-LMMT34	KX179477.1	99.56
WS7-iq	<i>Alternaria tenuissima</i> strain NX-ZSX-28	MK798773.1	100
WS8-iq	<i>Alternaria brunsii</i> strain FR3	MW237674.1	99.34
WS9-iq	<i>Alternaria infectoria</i> strain CN011A5	ON074757.1	99.53
WS10-iq	<i>Alternaria infectoria</i> strain CN011A5	ON074757.1	99.59

Group D: this cluster contain *A.infectoria* solely. Confirming the reality that this fungus possess a different genomic diversis that might affect its host range or pathogenic capabilities (Jayawardena *et al.*, 2021).

Group E: consist of *A. alternata* *A.infectoria*. Laying these species together may reflect genetic homology between them, suggesting possible hybridisation or shared ancestral history (Barzee *et al.*, 2021; Jayawardena *et al.*, 2021).

Group F: The last group includes *A. brunsii*. This is genetically distinguished from the other groups to fully comprehend its pathogenic capability and host specificity; further studies may be required.

It is important to note that while both ITS sequencing and ISSR markers classified the isolates into six groups, these classifications are distinct. The ITS groups (A–F) represent phylogenetic relationships at the species level, whereas the ISSR groups reflect intra-specific genetic variation among the isolates. Although some overlap exists, the ISSR clustering provides finer resolution of genetic diversity beyond that provided by ITS. The phylogenetic tree constructed from genomic information showed a clear relationship among *Alternaria* species. Classifying into separate groups supports the idea that *Alternaria* species exhibit considerable genetic variability, which can help clarify their environmental roles, pathogenicity, and potential management strategies in agricultural settings. Further studies on these groups could explore how specific environmental conditions or host plants influence the genetic divergence observed in these species.

Phylogenetic tree analysis

presents a comprehensive overview of the most closely related strains of *Alternaria* spp. (Fig. 3, Table 4) It includes important details, such as their accession numbers, which are crucial for understanding their genetic relationships. The results showed a great similarity between *A. tenuissima* and *A. angustivoidea*, and

also indicated that there is a separate group of *A. infectoria* with a Distinctive genetic pattern. There is an indication of a common evolutionary history between *A. infectoria*, *A. alternata*, and *A. brunsii*. These locally obtained strains were compared to those in the National Center for Biotechnology Information (NCBI) GenBank database. A thorough examination of the data reveals genetic similarities between the local strains and their corresponding counterparts in GenBank.

This high genomic variability detected in this study indicates that *Alternaria* species are highly adaptable to diverse environmental conditions and can evolve rapidly, which could make controlling fungal diseases more difficult. This spectrum of genetic diversity is consistent with earlier research on *Alternaria* species, which has reported identical levels of diversity across different geographic areas (Dettman and Eggertson., 2021). In a study conducted on some *Alternaria* species were obtained from ornamental plants, in which the researchers investigated the genetic variability and population structure of such species the results detected high degree of genetic variability among three species included *A.solani*, *A. alternata*, and *A.linariae*, with remarkable verities according to the geographic areas and species of plant host (Dettman *et al.*, 2023). Another study by Ozkilinc e *et .al.* (2018) also investigated species divisions within the genus *Alternaria*. They noticed that whereas some pose distinct host specialisation and species borders, others exhibit higher genetic variability and affect a wider variety of hosts (Taha and Ramadan 2017).

In this study, we relied on ISSR markers to assess genetic diversity, a method commonly used to examine fungal genetic variation. While ITS sequencing and ISSR markers provided valuable insights into genetic diversity and isolate identification, it is important to acknowledge their limitations within the complex *Alternaria* genus. To achieve more robust species-level delineation and confirm potential novel genetic groups,

future studies could incorporate additional multilocus markers, such as glyceraldehyde-3-phosphate dehydrogenase (gpd), Alt-a1, or endopolygalacturonase (endoPG). This would provide higher phylogenetic resolution and further validate the current species assignments. The findings revealed that the isolates grouped into discrete genetic groups, confirming that the species comprise a variety of genetic groups. Based on genetic similarity, phylogenetic analysis demonstrated that the isolates were classified into six groups. Despite some isolates sharing similar intermediate attributes, perhaps due to hybridisation or considerable morphological flexibility, these clusters mainly complemented morphological identification. The observed variability among species at the molecular level also suggests that various strains within a species may exhibit substantial differences in pathogenicity and virulence. Such diversity is significant, especially in plant disease control, as certain strains may be more virulent or able to overcome host defences (Li *et al.*, 2022; Hmood and Qassim, 2023). Understanding the genetic makeup of the *Alternaria* genus is vital for developing targeted control strategies and enhancing disease management practices in ornamental plants (Muhammadi and Bahramikia, 2019; Daughtrey and Buitenhuis, 2020). Moreover, the present phylogenetic analyses helped elucidate the ancestral relatedness among *Alternaria* species. The discrete genetic clusters identified in the phylogenetic tree correspond to several evolutionary divisions, which may reflect differences in environmental conditions or host preferences. This provides valuable information for further studies on the development of fungal pathogens and their interactions with host plants (Armitage *et al.*, 2020; Dettman *et al.*, 2023; Al-Nuaimy *et al.*, 2025). Future studies on the pathogenicity and resistance mechanisms of *Alternaria* species could be guided by the obtained phylogenetic tree, which also provides a better understanding of their ancestral history. Although this study identified several *Alternaria* species, the lack of pathogenicity tests (Koch's postulates) means these isolates are considered associated with the symptoms. Future studies should focus on confirming their specific causal roles. It is important to acknowledge that our results are specific to the isolates collected from Mosul, Iraq. The future research involving a wider range of ecological zones is suggested to confirm these patterns globally.

Conclusion

This molecular study successfully demonstrated the effectiveness of ITS-based PCR amplification and phylogenetic analysis for accurate identification and genetic characterization of *Alternaria* species associated with ornamental plant diseases. The research revealed sig-

nificant findings regarding the diversity and distribution of *Alternaria* pathogens across different host plants. The research revealed significant findings regarding the diversity and distribution of *Alternaria* pathogens across different host plants. The distinct genetic groups suggests potential variations in pathogenicity and fungicide sensitivity. Therefore, management strategies should be tailored to the specific genetic profile of the prevalent strains in each region. Future research should focus on pathogenicity tests and mycotoxin profiling to fully understand the functional roles of the identified isolates.

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Conflict of interest

The authors declare that they have no conflict of interest.

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