Plant Protection (Scientific Journal of Agriculture)

49(2), Summer, 2026

10.22055/ppr.2026.52451.1896

In silico and experimental evaluation of ITS1/ITS4 PCR for size-based discrimination between *Alternaria* and *Juniperus* amplicons

F. Shokouhifar*[1], M. Mamarabadi[1], B. Zaferanloo[2]

1. ***Corresponding Author:** Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran (shokouhifar@um.ac.ir)
2. Department of Chemistry & Biotechnology, School of Science, Computing and Engineering Technologies, Swinburne University of Technology, Melbourne, Australia

Received: 22 July2 026 Revised: 24 September 2026 Accepted: 11 October 2026

Abstract

The genus *Alternaria* comprises endophytic and opportunistic pathogenic fungi frequently associated with *Juniperus* trees. The use of ITS-based molecular markers for rapid and accurate differentiation, independent of culture-based and classical plant pathology identification methods, has received considerable attention. Because ITS sequences occur in both plants and fungi, reliably distinguishing detectable fragments from fungal and plant isolates is critical. However, the standard ITS barcode has limited resolution for species-level identification. In this study, we conducted an integrated *in silico* and experimental evaluation of the ITS region for discriminating *Alternaria* from *Juniperus* host tissues. For the *in silico* component, we constructed validated ITS-rDNA reference sequences for *Juniperus* spp. (4,231 bp) and *Alternaria alternata* (2,911 bp) using reference-guided assembly of publicly available sequences, including 193 records from Iranian *Juniperus* species and 8,050 *A. alternata* records. Alignment of the two reference sequences revealed a marked length difference: the complete ITS region (ITS1+5.8S+ITS2) is approximately 1,093 bp in juniper but only approximately 482 bp in *Alternaria*, representing a difference of more than 600 bp. *In silico* mapping of 14 universal fungal primers onto both reference sequences identified primer-binding sites and predicted amplicon sizes. Among the primer pairs, ITS1/ITS4 was selected for experimental validation because of its optimal amplicon size range, fully conserved binding sites, and the clear ~600 bp size difference between the host and fungal products. For the experimental component, PCR with ITS1/ITS4 primers on pure *Alternaria* DNA produced a single band of approximately 570 bp. In contrast, juniper leaf DNA produced a single band of approximately 1.2 kb, indicating a detectable size difference between the plant- and fungal-derived amplifiable fragments under the tested conditions. This simple, low-cost, culture-free method provides a preliminary size-based screening tool for detecting a fungal amplicon in juniper tissues. However, species identification of fungal isolates, and even confirmation of their assignment to a specific fungal genus, requires additional markers.

Keywords: Agarose gel electrophoresis, amplicon size, in silico analysis, Juniperus excelsa, PCR-based detection

Associate editor: S. Abbasi (Ph.D.)

Citation: Shokouhifar, F., Mamarabadi, M., & Zaferanloo, B. (2026). *In silico* and experimental evaluation of ITS1/ITS4 PCR for size-based discrimination between *Alternaria* and *Juniperus* amplicons. *Plant Protection (Scientific Journal of Agriculture), 49*(2), 161-189. https://doi.org/ 10.22055/ppr.2026.52451.1896

گیاه‌پزشکی (مجله علمی کشاورزی)
جلد ۴۹، شماره ۲، تابستان ۱۴۰۵

10.22055/ppr.2026.52451.1896

ارزیابی نرم افزاری و آزمایشگاهی PCR با آغازگرهای ITS1/ITS4 برای تفکیک مبتنی بر طول قطعه قابل تکثیر در *Alternaria* و *Juniperus*

فرهاد شکوهی فر*[۱]، مجتبی ممرآبادی[۱]، بیتا زعفرانلو[۲]

۱- * **نویسنده مسوول**: گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه فردوسی مشهد، مشهد، ایران

۲- گروه شیمی و بیوتکنولوژی، دانشکده علوم، محاسبات و فناوری‌های مهندسی، دانشگاه صنعتی سوینبرن، ملبورن، استرالیا

تاریخ دریافت: ۱۴۰۵/۰۴/۰۱ تاریخ بازنگری: ۱۴۰۵/۰۷/۰۲ تاریخ پذیرش: ۱۴۰۵/۰۷/۱۹

چکیده

جنس *Alternaria* شامل قارچ‌های اندوفیت و بیماری‌زای فرصت‌طلب است که اغلب با درختان ارس مرتبط هستند، استفاده از نشانگرهای مولکولی مبتنی بر منطقه ITS برای تمایز سریع و دقیق مستقل از روش های کشت و شناسائی کلاسیک بیماری شناسی گیاهی بسیار مورد توجه قرار گرفته است. نظر به حضور توالی ITS در فرمانروا گیاهان و قارچ ها، تمایز قابل اعتماد قطعات قابل ردیابی این منطقه در جدایه‌های قارچ و گیاه اهمیت بالائی دارد. در این مطالعه با ارزیابی درون‌رایانه‌ای توالی‌های مرجع ITS-rDNA معتبری برای گونه های *Juniperus* spp. (۴۲۳۱ جفت‌باز) و *Alternaria alternata* (۲۹۱۱ جفت‌باز) با استفاده از روش مونتاژ هدایت‌شده با توالی مرجع از داده‌های قابل دسترس عموم شامل ۱۹۳ رکورد از گونه‌های ارس ایران و ۸۰۵۰ رکورد از *A. alternata* ساخته شد. هم‌ترازی دو توالی مرجع اختلاف طول قابل‌توجهی را نشان داد به‌طوری‌که ناحیه کامل ITS (ITS1+5.8S+ITS2) در ارس حاوی حدود ۱۰۹۳ جفت‌باز و همین ناحیه در قارچ *Alternaria* دارای حدود ۴۸۲ جفت‌باز است که اختلافی بیش از ۶۰۰ جفت‌باز را نشان می دهد. مکانیابی رایانه‌ای از ۱۴ آغازگر عمومی قارچی بر روی هر دو توالی مرجع، جایگاه‌های اتصال آغازگرها را شناسایی و اندازه قطعات تکثیرشده را پیش‌بینی کرد. از میان جفت آغازگرها، ITS1/ITS4 برای تأیید آزمایشگاهی بر اساس محدوده اندازه مطلوب قطعه، جایگاه‌های اتصال کاملاً حفاظت‌شده و اختلاف اندازه واضح حدود ۶۰۰ جفت‌باز بین محصولات میزبان و قارچ انتخاب شد. در بخش آزمایشگاهی، واکنش زنجیره‌ای پلیمراز با آغازگرهای ITS1/ITS4 بر روی DNA خالص *Alternaria* یک باند حدود ۵۷۰ جفت‌باز و بر روی DNA برگ ارس یک باند حدود ۱/۲ کیلوباز تولید کرد که نشان‌دهنده اختلاف اندازه قابل تشخیص برای تفکیک دو قطعه قابل تکثیر از محتوی ژنومی گیاه و قارچ در شرایط آزمایش‌شده است. این روش ساده، کم‌هزینه و بدون نیاز به کشت، یک ابزار غربالگری اولیه مبتنی بر اندازه برای تشخیص یک آمپلیکون قارچی در بافت‌های ارس فراهم می کند، اما تشخیص گونه جدایه‌ها قارچی و حتی تایید تعلق آن‌ها به جنس قارچی خاص نیازمند نشانگرهای تکمیلی است.

کلید واژه‌ها: *ارس (**Juniperus excelsa**)، الکتروفورز ژل آگارز، اندازه آمپلیکون، تحلیل درون‌رایانه‌ای، تشخیص مبتنی بر PCR*

دبیر تخصصی: دکتر سعید عباسی

Introduction

Juniper trees (*Juniperus* spp.) are evergreen, drought-tolerant conifers widely distributed across semi-arid and mountainous regions of the Northern Hemisphere (Caudullo et al., 2017). In Iran, the *Juniperus excelsa* complex includes *J. excelsa* (M. Bieb.), *J. polycarpos* var. *polycarpos* (K. Koch), *J. polycarpos* var. *turcomanica* (B. Fedtsch.), and *J. seravschanica* (Kom.), which form valuable natural forests, particularly in the Hezar-Masjed region of northeastern Iran (Adams & Hojjati, 2012; Jafary et al., 2023). These forests are exposed to multiple environmental stresses, including shoot dieback, reduced regeneration, and climate-induced drought (Fatemi et al., 2018; Jafary et al., 2023; Zamani et al., 2025). Climate change is expected further to reduce suitable habitats for *J. excelsa* in Iran (Fatemi et al., 2018; Naghipour et al., 2021). Therefore, understanding the biotic and abiotic factors affecting juniper health, including the role of endophytic microorganisms, is important (Hoffman & Arnold, 2008).

Recent studies have documented the vulnerability of juniper forests in northern Khorasan to branch tip dieback (shoot blight). In a survey of 40 *J. excelsa* trees across three elevation gradients (low: 1596–1634 m; medium: 1778–1805 m; high: 1869–1895 m), the medium-elevation range was identified as a critical zone for disease outbreaks, with the highest disease index (mean = 3.75 out of 5), whereas elevations above 1869 m represented a relatively safe zone (mean disease index = 1.95) (Shokouhifar et al., 2026). The study also showed that elevation, rather than temperature alone, drove dieback severity, while summer precipitation was negatively correlated with the disease index ($\rho = -0.452$, $p = 0.003$), indicating that drought stress increases dieback severity. These findings indicate a need for rapid, simple, and reliable molecular tools to monitor potential pathogens such as *Alternaria* in vulnerable mid-elevation populations, where early detection could support targeted conservation and management interventions.

Endophytic fungi live asymptomatically within plant tissues (Lewis, 1924) and can improve host resistance to abiotic stress and pathogens by producing secondary metabolites (Singh & Sahni, 2025). Studies published between 2000 and 2025 have shown that junipers host a diverse range of endophytic fungi, mainly from the classes *Dothideomycetes*, *Sordariomycetes*, *Eurotiomycetes*, and *Agaricomycetes* (Abo Nouh et al., 2025; Al-Nadabi et al., 2026; Eo et al., 2018). Dominant genera include *Alternaria*, *Cladosporium*, *Phoma*, *Epicoccum*, *Aureobasidium*, *Penicillium*, and *Trichoderma*. Among these, *Alternaria* has consistently been reported as the most frequent endophyte in junipers worldwide (Gherbawy & Elhariry, 2016; Vigneshwari et al., 2020; Abo Nouh et al., 2025; Al-Nadabi et al., 2026). In addition, *Alternaria alternata* can act as an opportunistic pathogen, causing necrosis and shoot blight under favorable conditions (Nadziakiewicz et al., 2018; Behnke-Borowczyk et al., 2019; Mazur et al., 2019). The economic importance of this pathogen is well documented in agricultural systems, where it causes early blight and requires effective management strategies (Habibi et al., 2022). Additionally, host plant anatomical traits can significantly influence resistance to *Alternaria* infections (Nasr Esfahani et al., 2017). Therefore, rapid and accurate discrimination between fungal and host amplicons in juniper tissues, especially in the critical mid-elevation zone, may be useful for preliminary screening. However, detecting *Alternaria* specifically requires sequencing or more specific markers.

The internal transcribed spacer (ITS) region of ribosomal DNA (ITS1, 5.8S, ITS2) is the standard DNA barcode for fungi (Hillis

& Dixon, 1991). Universal primers ITS1 and ITS4 amplify this region across a wide range of fungal taxa (White et al., 1990). Because it is easy to amplify and is supported by extensive reference databases, ITS is widely used in fungal diversity studies, metabarcoding, and diagnostics (Tedersoo & Lindahl, 2016; Usyk et al., 2017). However, ITS has limited species-level resolution in many ascomycete genera, including *Alternaria* (Bradshaw et al., 2020). Previous studies have shown that ITS alone cannot discriminate closely related *Alternaria* species and that multi-locus approaches, for example using *TUB2*, *GAPDH*, and *EF-1α*, are necessary (Jasalavich et al., 1995; Woudenberg et al., 2015; Jia et al., 2024). Practical surveys have also confirmed that ITS-based identification of Ascomycota often requires complementary morphological and additional genetic data (Baturo-Cieśniewska et al., 2020). Recent phylogenomic studies have further shown that even genome-wide data may not fully resolve some species boundaries within section *Alternaria* (Dettman & Eggertson, 2021). As an alternative approach, whole-genome analysis has been used to identify species-specific target sequences for *Alternaria*, enabling rapid detection with CRISPR-Cas12a (Yang et al., 2025).

Despite these limitations, the ITS region may still be useful for detecting the presence of *Alternaria* in juniper host tissue, provided that a size difference exists between the host-derived and fungal amplicons. In plants, the entire ITS region (ITS1, 5.8S, ITS2) is typically 600–700 bp, whereas in *Alternaria* it is approximately 500–580 bp. This size difference could allow rapid screening on agarose gels. However, a systematic *in silico* and experimental evaluation of this approach, specifically for Iranian juniper species and *Alternaria* isolates from these hosts, is lacking. Moreover, the specificity of ITS primers against other common endophytic genera (*Fusarium*, *Penicillium*, *Trichoderma*) has not been tested in this plant-fungus system. A large-scale comparative study of foliar endophytes in disjunct tree species showed that host identity and local environment explain most of the variation in endophyte communities, while historical geographic isolation plays a minor role (Zhou et al., 2023). This finding suggests that geographic origin may influence the clustering of *Alternaria* isolates, an aspect considered in the broader context of this research. Therefore, the aims of the present study are: (1) to construct validated ITS reference sequences for Iranian juniper species (*J. excelsa*, *J. polycarpos*) and *A. alternata* using publicly available NCBI records; (2) to align and compare the two reference sequences to identify length differences and conserved regions; (3) to map universal fungal primers onto both reference sequences and predict amplicon sizes; and (4) to experimentally validate a PCR-based method for size-based discrimination between *Alternaria* and *Juniperus* amplicons under pure-template conditions. The following hypotheses are tested: (i) a size difference of ≥50 bp exists between the ITS amplicons of *Juniperus* and *A. alternata*, allowing gel-based separation; (ii) the universal ITS1/ITS4 primers can amplify both templates but produce products of distinct sizes; and (iii) although the ITS region is insufficient for species-level identification, it can produce distinguishable amplicon sizes from *Alternaria* and *Juniperus* DNA under the tested conditions, without confirming the presence of *Alternaria* in natural samples. Addressing these questions may provide a rapid, low-cost, and reasonably reliable method for size-based discrimination between *Alternaria* and *Juniperus* amplicons, supporting preliminary screening and future conservation applications after validation, particularly in

the critical mid-elevation zone (Shokouhifar et al., 2026).

Materials and Methods

Retrieval and filtering of ITS sequences from NCBI

All available ITS-rDNA sequences of *Juniperus* spp. and *Alternaria* spp. were retrieved from the NCBI nucleotide database using the search terms (*Juniperus* [Organism]) AND ("internal transcribed spacer" OR ITS) and (*Alternaria* [Organism]) AND ("internal transcribed spacer" OR ITS). For *Alternaria*, we further refined the search to include only sequences longer than 500 bp and sequences derived from *Juniperus* hosts where host information was available. We initially retrieved 643 ITS-rDNA records for the genus *Juniperus* worldwide. After filtering for sequences longer than 500 bp and removing low-quality and chloroplast-derived sequences, 483 records were retained. Among these, 193 accessions belonged to *Juniperus* species reported from various regions of Iran (Table 1). For *Alternaria*, the search focused on four species: *A. alternata*, *A. tenuissima*, *A. arborescens*, and *A. gaisen*, yielding 17,744 sequences, of which 15,231 belonged to *A. alternata*. After filtering for sequences ≥500 bp in length, 8,050 *A. alternata* sequences were retained for reference assembly. We downloaded all sequences in FASTA format with full GenBank annotations.

Construction of reference sequences

We constructed reference sequences by reference-guided assembly in CLC Genomics Workbench v21.05 (QIAGEN). For *Juniperus*, we selected the longest ITS sequence with complete annotations for SSU, ITS1, 5.8S, ITS2, and LSU (accession ON685361; *Juniperus navicularis*) as the initial reference. We used additional sequences (FJ948470, FJ948469, GU197870, GU139571, AF174627, AY521466, and EU277699) to verify the annotation boundaries. All 193 ITS records from Iranian *Juniperus* species (Table 1) were aligned to the reference sequence using reference-guided assembly, and a consensus sequence was generated that included all conflicts reported among *Juniperus* species from Iran. We used high-quality sequences (570 records after removing short and chloroplast-derived reads) to generate the final consensus. The resulting reference sequence (SUB16216725) was 4,231 bp long and included partial 18S, ITS1, 5.8S, ITS2, and partial 28S regions.

For *A. alternata*, we used the same approach with the longest, best-annotated records (accessions PQ217832, LC749799, AY154682, and OM337556) and 8,050 filtered sequences. The consensus sequence (SUB16216726) was 2,911 bp long. We annotated both reference sequences using ITSx on the Galaxy platform to define the region boundaries (partial 18S, ITS1, 5.8S, ITS2, and partial 28S). Because NCBI assembled these reference sequences from multiple records, it did not assign accession numbers. The full sequences are provided in supplementary Fig. S1 (*Juniperus*) and Fig. S2 (*A. alternata*).

BLAST validation of reference sequences

To confirm taxonomic identity and assess the integrity of the assembled reference sequences, we conducted independent BLASTN searches against the NCBI non-redundant nucleotide (core_nt) database using default algorithm parameters. The reference sequences for *Juniperus* spp. (4,231 bp) and *A. alternata* (2,911 bp) were used as separate queries. We filtered significant alignments using an expectation value (E-value) threshold of 0.0 and evaluated them by percent identity and query coverage. We validated taxonomy by analyzing the hierarchical distribution of the top 100 hits, from species-level to order-level matches, to assess phylogenetic consistency and identify outliers that could indicate database misannotation or contamination.

Alignment and comparison of reference sequences

We aligned the ITS reference sequences of *A. alternata* and *Juniperus* using CLC Genomics Workbench v21.05 to identify conserved and variable regions. The lengths of ITS1, 5.8S, and ITS2 were determined for each reference sequence, and the total length of the complete ITS region (ITS1+5.8S+ITS2) was calculated. We visualized the alignment schematically (Fig. 4) to show structural differences between the reference sequences, including insertions/deletions (indels) and conserved regions.

***In silico* mapping of universal primers**

A set of universal fungal primers targeting the ITS1 and ITS2 regions (Table 6) was compiled from the literature (Vilgalys & Hester, 1990; White et al., 1990; Gardes & Bruns, 1993; Wang et al., 2015; Tedersoo & Lindahl, 2016; Usyk et al., 2017; Tedersoo et al., 2018). Primer sequences and their 3′-end coordinates on the *Alternaria* and *Juniperus* reference sequences were determined using BLASTN and manual alignment in CLC Genomics Workbench. For each primer, we recorded mismatches with the reference consensus, paying particular attention to the 3′-terminal nucleotide because of its important role in DNA polymerase extension. The frequency of alternative nucleotides at each position was calculated based on coverage by the 8,050 *Alternaria* sequences, that is, the number of sequences covering each position rather than the total number of sequences. We applied the same mapping procedure to *Juniperus*.

Fungal isolate and plant material

The *Alternaria* isolate used in this study was previously obtained from symptomatic twigs of *J. excelsa* in the Hezar-Masjed forests (Khorasan-Razavi Province, Iran) and morphologically identified as *Alternaria* sp. based on colony characteristics and conidial morphology (Charesaz et al., 2025). The isolate, designated Iso-10/S2, was grown on potato dextrose agar (PDA) at 28 °C for 7 days before DNA extraction and morphological characterization (Fig. 7).

For the plant host control, healthy leaves and twigs were collected from a *J. excelsa* tree near Sorkhcheshmeh in the Hezar-Masjed region (coordinates: 37°1′36.28″N, 56°53′44.18″E; elevation: 1,778.6 m) (Fig. 8). The tree appeared visually healthy, with a fully green canopy and no visible signs of dieback or necrosis. Leaf and twig samples were surface-sterilized with 70% ethanol, rinsed with sterile water, and subsequently used for DNA extraction.

DNA extraction

For the pure *Alternaria* isolate, we harvested mycelium from 7-day-old PDA cultures, freeze-dried it, and ground it in liquid nitrogen. We extracted DNA using a modified CTAB method (Doyle & Doyle, 1990). For plant samples, we ground approximately 100 mg of juniper leaves and twigs in liquid nitrogen and extracted total genomic DNA using the same CTAB buffer method. We measured DNA concentration and purity using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and assessed DNA integrity by agarose gel electrophoresis. We stored purified DNA at –20 °C until use.

PCR amplification and gel electrophoresis

PCR was performed using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). Each 25 µL reaction contained 12.5 µL of 2× PCR Master Mix (ApplChem, Denmark), 1 µL of each primer (10 µM), 2 µL of template DNA (10–50 ng), and nuclease-free water to a final volume of 25 µL. PCR cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, with a final

extension at 72 °C for 10 min. Each run included a negative control without template DNA. Amplicons (5 µL) were separated on 1.5% agarose gels in 1× TAE buffer at 85 V for 45 min, stained with GelRed (1 µL dye per 30 mL gel), and visualized under UV light using a gel documentation system. We used a 1 kb DNA ladder as a size marker.

Data availability

All ITS sequences used in this study are available from NCBI GenBank under the accession numbers listed in Table 1 for *Juniperus* and Table 2 for *Alternaria*. The constructed reference sequences were submitted to NCBI under submission IDs SUB16216725 (*Juniperus*) and SUB16216726 (*A. alternata*). However, under current NCBI policies, these assembled reference sequences have not been released as independent database entries. Therefore, the full-length annotated sequences are provided in supplementary Fig. S1 (*Juniperus*) and Fig. S2 (*A. alternata*) in FASTA format. Alignments are available upon request. We did not include phylogenetic trees in this study and will present them elsewhere.

Results

Workflow for developing PCR-based markers to track *Alternaria* in *Juniperus* plants

The primary objective of this study was to develop a simple, reliable, and low-cost PCR-based method for size-based discrimination between *Alternaria* and *Juniperus* amplicons by exploiting the difference in ITS amplicon size between the fungal endophyte and its plant host. To achieve this, we followed a stepwise approach comprising: (i) construction and validation of ITS reference sequences for *Juniperus* spp. and *Alternaria alternata*; (ii) alignment and comparison of the two reference sequences to identify length differences and conserved regions; (iii) *in silico* mapping of universal fungal primers to predict amplicon sizes and assess primer-binding efficiency; and (iv) experimental PCR validation using pure DNA templates.

Fig. 1 summarizes the study workflow. We retrieved ITS sequences from NCBI, filtered them, and assembled them into high-quality reference sequences using CLC Genomics Workbench v21.05. The *Juniperus* reference sequence (4,231 bp, SUB16216725) was constructed from 193 records belonging to Iranian *Juniperus* species, whereas the *A. alternata* reference sequence (2,911 bp, SUB16216726) was assembled from 8,050 sequences. We validated both reference sequences by BLASTing them against the NCBI core_nt database. Alignment of the two reference sequences revealed a marked length difference: the complete ITS region (ITS1+5.8S+ITS2) was approximately 1,093 bp in juniper but only approximately 482 bp in *A. alternata*, a difference of more than 600 bp. We used this difference to predict distinct amplicon sizes for the commonly used ITS1/ITS4 primer pair: approximately 570 bp for *Alternaria* and approximately 1.2 kb for juniper.

In silico mapping of 14 universal primers onto both reference sequences confirmed that all primers had perfect or near-perfect 3′-end matches with *A. alternata*, with rare SNPs occurring at frequencies below 0.1%. On the *Juniperus* reference sequence, all forward primers except ITS1-F had perfect 3′-end matches. ITS1-F showed a mismatch at its 3′ end in juniper (A vs. G), which may prevent host amplification and make the ITS1-F/ITS4 pair a promising candidate for reducing host co-amplification, although it may still amplify other fungi. Finally, experimental PCR with ITS1/ITS4 primers on pure *Alternaria* DNA and juniper leaf DNA produced single bands of approximately 570 bp and approximately 1.2 kb, respectively. This confirmed that the approximately 600 bp size difference allows clear discrimination

between the two amplicons under the tested conditions, but does not permit specific identification of *Alternaria* on a standard agarose gel. Together, these results support a practical, culture-free preliminary screening method for detecting a fungal amplicon in juniper tissues, but not for confirming *Alternaria* colonization.

Construction and validation of ITS-rDNA reference sequences

Sequences retrieved from NCBI

We conducted a systematic search of the NCBI database to obtain reliable reference sequences for juniper species occurring in Iran, mainly *J. excelsa* and *J. polycarpos*, and for the fungal species *A. alternata*, the endophyte most frequently reported from juniper. We initially retrieved 643 ITS-rDNA records for the genus *Juniperus* worldwide. After filtering for sequences longer than 500 bp and removing low-quality and chloroplast-derived sequences, 483 records were retained. Of these, 193 accessions belonged to *Juniperus* species reported from different regions of Iran (Table 1). These sequences were not necessarily obtained from plants collected in Iran; rather, they represented species whose occurrence in Iran has been confirmed (Kasaian et al., 2011; Adams & Hojjati, 2012; Hojjati et al., 2018).

The geographic region column indicates the origin of the NCBI records and may therefore include sequences from specimens collected outside Iran. For example, *J. excelsa* records include sequences from the USA (BAYLU herbarium), whereas *J. seravschanica* records are derived mainly from Oman and Iran. The reported length ranges are based on ITS sequences available in NCBI and may vary with the completeness of the ITS region in each accession. For the genus *Alternaria*, the search focused on four species: *A. alternata*, *A. tenuissima*, *A. arborescens*, and *A. gaisen*. This search returned 17,744 sequences, of which 15,231 belonged to *A. alternata* (Table 2). After filtering for sequences ≥500 bp, 8,050 *A. alternata* sequences were retained for reference assembly. The lengths of the *Alternaria* ITS sequences ranged from 500 to 2,338 bp.

Reference sequence construction for *Juniperus* spp.

The longest juniper ITS sequences were selected based on the presence of complete annotations for the SSU, ITS1, 5.8S, ITS2, and LSU regions. The longest and most comprehensively annotated sequence, ON685361 (*J. navicularis*), was selected as the initial reference. We used additional sequences (FJ948470, FJ948469, GU197870, GU139571, AF174627, AY521466, and EU277699) to confirm the annotation boundaries.

All 193 ITS records from Iranian *Juniperus* species were aligned to the reference sequence by reference-guided assembly in CLC Genomics Workbench v21.05 (QIAGEN). After removing short and chloroplast-derived reads, we used 570 high-quality records to generate the consensus sequence. The resulting reference sequence (SUB16216725) was 4,231 bp long and contained a partial 18S gene at positions –2276 to –719, ITS1 at positions –718 to –1, the 5.8S gene at positions 1 to 157, ITS2 at positions 158 to 375, and a partial 28S gene at positions 376 to 1948 (Table 3, Fig. 2). Nucleotide positions were numbered relative to the first base of the 5.8S region, designated as +1. The complete reference sequence is provided in Supplementary Fig. S1.

Reference sequence construction for *A. alternata*

Using the same approach, a reference ITS-rDNA sequence was constructed for *A. alternata*, the endophytic fungus most frequently reported from juniper trees. Among approximately 8,050 *A. alternata* ITS sequences available in NCBI (Table 3), the longest and best-annotated records, with accession numbers PQ217832, LC749799,

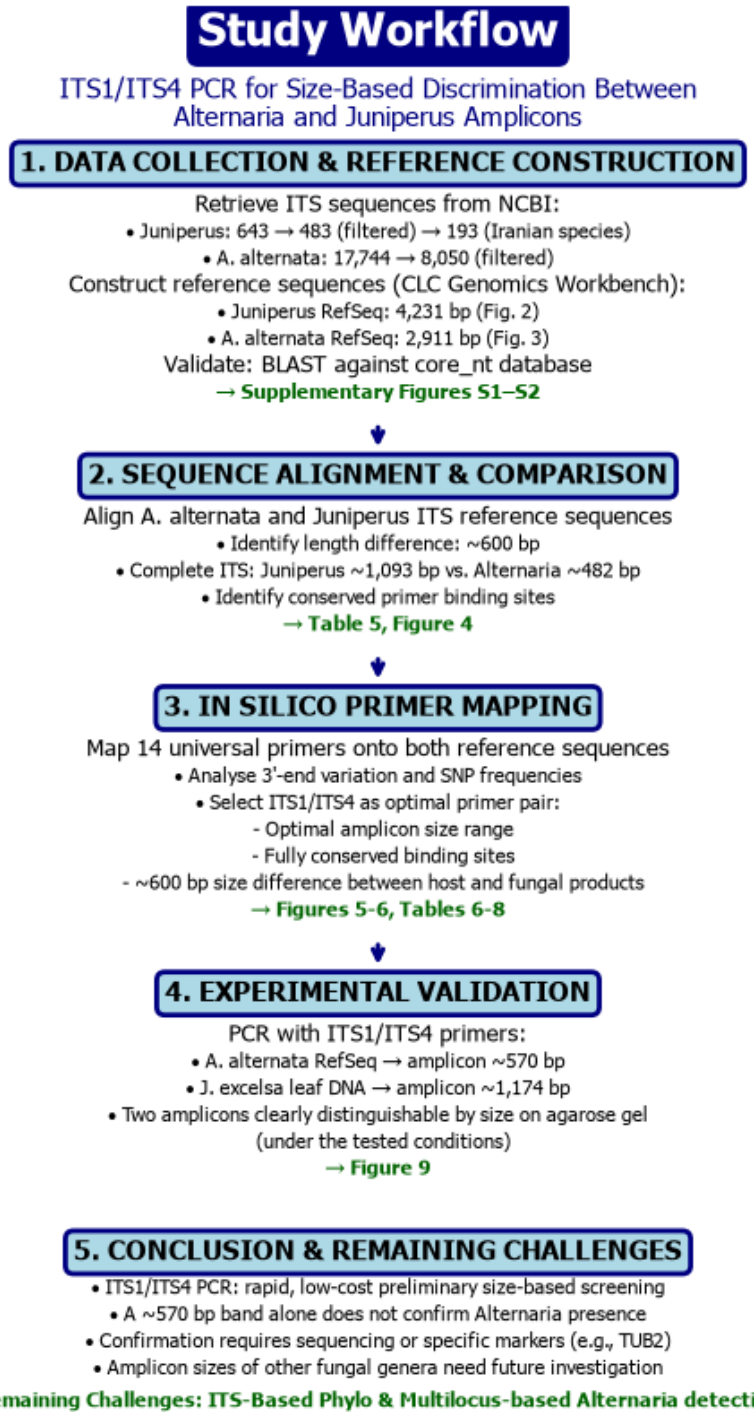


Figure 1. Schematic workflow of the study. The diagram summarizes the five main phases of the research. (1) Data collection and reference construction: ITS sequences were retrieved from NCBI (*Juniperus*: 643 initial records → 483 filtered → 193 from Iranian species; *A. alternata*: 17,744 records → 8,050 filtered). Reference sequences for *Juniperus* (4,231 bp, Fig. 2) and *A. alternata* (2,911 bp, Fig. 3) were assembled using CLC Genomics Workbench v21.05 and validated by BLAST against the core_nt database (Supplementary Figs. S1–S2). (2) Sequence alignment and comparison: Alignment of the two reference sequences showed a length difference of approximately 600 bp, with the complete ITS region measuring approximately 1,093 bp in juniper and approximately 482 bp in *Alternaria* (Table 5). (3) *In silico* primer mapping: Fourteen universal fungal primers were mapped onto both reference sequences to identify conserved regions and variation at the 3′ ends of the primers (Figs. 5–6, Tables 6–8). (4) Experimental validation: PCR using ITS1/ITS4 primers produced a

single band of approximately 570 bp from pure *Alternaria* DNA and a single band of approximately 1.2 kb from juniper leaf DNA. This approximately 600 bp difference allowed clear gel-based discrimination between the two amplicons under the tested conditions (Fig. 9). (5) Conclusions and remaining challenges: PCR with ITS1/ITS4 provides a rapid and low-cost method for preliminary size-based discrimination between *Alternaria* and *Juniperus* amplicons, but it cannot distinguish fungal genera or species. Future studies should evaluate ITS amplicon characteristics in other fungal genera and develop additional markers for species-level discrimination within *Alternaria* and confirmation of its presence. The diagram provides an overview of the combined bioinformatics and molecular approaches used to develop the PCR-based screening method for size-based discrimination between *Alternaria* and *Juniperus* amplicons in juniper tissues.

Table 1. Summary of ITS-rDNA sequences retrieved from NCBI for *Juniperus* species reported to occur in Iran, based on 193 accessions

***Juniperus* Species**	**Length (bp)**	**Records**	**Geographic Region**
J. communis	400 – 604	12	Iran, USA, Canada, Europe
J. communis var. oblonga	~400 – 604	5	Iran
J. communis var. saxatilis	~400 – 604	5	Iran
J. deltoides	1101 – 1282	17	Italy, Greece, Mediterranean
J. excelsa †	953 – 1315	27	Iran, USA (BAYLU)
J. foetidissima	1270 – 1271	6	Iran
J. oxycedrus	291 – 1275	13	Iran, USA, Mediterranean
J. polycarpos (unspecified)	1042 – 1292	5	Iran, USA (BAYLU)
J. polycarpos var. polycarpos	1042 – 1292	13	Iran, USA (BAYLU)
J. polycarpos var. turcomanica	1042 – 1292	13	Iran, USA (BAYLU)
J. sabina	441 – 1270	42	Iran, USA, Europe
J. seravschanica	471 – 512	35	Iran, Oman (based on isolates)
Total		193	

† **Taxonomic note:** The identification of *J. excelsa* in Iran remains contested. Several studies suggest that populations assigned to this species may instead represent regional variants within the *J. polycarpos* group, indicating the need for further integrative taxonomic investigation (Kasaian et al., 2011; Adams & Hojjati, 2012; Hojjati et al., 2018).

Table 2. Summary of ITS-rDNA sequences retrieved from NCBI for selected *Alternaria* species

Species	**Seq L. (bp)**	**Records**	**hosts**	**Geographic region**
A. alternata	500 – 2338	8051	apple, tomato, soil, air, etc.	China, USA, Japan, South Africa, Iran
A. tenuissima	500 – 1395	5097	grape, cereals, tomato, etc.	China, USA, South Africa, Iran, Iraq
A. arborescens	240 – 1501	492	grape, tomato, etc.	China, USA, South Africa, Italy, Iraq
A. gaisen	371 – 583	115	Ornamental plants and fruits	Japan, China, South Africa, Netherlands

AY154682, and OM337556, were selected and aligned. The resulting consensus sequence was 2,911 bp long and included a partial 18S gene at positions –958 to –165, ITS1 at positions –164 to –1, the 5.8S gene at positions 1 to 157, ITS2 at positions 158 to 318, and a partial 28S gene at positions 319 to 1950 (Fig. 3 and Table 4). We numbered nucleotide positions relative to the first base of the 5.8S region, designated as +1. We verified each region's boundaries using ITSx on the Galaxy platform, and the output fully matched the annotated positions.

BLAST-based validation of reference sequences

To confirm the taxonomic identity and integrity of the assembled ITS reference sequences, we performed separate BLASTN searches against the NCBI core_nt database.

***Juniperus* spp. reference sequence**

BLAST analysis of the 4,231 bp *Juniperus* spp. ITS reference sequence produced a single top hit with *J. navicularis* (accession ON685361.1), showing 99.39% identity over 100% query coverage (Max Score = 7486, E-value = 0.0). Apart from one anomalous alignment, all other significant hits (E-value = 0.0) belonged to the order Cupressales. We identified no additional congeneric matches beyond *J. navicularis*. However, sequences from *Chamaecyparis* and *Thuja* (Cupressaceae) showed 91.8–92.9% identity over full query coverage, while *Amentotaxus* (Taxaceae) showed approximately 93.4% identity with lower query coverage (84%), reflecting indel polymorphisms within the ITS spacers. One outlier alignment with *Dracaena sanderiana* (Asparagaceae) showed 99.27% identity over full query coverage. Because this match was phylogenetically inconsistent with the remaining results, we considered it a likely database misannotation. The other top 100 alignments formed a consistent Cupressaceae/Cupressales cluster. Overall, these results support placement of the query within Cupressaceae, with a strong affiliation to the genus *Juniperus*.

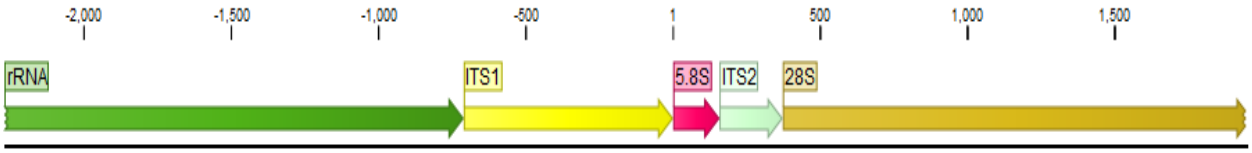


Figure 2. Schematic representation of the ITS-rDNA reference sequence for *Juniperus* spp. The reference sequence is 4,231 bp long, with nucleotide numbering based on the first base of the 5.8S region (+1). Negative values indicate upstream positions within the partial 18S region. The boundaries of ITS1, 5.8S, ITS2, and the partial flanking 18S and 28S regions are shown. We assembled the sequence from 570 ITS records retrieved from NCBI, and we provide the full reference sequence in Supplementary Fig. S1.

Table 3. Boundary positions of the ITS regions in the *Juniperus* reference sequence, with nucleotide numbering based on the first base of the 5.8S region as +1

Region	Start position (bp)	End position (bp)	Length (bp)
18S (partial)	-2276	-719	1558
ITS1	-718	-1	718
5.8S	1	157	157
ITS2	158	375	218
28S (partial)	376	1948	1573
Total reference sequence			4231

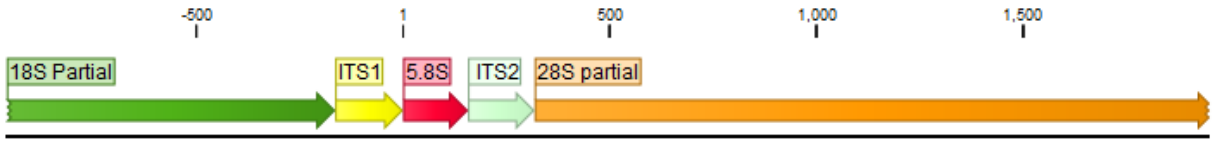


Figure 3. Schematic representation of the *Alternaria alternata* ITS-rDNA reference sequence. The reference sequence is 2,911 bp long and includes a partial 18S gene, ITS1, the 5.8S gene, ITS2, and a partial 28S gene. We numbered nucleotide positions relative to the first base of the 5.8S region, designated as +1. We assembled the sequence from 8,050 ITS records retrieved from NCBI, and the full reference sequence is provided in Supplementary Fig. S2.

Table 4. Boundary positions of the ITS regions in the *Alternaria alternata* reference sequence

Region	Start position (bp)	End position (bp)	Length (bp)
18S (partial)	-961	-165	797
ITS1	-164	-1	164
5.8S	1	157	157
ITS2	158	318	161
28S (partial)	319	1950	1632
Total reference sequence			2911

Alternaria alternata reference sequence

BLAST analysis of the 2,911 bp *A. alternata* ITS reference sequence produced an exact match with *A. alternata* isolate Karbala-1 (accession PQ217832.1), showing 100.00% identity over 100% query coverage (Max Score = 5250, E-value = 0.0). Subsequent hits showed a clear taxonomic gradient. Other *A. alternata* strains reached up to 100% identity, although query coverage varied. Other *Alternaria* species, including *A. infectoria* and *A. solani*, showed 97.9–100% identity with lower query coverage (51–75%), consistent with divergence in the ITS1 and ITS2 spacer regions. Genera in other Pleosporales families, including *Leptosphaeria*, *Ophiosphaerella*, and *Phoma*, showed 88–93% identity over full or near-full query coverage. No phylogenetically anomalous or cross-kingdom hits were detected, and all significant alignments were confined to the class *Dothideomycetes* (order Pleosporales). These results confirm the reference sequence's species-level identity as *A. alternata*. Because these reference sequences were assembled from multiple records, NCBI did not assign accession numbers. The complete annotated sequences in FASTA format are therefore provided in Supplementary Fig. S1 (*Juniperus*) and Fig. S2 (*A. alternata*). The corresponding author can also provide the sequences upon request.

Sequence alignment and comparison of ITS regions

To visualize structural differences between the ITS regions of *Juniperus* and *A. alternata*, the two reference sequences were aligned and displayed as a schematic alignment (Fig. 4). The *Juniperus* ITS reference sequence spans 3,455 bp. In contrast, the *A. alternata* reference sequence spans 2,828 bp, with nucleotide numbering based on the first base of the 5.8S gene as +1. The alignment shows that the ITS1 region (yellow) contains a large insertion in *Juniperus* relative to *A. alternata*, accounting for most of the length difference (545 bp). The ITS2 region (yellow-green) contains a smaller 54 bp insertion. By contrast, the 5.8S gene (red) is identical in length and highly conserved between the two organisms. The flanking 18S (green) and 28S (orange) regions are shown partially and contain

several conserved stretches. The conservation track (0–100%) indicates greater variability in ITS1 and ITS2 than in the coding regions, with the highest variability occurring in ITS1. The 5.8S gene contains at least two fully conserved regions, while the 18S and 28S regions contain five and more than five conserved regions, respectively. These structural differences, particularly the major gaps within ITS1 and ITS2, are quantified in Table 5 and form the basis for size-based discrimination by diagnostic PCR.

Length comparison of ITS subregions

The alignment showed that the 5.8S rRNA gene was identical in length (157 bp) in both organisms and remained highly conserved at the sequence level, as expected for a functional rRNA-coding region. In contrast, the ITS1 and ITS2 spacer regions showed substantial differences in length between *Juniperus* and *A. alternata* (Table 5).

As shown in Table 5, the complete ITS region (ITS1+5.8S+ITS2) in *Juniperus* (approximately 1,093 bp) is more than twice as long as that in *A. alternata* (approximately 482 bp), giving a length difference of approximately 611 bp. Most of this difference is due to the considerably longer ITS1 region in *Juniperus* (718 bp) than in *A. alternata* (164 bp).

Fig. 4 shows the structural differences between the two reference sequences as a schematic alignment of the ITS-rDNA regions of *Juniperus* and *A. alternata*. The alignment shows two major insertion/deletion events within ITS1 and ITS2 that account for the ~600 bp length difference between the two organisms. This length difference underpins the size-based PCR assay described below.

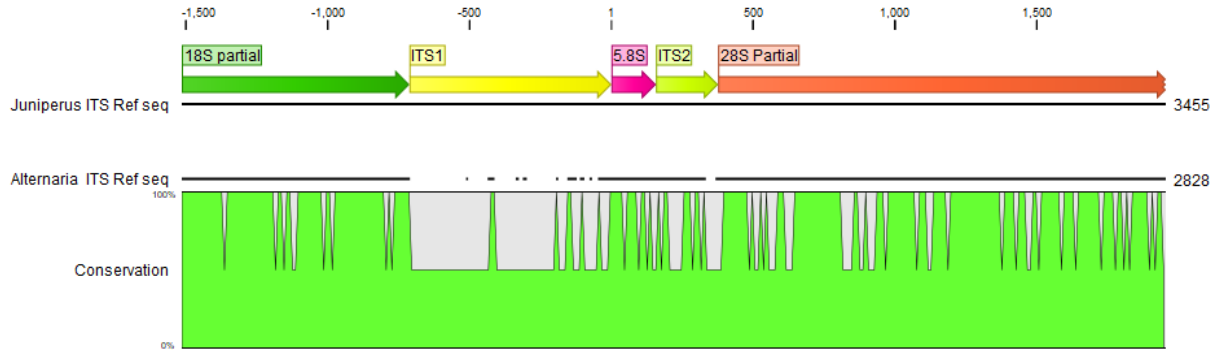


Figure 4. Schematic alignment of the ITS-rDNA reference sequences of *Juniperus* spp. (3,455 bp) and *Alternaria alternata* (2,828 bp). The alignment is numbered relative to the first nucleotide of the 5.8S gene (+1). Annotated regions include partial 18S (green), ITS1 (yellow), 5.8S (red), ITS2 (yellow-green), and partial 28S (red-yellow). The conservation track (0–100%) shows sequence conservation across the alignment, with darker shading indicating more conserved positions. Two major insertion/deletion (indel) gaps are present in the ITS1 and ITS2 regions of *Juniperus* relative to *A. alternata* and account for most of the difference in sequence length (Table 5). By contrast, the 5.8S gene is identical in length and highly conserved. The flanking 18S and 28S regions contain several conserved stretches, whereas the ITS1 and ITS2 spacers show greater sequence variability, consistent with their faster evolutionary rate. We generated the alignment using CLC Genomics Workbench v21.05.

Table 5. Comparison of ITS subregion lengths in the *Alternaria alternata* and *Juniperus* reference sequences

Region	*Alternaria alternata* (bp)	*Juniperus* (bp)	Length difference (bp)
ITS1	164	718	554
5.8S	157	157	0
ITS2	161	218	57
(ITS1+5.8S+ITS2)	482	1,093	611

Conserved and variable regions

Examination of the aligned sequences showed that the 5.8S gene was highly conserved, whereas the ITS1 and ITS2 regions showed substantial variation in both length and nucleotide composition. The ITS1 region of *Juniperus* contained multiple insertions (indels) relative to *A. alternata*, accounting for most of the difference in sequence length. By comparison, the ITS2 region showed a smaller length difference of 57 bp.

Within the *A. alternata* reference sequence, several fully conserved regions were identified across all 8,050 sequences used for assembly. These invariant regions included the following: (1) the 18S tail at positions –249 to –242, –236 to –233, –229 to –225, and –216 to –215; (2) the 5.8S region at position 70; and (3) the 28S region at positions 381–383 and 386–407. Because these regions showed no nucleotide variation, they may provide suitable targets for the future design of highly specific *A. alternata* primers with perfect 3′-end matches. Future studies will investigate these candidate regions.

***In silico* primer mapping**

To assess the reliability of commonly used universal fungal primers for amplifying A. alternata and to identify mismatches that could affect amplification efficiency, we performed a detailed in silico analysis. All 8,050 *A. alternata* ITS sequences longer than 500 bp available in NCBI as of June 2026 were retrieved and assembled against the *A. alternata* reference sequence (SUB16216726) using reference-guided assembly. We then generated a consensus sequence, retaining all conflicting positions as single nucleotide polymorphism (SNP) annotations. The analysis focused on positions –252 to +423, with numbering based on the first base of the 5.8S rRNA gene as +1. This region includes the 3′ end of the 18S rRNA gene (positions –252 to –165), the entire ITS1 region (positions –164 to –1), the complete 5.8S gene (positions 1–157), ITS2 (positions 158–318), and the 5′ end of the 28S rRNA gene (positions 319–423) (Fig. 5A).

The binding sites of 16 universal primers listed in Table 6 were examined, and 14 were successfully mapped to the *A. alternata* ITS reference sequence. We checked each primer alignment for mismatches, paying particular attention to the 3′-terminal nucleotide because it is critical for DNA polymerase extension. We calculated nucleotide frequencies at each position using the actual sequence coverage at that site, rather than the full set of 8,050 records, because not all sequences extended across the entire analyzed region.

Primer mapping on *Alternaria alternata*

Forward primers annealing to the 18S tail are shown in Fig. 5B. The primers ITS1-F, ITS5, SR6R, ITS1, and ITS1catta all bind within the final 66 bp of the 18S rDNA, at positions –232 to –165. Their 3′ ends are located at positions –211 (ITS1-F), –197 (ITS5 and SR6R), –176 (ITS1), and –165 (ITS1catta), respectively. Alternative nucleotides occurred at low frequencies at these 3′-terminal positions (<0.3%). The 3′ end of ITS1-F showed relatively greater variation, although only 1,060 reads covered this position and the consensus nucleotide remained predominant. These results suggest that all tested forward primers should efficiently amplify *A. alternata* when paired with suitable reverse primers.

Primers annealing within the 5.8S region are shown in Figs. 5C1 and 5C2. In the first part of the 5.8S gene (positions 31–51; Fig. 5C1), four reverse primers, ITS2, ITS2-R, ITS2-Melampsora, and 5.8S, bind within this region. Two forward primers, ITS3 and 5.8SR, also bind in the same region. Although sequence variation was detected at several positions, as indicated in red in Fig. 5C1, the 3′ ends remained highly conserved. In the central part of the 5.8S region

(positions 85–103), the reverse primer gITS7ngs binds as shown in Fig. 5C2. This primer contains four degenerate positions (R89, R95, R97, and Y99) that are compatible with the corresponding nucleotides in the reference sequence (A89, G95, A97, and C99). Its 3′-terminal nucleotide at position 103 aligns with G and showed the following variation: gaps (1), A (1), C (2), G (8,036), and T (1), corresponding to a frequency of 99.9% for G.

Reverse primers annealing to the beginning of the 28S region are shown in Fig. 5D. The primers ITS4 and ITS4ngsUni bind near the 5′ end of the 28S rDNA. The 3′ end of ITS4 at position 357 has a consensus G, with the following variation: gaps (2), A (18), C (8), G (5,364), T (2), and N (1). The ITS4 sequence matched the reference sequence completely. ITS4ngsUni contains four degenerate positions (H363, N365, S370, and S372) that, according to IUPAC nucleotide codes, are compatible with the corresponding reference nucleotides (C363, A365, C370, and G372). Its 3′ end showed a similar pattern of sequence variation.

Overall, the *in silico* analysis showed that all tested universal ITS primers had perfect or near-perfect matches at their 3′ ends when aligned with the *A. alternata* reference sequence, suggesting reliable amplification of *Alternaria* DNA. The rare SNPs detected within the primer-binding regions occurred at very low frequencies and are therefore unlikely to affect PCR amplification substantially.

Table 6. Universal fungal primers targeting the ITS1 and ITS2 regions (adapted from Wang et al., 2015; Usyk et al., 2017; Tom Bruns Laboratory website; R. Vilgalys Laboratory website)

Region	Direction	Primer name	Primer sequence (5′→3′)	Reference
ITS1	Forward	ITS1	TCCGTAGGTGAACCTGCGG	(White et al., 1990)
ITS1	Forward	ITS1-F	CTTGGTCATTTAGAGGAAGTAA	(Gardes & Bruns, 1993)
ITS1	Forward	ITS1-P	TTATCATTTAGAGGAAGGAG	Bruns lab website
ITS1	Forward	ITS1-P2	CTTTATCATTTAGAGGAAGGAG	(Taylor et al., 2004)
ITS1	Forward	ITS5	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)
ITS1	Forward	SR6R	AAGWAAAAGTCGTAACAAGG	(Vilgalys & Hester, 1990)
ITS1	Reverse	ITS2	GCTGCGTTCTTCATCGATGC	White et al. (1990)
ITS1	Reverse	ITS2-Melampsora	CACTGTGTTCTTCATCGACG	Bruns lab website
ITS1	Reverse	ITS2-R	TGTGTTCTTTCATCGATG	Bruns lab website
ITS1	Reverse	5.8S	CGCTGCGTTTCTTCATCG	Vilgalys & Hester (1990)
ITS2	Forward	ITS3	GCATCGATGAAGAACGCAGC	White et al. (1990)
ITS2	Forward	ITS3-R	ATCGTAGAAGAACACAG	Bruns lab website
ITS2	Forward	5.8SR	TCGATGAGAAGACGCAGCG	Vilgalys & Hester (1990)
ITS2	Reverse	ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITS2	Reverse	ITS4-B	CAGGAGACTTGTACACGGTCCAG	Gardes & Bruns (1993)
ITS1	Forward	ITS1catta	ACCWGCGGARGGATCATTA	(Tedersoo & Anslan, 2019)
ITS1	Reverse	ITS2ngs	TTYRCKRCGTTCTTCATCG	Tedersoo et al. (2018)
ITS2	Forward	gITS7ngs	GTGARTCATCRARTYTTTG	Tedersoo & Lindahl (2016)
ITS2	Reverse	ITS4ngsUni	CCTSCSCTTANTDATATGC	Tedersoo & Lindahl (2016)

Primer mapping on *Juniperus*

To determine whether the universal primers used for *A. alternata* detection could also bind to the ITS region of the host plant (*Juniperus* spp.), all 16 universal primers listed in Table 6 were aligned against the *Juniperus* ITS reference sequence (SUB16216725). Assessing primer binding to the host sequence is important for interpreting co-amplification in diagnostic PCR and for identifying primer combinations that reduce host interference. Multiple sequence alignment showed that 7 of the 16 primers had binding sites with at least 8 complementary nucleotides (Fig. 6A). Particular attention was given to the 3′-terminal nucleotide because of its importance for polymerase extension. In the *A. alternata* analysis, 3′-end mismatches were considered undesirable because they could reduce fungal amplification and increase the risk of false negatives. In the host sequence, however, a 3′-end mismatch is advantageous because it can reduce or prevent amplification of plant DNA, thereby improving fungal specificity.

Four forward primers, ITS1-F, ITS5, SR6R, and ITS1, mapped to the terminal region of the 18S rDNA at positions –780 to –724, relative to the first base of the 5.8S region (+1) (Fig. 6B). No sequence conflicts were detected within this region. The 3′ end of ITS1-F is located at position –759, where the reference sequence contains G but the primer terminates with A, resulting in a mismatch. This mismatch is expected to prevent efficient amplification of juniper DNA. ITS5 and SR6R share the same 3′-terminal position at –745, where both the reference sequence and primers contain G, indicating a perfect match and allowing primer extension. The 3′ end of ITS1 is located at position –724, where the terminal G also matches the reference sequence.

The forward primer gITS7ngs (19 bp) binds to positions 85–103 within the 5.8S gene (Fig. 6C). Its 3′-terminal nucleotide at position 103 is G and matches the reference sequence, indicating that this primer can also support amplification.

Two reverse primers, ITS4 and ITS4ngsUni, bind within the first half of the 28S rDNA at positions 412–431 (Fig. 6D). Both have a 3′-terminal G at position 412 that matches the reference sequence, suggesting efficient primer extension.

All combinations of the functional forward primers (ITS5, SR6R, ITS1, and gITS7ngs) with the functional reverse primers (ITS4 and ITS4ngsUni) are therefore expected to produce amplicons. Table 7 lists their predicted lengths, calculated from the reference sequence using the exact 3′-end positions and primer lengths. Notably, the widely used ITS1-F/ITS4 pair is unlikely to amplify the juniper host because ITS1-F contains a 3′-end mismatch. This characteristic may help reduce host DNA co-amplification during fungal detection. Other universal primer pairs, such as ITS1/ITS4, are expected to co-amplify the host and produce a larger band of approximately 1,174 bp, compared with the approximately 570 bp *Alternaria* product. This difference in amplicon size allows gel electrophoresis to distinguish the two products readily.

Predicted amplicon sizes for selected primer pairs

Using the primer-binding positions identified in the previous sections, the expected amplicon sizes for selected universal primer pairs were calculated for both *A. alternata* and *Juniperus* (Table 8). The commonly used ITS1/ITS4 primer pair was predicted to produce clearly different amplicon sizes in *Alternaria* (approximately 570 bp) and *Juniperus* (approximately 1,174 bp), providing a clear basis for size-based discrimination. The ITS1-F/ITS4 pair is not expected to amplify *Juniperus* because of the 3′-end mismatch, making it a promising option for fungal detection with reduced host interference.

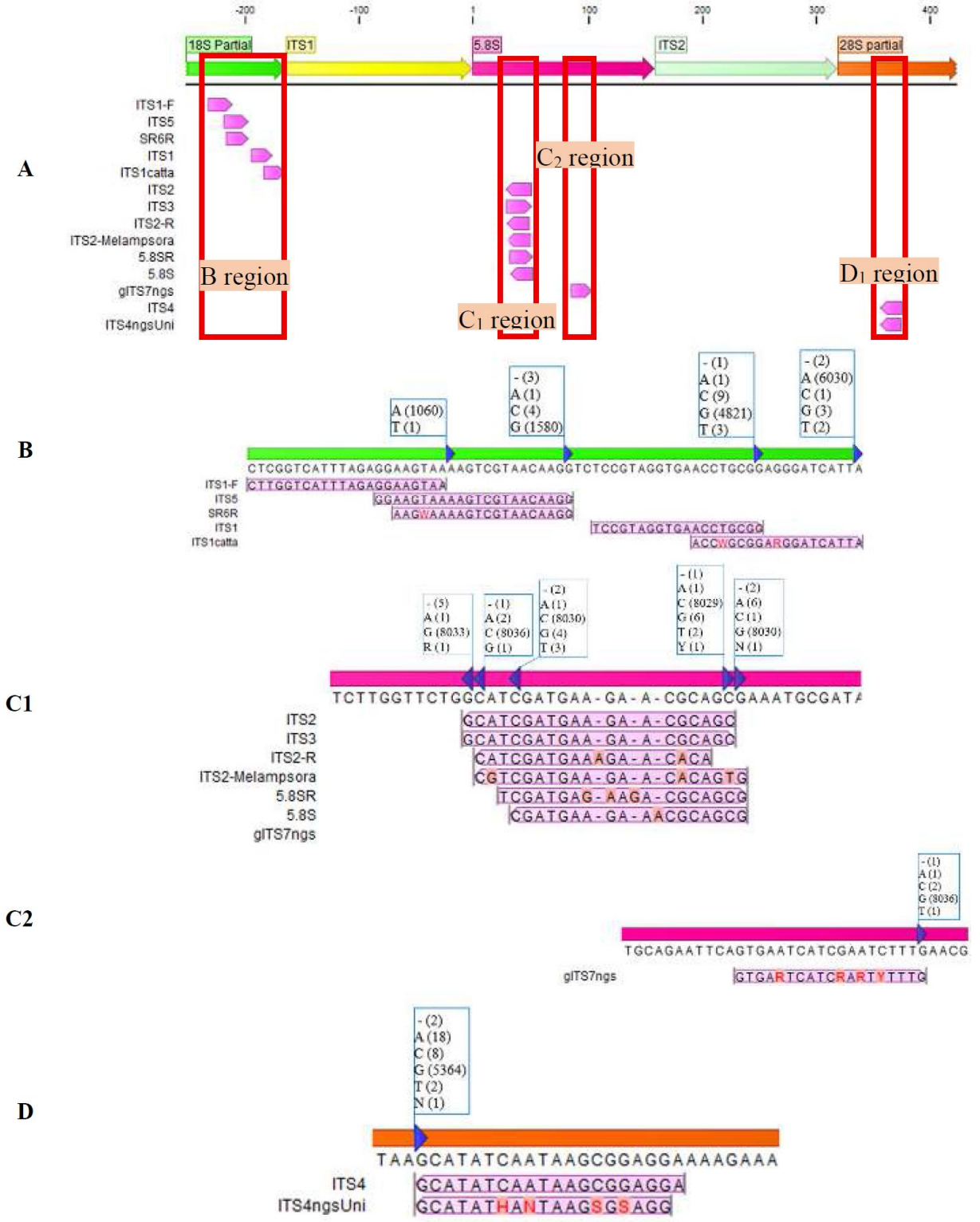


Figure 5. ***In silico*** **mapping of universal fungal primer-binding sites onto the consensus ITS region of** ***Alternaria alternata*****. (A) Schematic representation of the complete ITS region from positions –252 to +423, numbered relative to the first base of the 5.8S region as +1. The reference sequence includes the 3′ end of 18S rDNA, the complete ITS1 region, 5.8S, ITS2, and the 5′ end of 28S rDNA. The binding positions of 14 universal primers**

listed in Table 6 are indicated by arrows. The regions expanded in the subfigures are marked as B (18S tail), C1 and C2 (5.8S), and D (28S start). (B) Detailed alignment of the 18S tail (positions –232 to –165) showing the forward primers ITS1-F, ITS5, SR6R, ITS1, and ITS1catta. Nucleotide variation observed across the assembly of 8,050 sequences is reported below each primer sequence as counts (e.g., A = 1060, T = 1). The 3′-terminal nucleotide of each primer is boxed. Rare alternative nucleotides occur at frequencies below 0.3%. (C1) Alignment of the first part of the 5.8S gene (positions 31–51), showing the binding sites of the reverse primers ITS2, ITS2-R, ITS2-Melampsora, and 5.8S, together with the forward primers ITS3 and 5.8SR. Mismatches relative to the reference sequence are shown in red. The 3′-terminal nucleotides are highly conserved, with frequencies of 99.7–99.99%. (C2) Expanded view of the central part of the 5.8S region (positions 85–103), showing the reverse primer gITS7ngs. This primer contains four degenerate positions (R, R, R, Y) that are compatible with the corresponding reference nucleotides (A, G, A, C). Its 3′-terminal G occurs in 99.9% of the sequences. (D) Alignment of the 5′ end of the 28S rDNA (positions 319–357), showing the reverse primers ITS4 and ITS4ngsUni. ITS4 shows a complete match with the reference sequence, whereas ITS4ngsUni contains degenerate bases (H, N, S, S) that are compatible with the corresponding reference nucleotides (C, A, C, G). The 3′ ends of both primers align with a G present in 99.5% of the sequences. All primers are shown in the 5′→3′ direction. Rare SNPs occurred at very low frequencies and did not affect the 3′ termini, supporting reliable amplification of *Alternaria* DNA by these primers.

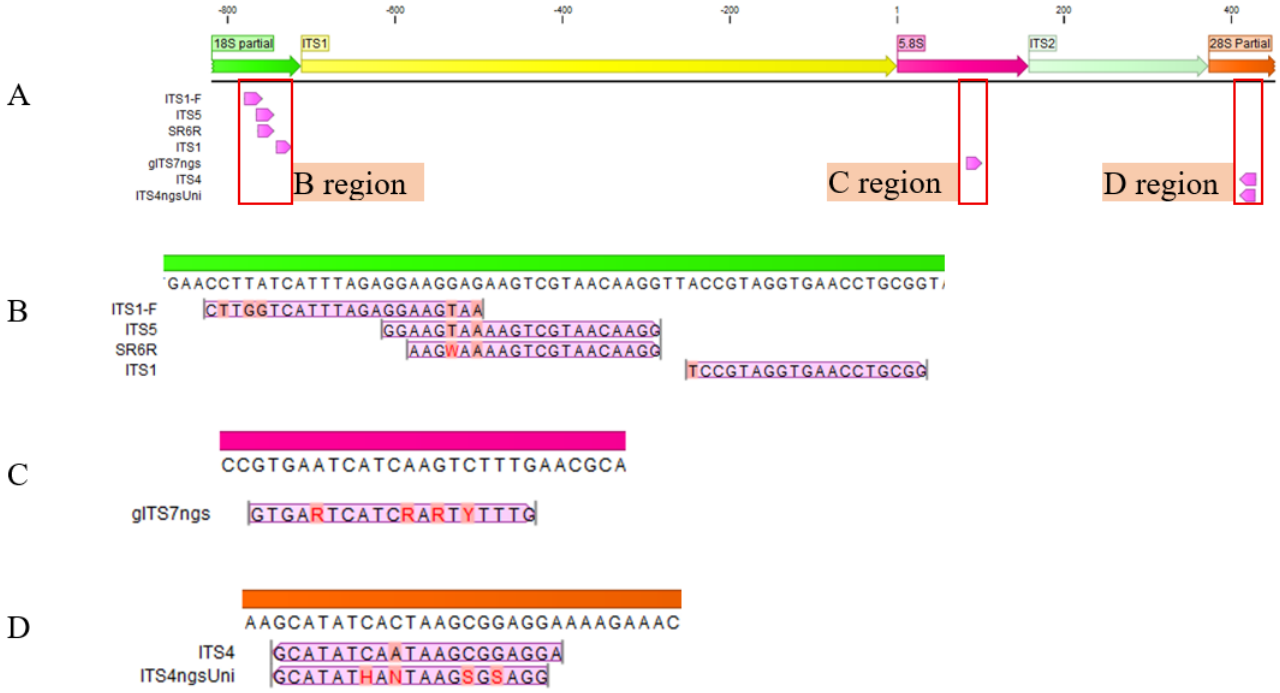


Figure 6. Mapping of universal fungal primer-binding sites onto the *Juniperus* ITS reference sequence (SUB16216725). Schematic representation of the complete ITS region, including the 3′ end of 18S rDNA, ITS1, 5.8S, ITS2, and the 5′ end of 28S rDNA. The binding positions of seven primers with ≥8 complementary nucleotides are indicated by arrows. (A) Expanded view of the 18S tail (positions –780 to –724), showing the forward primers ITS1-F, ITS5, SR6R, and ITS1. The 3′-terminal nucleotide of each primer is boxed. ITS1-F contains a mismatch at the 3′ end (A vs. reference G), which is expected to prevent efficient amplification, whereas ITS5, SR6R, and ITS1 show perfect 3′-end matches. (B) The 5.8S region (positions 85–103), showing the forward primer gITS7ngs. Its 3′-terminal G matches the reference sequence. (C) The beginning of the 28S region (positions 412–431), showing the reverse primers ITS4 and ITS4ngsUni. Both primers have perfect 3′-end matches (G). All primers are shown in the 5′→3′ direction. Table 7 presents expected amplicon sizes for functional primer pairs.

Table 7. Predicted amplicon sizes for the *Juniperus* ITS region using functional universal primer pairs

Forward primer	Reverse primer	Amplicon size (bp)
ITS5	ITS4	~1196
ITS5	ITS4ngsUni	~1197
SR6R	ITS4	~1196
SR6R	ITS4ngsUni	~1195
ITS1	ITS4	~1174
ITS1	ITS4ngsUni	~1173
gITS7ngs	ITS4	~347
gITS7ngs	ITS4ngsUni	~346

Note: ITS1-F is not included because it has a mismatch at its 3′ end on the *Juniperus* reference sequence and is therefore not expected to amplify host DNA.

Table 8. Predicted amplicon sizes for selected universal primer pairs using *Alternaria alternata* and *Juniperus* spp. DNA

Primer pair	*Alternaria* amplicon (bp)	*Juniperus* amplicon (bp)
ITS1-F / ITS4	608	No amplification (3′ mismatch)
ITS1 / ITS4	570	1174
ITS5 / ITS4	594	1198
SR6R / ITS4	592	1196
gITS7ngs / ITS4	292	347
ITS3 / ITS4	346	– (no functional forward binding)

Notes: *Juniperus* amplicon sizes are rounded to the nearest base pair. The ITS1-F/ITS4 pair is not expected to amplify juniper DNA because the 3′ end of ITS1-F mismatches the *Juniperus* reference sequence. This mismatch makes the pair a promising candidate for fungal detection with reduced host interference. In contrast, the ITS1/ITS4 pair amplifies both templates, but the approximately 600 bp difference in amplicon size allows the two products to be readily separated on a standard agarose gel.

Experimental validation of ITS1/ITS4 PCR for detecting *Alternaria* in juniper tissues

Based on the *in silico* analyses, we selected the ITS1/ITS4 primer pair for experimental validation. The selection was based on three key factors: (i) the predicted amplicon sizes of approximately 570 bp in *Alternaria* and 1,174 bp in *Juniperus*, which allow clear separation on standard agarose gels; (ii) fully conserved primer-binding sites in both organisms, supporting reliable amplification; and (iii) an approximately 600 bp difference in amplicon size, enabling clear discrimination between fungal and host DNA in a simple PCR-based assay.

Fungal isolate and plant material

The *Alternaria* isolate used in this study was previously obtained from symptomatic twigs of *Juniperus excelsa* in the Hezar-Masjed forests (Khorasan-Razavi Province, Iran) and morphologically identified as *Alternaria* sp. based on colony characteristics and conidial morphology (Charesaz et al., 2025). The isolate, designated Iso-10/S2, showed typical *Alternaria*-like colony morphology on potato dextrose agar (PDA) after 7 days of incubation at 28 °C, forming a compact, velvety, grey colony with a regular, entire margin (Fig. 7A). Microscopic examination showed dark-pigmented,

multicellular conidia consistent with the genus *Alternaria* (Fig. 7B).

For the plant host control, healthy leaves and twigs were collected from a *J. excelsa* tree near Sorkhcheshmeh in the Hezar-Masjed region (coordinates: 37°1′36.28″N, 56°53′44.18″E; elevation: 1,778.6 m) (Fig. 8A). The tree appeared healthy, with a fully green canopy and no visible signs of dieback or necrosis (Fig. 8B). Leaf and twig samples (Figs. 8C and 8D) were collected, surface-sterilized, and used for DNA extraction.

PCR amplification and gel electrophoresis

To experimentally validate the *in silico* predictions, PCR was performed using the universal fungal primers ITS1 and ITS4 with two DNA templates: DNA extracted from the pure *Alternaria* isolate described above and total DNA extracted from healthy juniper leaves collected in the Hezar-Masjed mountains (Shokouhifar et al., 2026). We also included a negative control without template DNA. PCR products were separated on a 1.5% agarose gel alongside a 1 kb DNA size marker (Fig. 9).

In well A, containing DNA from the pure *Alternaria* isolate, a single sharp band was observed between the 500 bp and 750 bp marker bands. The *in silico* analysis predicted an ITS1/ITS4 amplicon of 570 bp for *Alternaria*, which agrees closely with the observed gel position. The band is indicated in yellow in Fig. 9, while marker bands at 250 bp, 500 bp, 750 bp, 1 kb, and 1.5 kb are shown in white. In well J, containing total DNA from juniper leaves, we observed a single band of approximately 1.2 kb between the 1 kb and 1.5 kb marker bands. This result closely matches the predicted *Juniperus* ITS amplicon size of 1,174 bp (Table 8). The negative control (well C–) showed no amplification, indicating that no detectable contamination occurred during PCR.

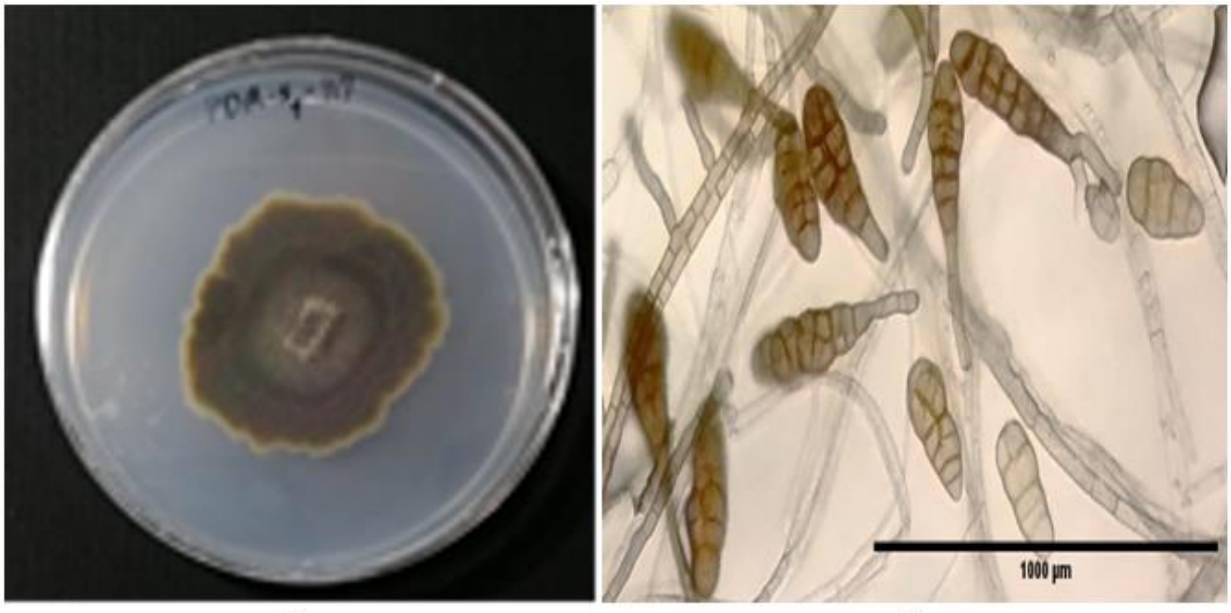


Figure 7. Morphological characteristics of the *Alternaria* isolate used in this study (Iso-10/S2; Charesaz et al., 2025; Charesaz et al., 2026). (A) Colony morphology on potato dextrose agar (PDA) after 7 days of incubation at 28 °C, showing a compact, velvety, grey colony with a regular, entire margin, consistent with *Alternaria*. (B) Microscopic image of *Alternaria* conidia (scale bar = 1,000 µm), showing dark-pigmented, multicellular conidia arranged in chains and containing transverse and longitudinal septa, characteristic of the genus *Alternaria*.



A







B **C** **D**

Figure 8. Sampling location and plant material used for experimental validation. (A) Location of the sampled *Juniperus excelsa* tree near Sorkhcheshmeh in the Hezar-Masjed region, Khorasan-Razavi Province, Iran (coordinates: 37°1′36.28″N, 56°53′44.18″E; elevation: 1,778.6 m). The map was prepared using Google Earth. (B) Overall view of the healthy tree, showing a fully green canopy with no visible symptoms of dieback. (C) Leaf samples collected from the tree. (D) Twig samples collected from the same tree.

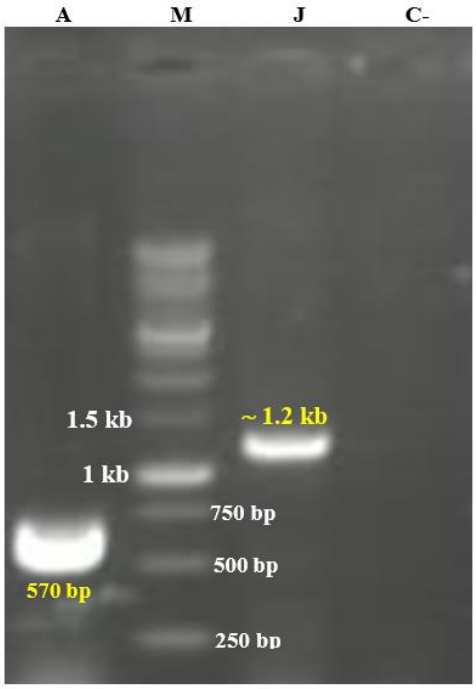


Figure 9. Experimental validation of ITS1/ITS4 PCR using DNA from an *Alternaria* isolate and *Juniperus* leaf tissue. PCR products were separated on a 1.5% agarose gel alongside a 1 kb DNA size marker. Marker bands at 250 bp, 500 bp, 750 bp, 1 kb, and 1.5 kb are indicated in white in well M. Well A: DNA extracted from a pure *Alternaria* culture previously isolated from juniper. A single sharp band, highlighted in yellow, is visible between the 500 bp and 750 bp markers and corresponds to the predicted 570 bp amplicon. Well J: Total DNA extracted from a juniper leaf collected in the Hezar-Masjed mountains. A single band of approximately 1.2 kb is visible, consistent with the predicted *Juniperus* ITS amplicon size of 1,174 bp. Well C–: Negative control without template DNA. The approximately 600 bp difference in amplicon size allows clear separation of the fungal and host products under the tested conditions.

Discussion

Validated ITS-rDNA reference sequences were constructed for *Juniperus* spp. and *A. alternata* and used to evaluate a simple, low-cost PCR-based method for distinguishing fungal and host amplicons. The results showed that the universal ITS1/ITS4 primer pair produced clearly different amplicon sizes from *Alternaria* (approximately 570 bp) and *Juniperus* (approximately 1,174 bp). The resulting difference of approximately 600 bp allowed the two products to be readily distinguished under the tested conditions, although this size difference does not specifically identify *Alternaria*. The method may therefore be useful as a preliminary, culture-free screening approach for detecting a fungal amplicon in juniper tissues. However, it cannot be used to confirm *Alternaria* colonization or applied as a routine monitoring tool by forest protection agencies without further validation and the use of additional genus- or species-specific markers.

We evaluated the ITS region as a tool for preliminary size-based screening between *Alternaria* and *Juniperus* amplicons rather than as a genus-level detection method. Alignment of the two reference sequences showed that the ITS1 and ITS4 primer-binding sites were fully conserved in both *Alternaria* and *Juniperus*, including identical 3′-terminal nucleotides. This conservation supports reliable amplification under standard PCR conditions. The approximately 600 bp difference between the fungal and host amplicons is also large enough to allow

clear visual separation on a 1.5% agarose gel, even with non-specific background bands.

This result is consistent with previous studies that have used ITS length variation for diagnostic purposes in other plant-fungus systems (Tedersoo & Lindahl, 2016; Usyk et al., 2017). To our knowledge, however, this is the first systematic evaluation of ITS-based size discrimination between *Alternaria* and *Juniperus* amplicons in juniper tissues, using reference sequences constructed from a large set of publicly available records, including 8,050 *A. alternata* sequences and 193 sequences from *Juniperus* species reported from Iran.

However, the ITS region has important limitations for species-level identification. Although it is useful for preliminary size-based screening, it should not be interpreted as a genus-level detection marker. Both the *in silico* analysis and previous studies indicate that ITS cannot reliably distinguish closely related *Alternaria* species. Phylogenetic analysis of 37 *Alternaria* isolates obtained from juniper hosts showed that ITS sequences clustered mainly according to geographic origin and host species rather than nominal species. This pattern agrees with previous reports showing that the ITS region is too conserved to discriminate among closely related species in several ascomycete genera, including *Alternaria* (Jasalavich et al., 1995; Woudenberg et al., 2015; Baturo-Cieśniewska et al., 2020).

Furthermore, the approximately 570 bp band generated by ITS1/ITS4 PCR is not specific to *A. alternata* or even to the genus *Alternaria*. Other common juniper endophytes, including *Penicillium*, *Fusarium*, and *Trichoderma*, may produce amplicons of similar size (approximately 520–590 bp) with the same primer pair. The method is therefore suitable only for preliminary screening, and amplicon sequencing is required for definitive identification.

In silico primer mapping showed that ITS1-F contains a mismatch at its 3′ end on the *Juniperus* reference sequence (A vs. G), which is expected to reduce or prevent host amplification. This feature makes the ITS1-F/ITS4 pair a promising option for reducing host co-amplification, although the pair may still amplify other fungal taxa. Such a reduction in host amplification could be useful in metabarcoding applications, where host DNA may account for a large proportion of sequencing reads (Yang et al., 2025). Nevertheless, ITS1-F/ITS4 does not provide genus- or species-specific detection, and taxonomic confirmation still requires sequencing.

Several limitations should be considered. First, we validated the assay by PCR using only one pure *Alternaria* isolate and one juniper leaf sample. Although the results agreed with the *in silico* predictions, further validation should include more fungal isolates from different geographic regions and juniper samples from multiple locations. Second, ITS1/ITS4 was not experimentally tested against other common juniper endophytes, such as *Fusarium*, *Penicillium*, and *Trichoderma*. Predicted amplicon sizes for these genera are similar to those of *Alternaria* (approximately 520–590 bp), and small differences in fragment length may not provide reliable taxonomic discrimination without sequencing. Third, the phylogenetic assessment was restricted to ITS sequences available in public databases. Additional isolates from under-sampled regions, including Central Asia and North Africa, may reveal further phylogeographic structure. More generally, ITS-based identification often requires complementary morphological

evidence and additional genetic markers because public databases may contain misannotated records and because substantial intraspecific variation can occur (Baturo-Cieśniewska et al., 2020).

The limitations identified in this study, particularly ITS's inability to resolve *Alternaria* species and the potential for cross-amplification with other fungal genera, provide a clear direction for further work. In the next phase of this research, we will develop and validate species-specific primers targeting the β-tubulin (TUB2) gene because this marker can help distinguish closely related *Alternaria* species (Samson et al., 2004; Zhao et al., 2014; Dettman & Eggertson, 2022). Future studies will also test the assay on naturally infected tissues and mixed endophyte communities using a panel of isolates representing different fungal genera.

The ITS1/ITS4 PCR assay described here offers a simple and low-cost method for preliminary screening. In DNA extracted from symptomatic juniper twigs or leaves, the presence of an approximately 570 bp band in addition to the host-derived band may indicate a fungal amplicon, but it does not confirm *Alternaria* colonization. The method does not require fungal culture and can be completed within one day, making it potentially useful for preliminary screening once adequately validated. For precise taxonomic identification, sequencing the smaller amplicon or using additional markers such as *TUB2* is required.

Given the reported diversity of *Alternaria* in Iran, including recently described species (Ahmadpour et al., 2025), further molecular surveillance may be useful after appropriate validation. However, the present data do not support claims of early pathogen detection. This study should therefore be regarded as a proof of concept for size-based discrimination using pure DNA templates. Validation on naturally infected tissues and mixed endophyte communities is still required before the assay can be considered for routine monitoring.

Under the tested conditions, the approximately 570 bp *Alternaria* amplicon was clearly distinguishable from the approximately 1,174 bp *Juniperus* amplicon. However, the amplicon sizes produced by other fungal genera that may occur in juniper tissues have not yet been determined experimentally and should be examined in future studies. Because ITS1/ITS4 does not provide reliable genus- or species-level discrimination, additional markers will be necessary to confirm the presence of *Alternaria* and to distinguish among *Alternaria* species.

Conclusion

High-quality ITS-rDNA reference sequences were constructed and validated for *Juniperus* spp. (4,231 bp) and *A. alternata* (2,911 bp) using a large set of publicly available sequences. Alignment of the two reference sequences revealed an approximately 600 bp difference in ITS region length, which formed the basis of a simple PCR approach for size-based discrimination between *Alternaria* and *Juniperus* amplicons.

Experimental validation showed that the universal ITS1/ITS4 primer pair produced clearly different amplicon sizes, approximately 570 bp from *Alternaria* DNA and approximately 1.2 kb from Juniper leaf DNA. This difference allowed the two products to be readily distinguished under the tested conditions, although amplicon size alone cannot confirm *Alternaria* colonization. The ITS region also lacks

sufficient resolution for species-level identification within *Alternaria* and should not be considered a genus-specific detection marker. Its value in the present study is therefore limited to preliminary size-based screening. In contrast, the ITS1-F/ITS4 primer pair is not expected to amplify *Juniperus* DNA because ITS1-F contains a 3′-end mismatch on the host reference sequence. This pair may therefore help reduce host co-amplification, although amplification of other fungal taxa remains possible.

ITS cannot distinguish among *Alternaria* species or reliably separate fungal genera, which defines an important direction for further research. Species-specific primers targeting the β-tubulin (*TUB2*) gene will require development and validation to more precisely identify *Alternaria*. The conserved regions identified within the *A. alternata* assembly may also provide suitable targets for designing additional primers with greater taxonomic specificity.

Overall, the ITS1/ITS4 assay provides a simple, rapid, and low-cost method for preliminary size-based screening of fungal and host amplicons. The approximately 570 bp *Alternaria* product can be distinguished from the approximately 1,174 bp *Juniperus* product, but this distinction does not confirm *Alternaria* and cannot support early pathogen detection without sequencing or additional specific markers. Future studies should determine the ITS amplicon characteristics of other fungal genera associated with juniper and develop markers that can distinguish *Alternaria* species. The reference sequences and conserved regions identified in this study provide useful resources for further assay development and comparative analysis of *Alternaria* associated with *Juniperus*.

Acknowledgments

The Center for International Scientific Studies and Collaborations (CISSC), Ministry of Science, Research and Technology, supported this work under project number 4030643. The authors thank the Vice Chancellor for Research and Technology of Ferdowsi University of Mashhad (FUM), the Community Relations Department, the Vice Chancellor for Research and Technology of the Faculty of Agriculture, and Zista Laboratory for their administrative support and for providing the laboratory facilities required to conduct this project.

References

Abo Nouh, F. A., Gezaf, S. A., Fathy, H. M., Abdel-Monem, R. S., Elsayed, K. A., Thabet, O. F., Adel, M. A., Abdel-Salam, A. S., Elshazly, A. M., Ahmed, E. M., Allam, M. M., & Abdel-Azeem, A. M. (2025). Exploring Endophytic Fungal Biodiversity in Different Coniferous Species. In *Advances in Lichens and Non-Flowering Plants Fungal Endophytes Research: Volume 1: Biodiversity and Ecological Perspective* (pp. 253-275). Cham: Springer Nature Switzerland. https://doi.org/10.1007/978-3-032-01506-8_12

Adams, R. P., & Hojjati, F. (2012). Taxonomy of *Juniperus* in Iran: Insight from DNA sequencing. *Phytologia*, *94*(2), 219-227.

Ahmadpour, A., Ghosta, Y., Alavi, Z., Alavi, F., Hamidi, L. M., & Rampelotto, P. H. (2025). Morphological and molecular characterization of a new section and two new species of *Alternaria* from Iran. *Life, 15*(6), 870. https://doi.org/10.3390/life15060870

Al-Nadabi, H., Hussain, S., Alkamyani, O. A., Al-Sidairi, A., Al-Hanai, A., Al-Sadi, A. M., Al-Zeidi, K., Al-Raeesi, A. A., Al-Jaradi, A., & Jellali, S. (2026). Uncovering the hidden leaf mycobiota of native juniper (*Juniperus seravschanica*) in Oman's arid highlands. *All Life*, *19*(1), 2657063. https://doi.org/10.1080/26895293.2026.2657063

Baturo-Cieśniewska, A., Pusz, W., & Patejuk, K. (2020). Problems, limitations, and challenges in species identification of Ascomycota members on the basis of ITS regions. *Acta Mycologica, 55*(1), 5512. https://doi.org/10.5586/am.5512

Behnke-Borowczyk, J., Kwaśna, H., Szewczyk, W., & Zatorski, J. (2019). Mycobiota of juniper *Juniperus* x media with symptoms of dieback in sewage plant facilities area in Poznań. *Forest Research Papers*, *80*, 247-252. https://doi.org/10.2478/frp-2019-0024

Bradshaw, M., Grewe, F., Thomas, A., Harrison, C. H., Lindgren, H., Muggia, L., St. Clair, L. L., Lumbsch, H. T., & Leavitt, S. D. (2020). Characterizing the ribosomal tandem repeat and its utility as a DNA barcode in lichen-forming fungi. *BMC Evolutionary Biology*, *20*(1), 2. https://doi.org/10.1186/s12862-019-1571-4

Caudullo, G., Welk, E., & San-Miguel-Ayanz, J. (2017). Chorological maps for the main European woody species. *Data in brief*, *12*, 662-666. https://doi.org/10.1016/j.dib.2017.05.007

Charesaz, T., Mamarabadi, M., Shokouhifar, F., & Zaferanloo, B. (2025). Isolation of endophytes fungi belonging to genera of *Alternaria* and *Penicillium* from juniper trees of the Hezar masjid region of Khorasan. *The 3rd International and 4th National conference of Agriculture, Environment and Food security, Jiroft University, Kerman, Iran*.

Charesaz, T., Mamarabadi, M., Shokouhifar, F., & Zaferanloo, B. (2026). Isolation and morphological characterization of *Alternaria* endophytes from *Juniperus excelsa* in Iran and evaluation of ITS region for detection and species identification. *Iranian Journal of Forest.* Submitted.

Dettman, J. R., & Eggertson, Q. (2021). Phylogenomic analyses of *Alternaria* section *Alternaria*: A high-resolution, genome-wide study of lineage sorting and gene tree discordance. *Mycologia*, *113*(6), 1218-1232. https://doi.org/10.1080/00275514.2021.1950456

Doyle, J. J., & Doyle, J. L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, *12*, 13-15.

Eo, J. K., Park, H., & Eom, A. H. (2018). Diversity of endophytic fungi isolated from *Pinus densiflora* and *Juniperus rigida* distributed in Mt. Baekryeonsan and Mt. Johangsan, Korea. *The Korean Journal of Mycology*, *46*(4), 437-446. https://doi.org/10.4489/kjm.2013.41.3.137

Fatemi, S. S., Rahimi, M., Tarkesh, M., & Ravanbakhsh, H. (2018). Predicting the impacts of climate change on the distribution of *Juniperus excelsa* M. Bieb. in the central and eastern Alborz

Mountains, Iran. *Iforest-Biogeosciences and Forestry*, *11*(5), 643. https://doi.org/10.3832/ifor2559-011

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology*, *2*(2), 113-118. https://doi.org/10.1111/j.1365-294x.1993.tb00005.x

Gherbawy, Y. A., & Elhariry, H. M. (2016). Endophytic fungi associated with high-altitude *Juniperus* trees and their antimicrobial activities. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*, *150*(1), 131-140. https://doi.org/10.1080/11263504.2014.984011

Habibi, M. M., Mamarabadi, M., Taheri, P., & Ramezany, Y. (2022). The effect of essential oils derived from various plant species versus the Mancozeb+ Metalaxyl fungicide in inhibiting early blight of tomato. *Plant Protection (Scientific Journal of Agriculture)*, *45*(3), 71-89. https://doi.org/10.22055/ppr.2022.17813

Hillis, D. M., & Dixon, M. T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *The Quarterly review of biology*, *66*(4), 411-453. https://doi.org/10.1086/417338

Hoffman, M. T., & Arnold, A. E. (2008). Geographic locality and host identity shape fungal endophyte communities in cupressaceous trees. *Mycological Research*, *112*(3), 331-344. https://doi.org/10.1016/j.mycres.2007.10.014

Hojjati, F., Kazempour-Osaloo, S., Adams, R. P., & Assadi, M. (2018). Molecular phylogeny of *Juniperus* in Iran with special reference to the *J. excelsa* complex, focusing on *J. seravschanica*. https://doi.org/10.11646/phytotaxa.375.2.1

Jafary, H., Najafi, S., Zand, N., & Majidi, T. (2023). Investigation and identification of fungi associated with juniper tree decline. *Iranian Journal of Forest and Range Protection Research*, *21*(1), 123-134. https://doi.org/10.22092/ijfrpr.2023.354946.1497

Jasalavich, C. A., Morales, V. M., Pelcher, L. E., & Séguin-Swartz, G. (1995). Comparison of nuclear ribosomal DNA sequences from *Alternaria* species pathogenic to crucifers. *Mycological Research*, *99*(5), 604-614. https://doi.org/10.1016/s0953-7562(09)80720-x

Jaung, L. M., Lin, W. W., Chang, T. T., Fu, C. H., & Wang, L. H. (2022). Isolation and identification of fungi from damaged Dragon Juniper twig after a fire. *Taiwan Journal of Forest Science*, 55, 53-60.

Jia, A., Chen, B., Lu, H., Xing, Y., Li, B., & Fan, X. (2024). Multigene phylogeny and morphology reveal three new species of *Cytospora* isolated from diseased plant branches in Fengtai District, Beijing, China. *MycoKeys*, *101*, 163. https://doi.org/10.3897/mycokeys.101.116272

Kasaian, J., Behravan, J., Hassany, M., Emami, S. A., Shahriari, F., & Khayyat, M. H. (2011). Molecular characterization and RAPD analysis of *Juniperus* species from Iran. *Genetics and Molecular Research*, *10*(2), 1069-1074. https://doi.org/10.4238/vol10-2gmr1021

Lewis, F. J. (1924). An endotropic fungus in the coniferae. *Nature*, *114*(2876), 860-860. https://doi.org/10.1038/114860b0

Mazur, S., Nadziakiewicz, M., Kurzawińska, H., & Nawrocki, J. (2019). Effectiveness of mycorrhizal fungi in the protection of juniper, rose, yew and highbush blueberry against *Alternaria alternata*. *Folia Horticulturae*, *31*(1), 117-127. https://doi.org/10.2478/fhort-2019-0008

Nadziakiewicz, M., Kurzawińska, H., Mazur, S., & Tekielska, D. (2018). *Alternaria alternata*–the main causal agent of disease symptoms in juniper, rose, yew and highbush blueberry in nurseries in southern Poland. *Folia Horticulturae*, *30*(1), 15-25. https://doi.org/10.2478/fhort-2018-0002

Naghipour, A. A., Ashrafzadeh, M. R., & Haidarian, M. (2021). Assessing the potential distribution of *Juniperus excelsa* M. Bieb. under current and future climate scenarios in the Chaharmahal va Bakhtiari province, Iran. *Scientific Reports in Life Sciences*, *2*(3), 8-17. https://doi.org/10.22034/srls.2021.537634.1020

Nasr Esfahani, M., Alizadeh Moghaddam, G., & Karimkhah, M. A. (2017). -The leaf micro-morphological components with early blight resistant potatoes varieties. *Plant Protection (Scientific Journal of Agriculture)*, *39*(4), 51-64. https://doi.org/10.22055/ppr.2017.12549

Sharifi-Ghahfarrokhi, N. (2021). The influence of fungal cell envelope inhibitors on taxol production in plant symbiotic molds. *Master's thesis in Biotechnology and Microbiology. University of Shahrekord.* Supervised by M. Mobini Dehkordi and S. Farhadian. IranDoc. https://ganj.irandoc.ac.ir/#/articles/074d03b0510e67770c463e0e019d4a2a

Shokouhifar, F., Mamarabadi, M., & Zaferanloo, B. (2026). Diversity in branch tip dieback disease index of juniper trees (*Juniperus excelsa*) in the Hezar-Masjed forests in the north of Khorasan Razavi. *Iranian journal of Forest.* https://doi.org/10.22034/ijf.2026.577780.2113 (In Press)

Singh, D., & Sahni, S. (2025). Bioinformatics and omics tools in endophytic fungal research. In *Navigating Endophytic Research for Next-Generation Therapeutics* (pp. 381-402). Academic Press. https://doi.org/10.1016/b978-0-443-31484-1.00019-0

Taylor, D. L., Bruns, T. D., & Hodges, S. A. (2004). Evidence for mycorrhizal races in a cheating orchid. *Proceedings of the Royal Society B: Biological Sciences, 271*(1534), 35-43. https://doi.org/10.1098/rspb.2003.2557

Tedersoo, L., & Anslan, S. (2019). Towards PacBio-based pan-eukaryote metabarcoding using full-length ITS sequences. *Environmental Microbiology Reports, 11*(5), 659-668. https://doi.org/10.1111/1758-2229.12776

Tedersoo, L., & Lindahl, B. (2016). Fungal identification biases in microbiome projects. *Environmental Microbiology Reports, 8*(5), 774-779. https://doi.org/10.1111/1758-2229.12438

Tedersoo, L., Tooming-Klunderud, A., & Anslan, S. (2018). PacBio metabarcoding of Fungi and other eukaryotes: errors, biases and perspectives. *New Phytologist, 217*(3), 1370-1385. https://doi.org/10.1111/nph.14776

Usyk, M., Zolnik, C. P., Patel, H., Levi, M. H., & Burk, R. D. (2017). Novel ITS1 fungal primers for characterization of the mycobiome. *MSphere*, *2*(6), 10-1128. https://doi.org/10.1128/msphere.00488-17

Vigneshwari, A., Škrbić, B. D., Kredics, L., Abbas, L., Bakacsy, L., Vágvölgyi, C., & Szekeres, A. (2020). Bioprospecting and biodiversity investigations of endophytic fungi isolated from *Juniperus* communis. *Acta Biologica Szegediensis*, *64*(2), 129-138. https://doi.org/10.14232/abs.2020.2.129-138

Vilgalys, R., & Hester, M. (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology*, *172*(8), 4238-4246. https://doi.org/10.1128/jb.172.8.4238-4246.1990

Wang, X. C., Liu, C., Huang, L., Bengtsson-Palme, J., Chen, H., Zhang, J. H., Cai, D., & Li, J. Q. (2015). ITS 1: a DNA barcode better than ITS 2 in eukaryotes?. *Molecular Ecology Resources*, *15*(3), 573-586. https://doi.org/10.1111/1755-0998.12325

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, *18*(1), 315-322. https://doi.org/10.1016/b978-0-12-372180-8.50042-1

Woudenberg, J. H. C., Seidl, M. F., Groenewald, J. Z., De Vries, M., Stielow, J. B., Thomma, B. P., & Crous, P. W. (2015). *Alternaria* section *Alternaria*: Species, formae speciales or pathotypes? *Studies in Mycology*, *82*(1), 1-21. https://doi.org/10.1016/j.simyco.2015.07.001

Yang, Y., Gan, Y., Xu, W., Huang, Y., Xin, T., Tan, R., & Song, J. (2025). Analysis of whole-genome for *Alternaria* species identification. *Journal of Fungi*, *11*(3), 185. https://doi.org/10.3390/jof11030185

Zamani, S. M., Mojerlou, S., Alizadeh, M., & Ghanaei, S. (2025). First report of charcoal disease caused by *Biscogniauxia mediterranea* (De Not.) Kuntze on *Juniperus excelsa* M. Bieb. in Iran. *Trees*, *39*(1), 17. https://doi.org/10.1007/s00468-024-02585-8

Zhou, W., Shi, W., Soltis, P. S., Soltis, D. E., & Xiang, Q. Y. (2023). Foliar endophyte diversity in Eastern Asian-Eastern North American disjunct tree species–influences of host identity, environment, phylogeny, and geographic isolation. *Frontiers in Plant Science*, *14*, 1274746. https://doi.org/10.3389/fpls.2023.1274746

© 2026 by the authors. Licensee SCU, Ahvaz, Iran. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC 4.0 license) (http://creativecommons.org/licenses/by-nc/4.0/.