Contamination ID

# Evaluation of commercial assays for fungal speciation in environmental monitoring


## Abstract

Reliable fungal identification remains a challenge in environmental monitoring, where inconsistent resolution across methods can delay investigations and complicate data interpretation. This study evaluates the performance of three molecular assays targeting key genomic regions used for fungal identification: the D2 region of rDNA, the full internal transcribed spacer (ITS) region (ITS1 and ITS2), and a partial ITS region (ITS2 only).

A total of 27 fungal species were analyzed using sequencing-based workflows and compared against their respective reference libraries. The full ITS assay, supported by the Applied Biosystems™ MicroSEQ™ ID ITS Fungal Gene Library v1.0, showed consistent amplification, high sequencing success rates, and accurate species-level identification, with 23 species correctly identified at ≥99% sequence match. The Applied Biosystems™ Fast MicroSEQ™ D2 assay demonstrated comparable amplification and sequencing performance, with differences in species-level resolution observed for certain genera, reflecting variation in reference library coverage. The ITS2 assay showed lower overall performance, with reduced amplification success and identification rates, particularly for samples with low DNA input.

These results highlight the importance of target region selection and reference library design in fungal identification workflows. Among the methods evaluated, the full ITS region provided consistent species-level identification across the organisms tested, supporting its application in environmental monitoring programs.


## Introduction

There are an estimated 1.5 to 3.5 million fungal species across global ecosystems, healthcare, food production, and other industries, making fungi one of the largest groups of living organisms. In biopharmaceutical manufacturing, accurate fungal identification remains a persistent challenge in environmental monitoring programs, where inconsistent identification results and reliance on multiple methods can delay investigations and complicate data interpretation.

Commonly used approaches, including biochemical methods, MALDI-TOF, and targeted molecular assays, support routine identification but may not consistently provide species-level resolution. In response, some laboratories have developed in-house methods to achieve higher-resolution identification, which can introduce variability and require additional resources to implement and maintain.

The internal transcribed spacer (ITS) region, located between the small and large subunit rRNA genes, is widely used for fungal identification due to its combination of conserved and highly variable sequences. Commercial assays have traditionally targeted either the D2 or the ITS2 region of rDNA. Additional genomic targets, including beta-tubulin, calmodulin, and cytochrome oxidase genes, may also support species differentiation in specific applications [1].

In this study, assays targeting the full ITS region were evaluated alongside D2- and ITS2-based approaches to assess their performance for fungal species identification within environmental monitoring workflows.

## Materials and methods

Table 1 lists the 27 fungal species evaluated in this study, representing organisms commonly encountered in environmental monitoring programs, including those cited in sterility testing guidelines such as the USP <71>, JP 4.0.6, and EP 2.6.1.

Genomic DNA was either purchased from Westerdijk Fungal Biodiversity Institute (CBS) or extracted from single colonies or lyophilized cells obtained from American Type Culture Collection (ATCC) and CBS using the Applied Biosystems™ PrepMan™ Ultra Sample Preparation Reagent (Cat. No. 4318930). DNA extracted using the PrepMan Ultra reagent was diluted 10-fold prior to PCR amplification.

For purchased DNA samples, 0.2 ng per reaction was used for ITS PCR and 15 ng per reaction was used for D2 PCR reactions.

**Table 1. List of fungal species for D2, ITS, and ITS2 testing.**

| PrepMan Ultra reagent—extracted DNA | Reference DNA samples |
|---|---|
| *Alternaria alternata* | *Aspergillus brasiliensis* |
| *Chrysosporium keratinophilum* | *Aspergillus flavus* |
| *Cladosporium cladosporioides* | *Aspergillus fumigatus* |
| *Monascus ruber* | *Aspergillus niger* |
| *Penicillium chrysogenum* | *Aspergillus sclerotiorum* |
| *Rhizomucor pusillus* | *Aspergillus versicolor* |
| *Rhizopus oryzae* | *Beauveria bassiana* |
| *Sporothrix schenckii* | *Candida albicans* |
| *Trichophyton mentagrophytes* | *Candidozyma auris* (*Candida auris*) |
| *Umbelopsis ramanniana* | *Chaetomium globosum* |
| *Wickerhamomyces anomalus* | *Cladosporium halotolerans* |
| | *Cryptococcus neoformans* |
| | *Penicillium commune* |
| | *Penicillium expansum* |
| | *Pseudogymnoascus pannorum* |
| | *Saccharomyces cerevisiae* |

Amplification and cycle sequencing of the D2 region were performed using the Applied Biosystems™ Fast MicroSEQ™ D2 rDNA fungal identification and sequencing kits (PCR: Cat. No. 4382397; sequencing: Cat. No. 4347481). The ITS region was amplified and sequenced using the Applied Biosystems™ MicroSEQ™ ITS rDNA Fungal Identification Kit (Cat. No. A40005277). ITS2 testing was performed by a third-party vendor using an in-house workflow. A summary of the workflows and associated reference libraries used in this application note is provided in Table 2.

Each fungal species was tested in triplicate for both the ITS and D2 assays, while a single specimen was tested for the ITS2 assay. A sample was considered to meet acceptance criteria if it achieved a specimen score ≥37, a consensus read length >85%, and a ≥99% match to the reference library.

ITS analysis was performed using two reference libraries: the Applied Biosystems™ MicroSEQ™ ID Fungal ITS Core Library and the Applied Biosystems™ MicroSEQ™ ID Fungal ITS Extended Library.

**Table 2. Summary of workflow for fungi testing with D2, ITS, and ITS2 assays.**

| Process step | Process description | Fast D2 assay | ITS assay | ITS2 assay |
|---|---|---|---|---|
| DNA sample preparation | In-house extraction | Single colony (~3 mm) extracted using PrepMan Ultra reagent; DNA diluted 10x (or 100x for larger colonies) | Same as D2 | Same as D2 |
| | Extracted DNA | DNA diluted in nuclease-free water prior to PCR | Same as D2 | DNA diluted in TE buffer prior to PCR |
| PCR amplification | Target region and amplicon size | D2 region (95–465 bp) | Full ITS (ITS1 + ITS2; 158–1251 bp) | ITS2 (~350 bp) |
| Post-PCR preparation | PCR product dilution | Not required | 5x dilution with nuclease-free water | Not available |
| | PCR product clean-up | Applied Biosystems™ ExoSAP-IT™ *Express* PCR Product Cleanup | Same as D2 | Not available |
| Cycle sequencing | Sequencing chemistry | Fast MicroSEQ D2 rDNA Fungal Sequencing Kit | Applied Biosystems™ MicroSEQ™ ITS rDNA Fungal Sequencing Kit | Not available |
| Extension product clean-up | Purification of sequencing products | Edge BioSystems Optima™ DTR Ultra 96-well Plate | Same as D2 | Not available |
| Capillary electrophoresis | DNA sequence generation | Applied Biosystems™ SeqStudio™ Flex Genetic Analyzer (24-capillary, POP-6™ polymer) | Same as D2 | Not available |
| Data analysis | Library and identification approach | MicroSEQ ID Fungal Gene Library v2022 (Cat. No. A57314) | MicroSEQ ID Fungal ITS Library (Core + Extended or Core only) | Third-party vendor library |

## Results

### Amplification and sequencing of ITS region

All specimens were successfully amplified and sequenced using the MicroSEQ ITS assay. A total of 23 species were correctly identified with ≥99% sequence match when analyzed using both the MicroSEQ ID Fungal ITS Core Library and the MicroSEQ ID Fungal ITS Extended Library (Table 3).

Two species, *Pseudogymnoascus pannorum* and *Trichophyton mentagrophytes*, were identified as second-best matches (hit 2), with top hits corresponding to *P. carnis* and *T. interdigitale*, respectively. *Chrysosporium keratinophilum* and *Umbelopsis ramanniana* were not correctly identified using the combined libraries. Several species produced multiple hits with identical percentage matches (Table 3).

Reanalysis using the MicroSEQ ID Fungal ITS Core Library alone improved resolution for select species. Five species were resolved to a single, specific match, including *Aspergillus fumigatus*, *Aspergillus versicolor*, *Chaetomium globosum*, *Pseudogymnoascus pannorum*, and *Sporothrix schenckii* (Table 3). *Chrysosporium keratinophilum* was also correctly identified using the MicroSEQ ITS Core Library alone. *Umbelopsis ramanniana* remained unidentified due to the absence of forward sequence data across all replicates.

**Table 3. The fungi specimens with the correct hit as top match obtained from the analysis with both MicroSEQ ID Fungal ITS Core Library and the MicroSEQ ID Fungal ITS Extended Library.**

| Fungi species | Core and extended | | Core | |
|---|---|---|---|---|
| | **Hit** | **Percent match** | **Hit** | **Percent match** |
| *Alternaria alternata* | *Alternaria alternata** | 100% | *Alternaria alternata** | 100% |
| *Aspergillus brasiliensis* | *Aspergillus brasiliensis* | 100% | *Aspergillus brasiliensis* | 100% |
| *Aspergillus flavus* | *Aspergillus flavus** | 99.99–100% | *Aspergillus flavus** | 99.99–100% |
| *Aspergillus fumigatus* | *Aspergillus fumigatus** | 100% | *Aspergillus fumigatus* | 100% |
| *Aspergillus niger* | *Aspergillus niger** | 100% | *Aspergillus niger** | 100% |
| *Aspergillus sclerotiorum* | *Aspergillus sclerotiorum** | 99.99–100% | *Aspergillus sclerotiorum** | 99.99–100% |
| *Aspergillus versicolor* | *Aspergillus versicolor** | 99.97–99.99% | *Aspergillus versicolor* | 99.97–99.99% |
| *Beauveria bassiana* | *Beauveria bassiana* | 100% | *Beauveria bassiana* | 100% |
| *Candida albicans* | *Candida albicans* | 100% | *Candida albicans* | 100% |
| *Candidozyma auris* (*Candida auris*) | *Candidozyma auris* | 100% | *Candidozyma auris* | 100% |
| *Chaetomium globosum* | *Chaetomium globosum** | 100% | *Chaetomium globosum* | 100% |
| *Chrysosporium keratinophilum* | *Aphanoascus fulvescens* | 97.65–97.7% | *Chrysosporium keratinophilum* | 97.52–97.6% |
| *Cladosporium cladosporioides* | *Cladosporium cladosporioides** | 100% | *Cladosporium cladosporioides** | 100% |
| *Cladosporium halotolerans* | *Cladosporium halotolerans** | 100% | *Cladosporium halotolerans** | 100% |
| *Cryptococcus neoformans* | *Cryptococcus neoformans** | 100% | *Cryptococcus neoformans** | 100% |
| *Monascus ruber* | *Monascus ruber** | 99.99–100% | *Monascus ruber* (2/3)/ *purpureus* (1/3) | 100% |
| *Penicillium chrysogenum* | *Penicillium chrysogenum** | 99.80% | *Penicillium chrysogenum** | 99.80% |
| *Penicillium commune* | *Penicillium commune** | 100% | *Penicillium commune** | 100% |
| *Penicillium expansum* | *Penicillium expansum** | 99.98–100% | *Penicillium expansum** | 99.98–100% |
| *Pseudogymnoascus pannorum* | *Pseudogymnoascus pannorum*† | 99.62–99.65% | *Pseudogymnoascus pannorum* | 99.62–99.65% |
| *Rhizomucor pusillus* | *Rhizomucor pusillus* | 99.99–100% | *Rhizomucor pusillus* | 99.99–100% |
| *Rhizopus oryzae* | *Rhizopus arrhizus* (*R. oryzae*) | 99.99–100% | *Rhizopus arrhizus* (*R. oryzae*) | 99.99–100% |
| *Saccharomyces cerevisiae* | *Saccharomyces cerevisiae* | 99.58–99.61% | *Saccharomyces cerevisiae* | 99.58–99.61% |
| *Sporothrix schenckii* | *Sporothrix schenckii** | 99.72–99.82% | *Sporothrix schenckii* | 99.72–99.82% |
| *Trichophyton mentagrophytes* | *Trichophyton mentagrophytes*† | 99.52–99.54% | *Trichophyton mentagrophytes*† | 99.52–99.54% |
| *Umbelopsis ramanniana* | *Umbelopsis brunnea** | 95.24–99% | *Umbelopsis ovata* | 93.98–96.77% |
| *Wickerhamomyces anomalus* | *Wickerhamomyces anomalus* | 99.89–100% | *Wickerhamomyces anomalus* | 99.89–100% |

* Samples hit multiple species when searching against the ITS libraries.

† Samples matched with h2 in the ITS library.

Note: All specimens with at least 2/3 replicates pass the acceptance criteria, unless stated otherwise in the table.

A breakdown of identification outcomes showed that 25 species were correctly identified as a single, specific match using the combined MicroSEQ ID Fungal ITS Core and Extended Libraries, compared to 26 species using the MicroSEQ ITS Core Library alone. Correspondingly, two species were incorrectly identified using the combined libraries, while one species was incorrectly identified using the MicroSEQ ITS Core Library alone.

## Comparison of three molecular assays targeting the ITS, D2, and ITS2 regions

Species identification results for all tested specimens across the molecular assays are summarized in Table 4. ITS results are based on analysis using the MicroSEQ ITS Core Library. The relative ability of the three assays to achieve genus- and species-level resolution is illustrated in the heatmap in Figure 1.

**Table 4. Hit results of fungi specimens obtained with ITS, D2, and ITS2 assays.**

| Fungi species | ITS assay‡ | | D2 assay | | ITS2 assay | |
|---|---|---|---|---|---|---|
| | **Hit** | **% match** | **Hit** | **% match** | **Hit** | **% match** |
| *Alternaria alternata* | *Alternaria alternata** | 100% | *Alternaria alternata* | 100% | No data | No data |
| *Aspergillus brasiliensis* | *Aspergillus brasiliensis* | 100% | *Aspergillus brasiliensis** | 100% | No data | No data |
| *Aspergillus flavus* | *Aspergillus flavus** | 99.99–100% | *Aspergillus flavus** | 100% | *Aspergillus flavus** | 100% |
| *Aspergillus fumigatus* | *Aspergillus fumigatus* | 100% | *Aspergillus fumigatus* | 100% | *Aspergillus fumigatus* | 100% |
| *Aspergillus niger* | *Aspergillus niger** | 100% | *Aspergillus niger** | 100% | No data | No data |
| *Aspergillus sclerotiorum* | *Aspergillus sclerotiorum** | 99.99–100% | *Aspergillus sclerotiorum* | 100% | *Aspergillus sclerotiorum** | 100% |
| *Aspergillus versicolor* | *Aspergillus versicolor* | 99.97–99.99% | *Aspergillus versicolor* | 100% | *Aspergillus versicolor** | 100% |
| *Beauveria bassiana* | *Beauveria bassiana* | 100% | *Beauveria bassiana* | 100% | *Beauveria bassiana* | 99.43% |
| *Candida albicans* | *Candida albicans* | 100% | *Candida albicans* | 100% | *Candida albicans* | 100% |
| *Candidozyma auris* (*Candida auris*) | *Candidozyma auris* | 100% | *Candida auris* | 100% | *Candida auris* | 100% |
| *Chaetomium globosum* | *Chaetomium globosum* | 100% | *Chaetomium globosum** | 100% | *Chaetomium globosum** | 99.71% |
| *Chrysosporium keratinophilum* | *Chrysosporium keratinophilum* | 97.52–97.65% | *Chrysosporium keratinophilum*† | 97.46–97.49% | No data | No data |
| *Cladosporium cladosporioides* | *Cladosporium cladosporioides** | 100% | *Cladosporium cladosporioides** | 100% | No data | No data |
| *Cladosporium halotolerans* | *Cladosporium halotolerans** | 100% | *Cladosporium halotolerans** | 100% | *Cladosporium halotolerans* | 99.41% |
| *Cryptococcus neoformans* | *Cryptococcus neoformans** | 100% | *Cryptococcus neoformans* | 98.99–99% | *Cryptococcus neoformans* | 100% |
| *Monascus ruber* | *Monascus ruber** | 100% | *Monascus ruber* | 100% | No data | No data |
| *Penicillium chrysogenum* | *Penicillium chrysogenum** | 99.80% | *Penicillium chrysogenum** | 100% | No data | No data |
| *Penicillium commune* | *Penicillium commune** | 100% | *Penicillium commune** | 100% | *Penicillium commune** | 100% |
| *Penicillium expansum* | *Penicillium expansum** | 99.98–100% | *Penicillium expansum** | 100% | *Penicillium expansum** | 100% |
| *Pseudogymnoascus pannorum* | *Pseudogymnoascus pannorum* | 99.62–99.65% | *Geomyces pannorum* (current: *Pseudogymnoascus pannorum*)* | 100% | *Pseudogymnoascus appendiculatus* | 100% |
| *Rhizomucor pusillus* | *Rhizomucor pusillus* | 99.99–100% | *Rhizomucor miehei* | 95.87–95.95% | No data | No data |
| *Rhizopus oryzae* | *Rhizopus arrhizus* (*R. oryzae*) | 99.99–100% | *Rhizopus oryzae* | 99.87–100% | No data | No data |
| *Saccharomyces cerevisiae* | *Saccharomyces cerevisiae* | 99.58–99.61% | *Saccharomyces cerevisiae* | 100% | *Saccharomyces cerevisiae* | 99.76% |
| *Sporothrix schenckii* | *Sporothrix schenckii* | 99.72–99.82% | *Sporothrix schenckii* | 98.48–98.52% | *Sporothrix schenckii* | 100% |
| *Trichophyton mentagrophytes* | *Trichophyton mentagrophytes*† | 99.68–99.7% | *Trichophyton mentagrophytes*† | 99.26–99.28% | No data | No data |
| *Umbelopsis ramanniana* | *Umbelopsis ovata* | 93.98–96.77% | *Umbelopsis ovata* | 99.19–99.2% | No data | No data |
| *Wickerhamomyces anomalus* | *Wickerhamomyces anomalus* | 99.89–100% | *Wickerhamomyces anomalus* | 100% | No data | No data |

* Samples hit multiple species when searching against the ITS libraries.

† Samples matched with h2 in the ITS library.

‡ The ITS results were generated from the MicroSEQ ID Fungal ITS Core Library alone. Three replicates were performed for each species. The table provides the range of percent match for all 3 replicates. The hit is shown if the species is identified for at least 2 out of 3 replicates.

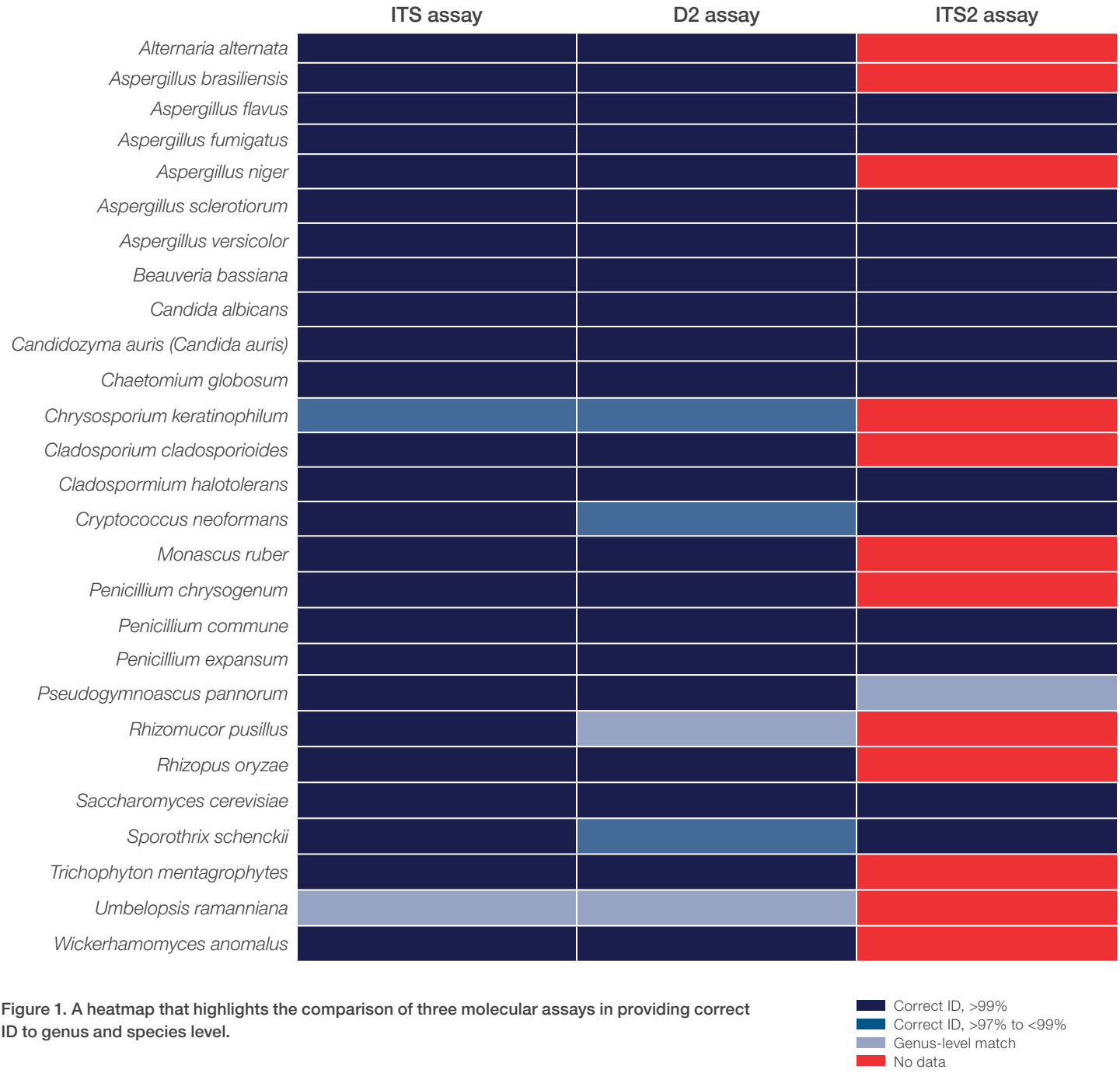


**Figure 1. A heatmap that highlights the comparison of three molecular assays in providing correct ID to genus and species level.**

The ITS assay correctly identified 25 of 27 species at ≥99% sequence match, while the D2 assay identified 22 species at the same threshold. Of the two species that did not meet the ≥99% match criterion for ITS, one species (*Chrysosporium keratinophilum*) was correctly identified at the species level with >97% match, and the other was correctly identified at the genus level (*Umbelopsis*) with >93% match.

The ITS2 assay demonstrated a lower identification rate, with 14 of 27 species identified at ≥99% match. Among the 13 species that were not identified, 12 did not generate sequence data, suggesting suboptimal amplification performance under the conditions evaluated.

## Conclusion

The MicroSEQ ID Fungal ITS Library is a curated reference database designed to reflect the diversity of the ITS region across fungal species. The MicroSEQ ID Fungal ITS Core Library represents a subset focused on commonly encountered environmental organisms. Analysis using the MicroSEQ ITS Core library alone was sufficient to correctly identify most species evaluated in this study. The MicroSEQ ITS Extended Library supports interrogation of a broader sequence space, which may result in multiple matches among closely related species with highly similar ITS sequences.

Comparison of the ITS and D2 assays demonstrated comparable amplification and sequencing performance across the species tested, with differences observed at the identification stage. In some cases, species that produced multiple matches with one assay were resolved to a single species using the other, reflecting differences in the targeted genomic regions and associated reference libraries. These findings highlight the complementary nature of ITS and D2 approaches for fungal identification.

Among the three methods evaluated, the ITS2 assay showed the lowest overall performance, with the highest number of specimens failing to generate sequence data. All failed specimens were prepared using the PrepMan Ultra reagent and had DNA concentrations below the input typically required for the ITS2 workflow. In contrast, specimens with higher DNA input were successfully sequenced, indicating that workflow requirements, particularly DNA input amounts, contributed to the observed performance differences.

In the results section, one observation was the failure of *Umbelopsis* species to meet the acceptance criteria for genus-level match (>97%) and species-level match (>99%). Despite this, the specimens demonstrated good quality metrics, with specimen scores ranging from 40–42 and clean EPG profiles, indicating that *Umbelopsis ramanniana* could be successfully amplified and sequenced using the in-house ITS assay. However, phylogenetic analysis based on ITS alone was insufficient to achieve species-level identification within the *Umbelopsis* genus. Some research groups have successfully used D2, as corroborated by our D2 data, and several other genes (*nSSU*, *nLSU*, *act1*, *MCM7*, *cox1*) to effectively resolve *Umbelopsis* [2,3].

Overall, the MicroSEQ ITS rDNA Fungal Identification Kit offers a robust framework for fungal identification. The D2 assay offers complementary capability, and both approaches showed effective performance across the organisms evaluated. Differences in identification outcomes reflect the targeted genomic regions and associated reference libraries. The ITS2 assay exhibited lower success rates under the evaluated conditions, largely due to DNA input requirements and workflow differences.

Within environmental monitoring workflows, the ITS assay delivered consistent species-level identification across a representative panel of commonly encountered fungi. The data demonstrate reproducible performance across replicates and the ability to resolve a broad range of organisms. When combined with the curated MicroSEQ ID ITS Fungal Gene Library, the ITS assay helps provide a consistent and scalable approach for fungal species identification.

Reference:

1. Clinical and Laboratory Standards Institute (CLSI) MM18-A:2008 Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing, 1st Edition. *Clinical and Laboratory Standards Institute.*

2. Ogawa, Y., Sugiyama, M., Hirose, D., Kusama-Eguchi, K., and Tokumasu, S. (2011). Polyphyly of intraspecific groups of Umbelopsis ramanniana and their genetic and morphological variation. *Mycoscience*, 52:91-98. doi: 10.1007/s10267-010-0074-3

3. Wang, Y., Liu, X., and Zheng, R. (2022). The Umbelopsis ramanniana Sensu Lato consists of five cryptic species. *Journal of Fungi*, 8, 895: 1-21. doi: 10.3390/jof8090895

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