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ITS3/ITS4 outperforms other ITS region and 18S rRNA gene primer sets for amplicon sequencing of soil fungi

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Abstract

The primer sets of 18S rRNA genes and Internal Transcribed Spacer (ITS) regions are universal primers for amplicon sequencing in soil fungal diversity studies. The selection of proper primer sets has been identified as one of the main drivers affecting effective cross-study comparisons in various types of soils. However, few studies have provided guidance for the selection of fungal primers in soil fungal biodiversity studies. The aim of this study was to evaluate and optimise fungal sequencing methods with three primer sets in desert, forest, grassland and farmland soils. Melting curve analyses were used to evaluate the specificity of each primer set in different soil ecosystems. The quality of fungal sequencing with three primer sets was assessed with the same conditions on the Illumina platform. The melting curve analysis showed that both the FR1/FF390 and the ITS3/ITS4 primer sets have better specificity than the ITS1/ITS primer set. 18S rRNA amplicon sequencing produced less fungal phylotypes compared to the amplicon sequencing data of the ITS1/ITS2 and ITS3/ITS4 primer sets. Our study suggests that the ITS3/ITS4 primer set was more suitable for the analysis of soil fungal biodiversity in desert, forest, grassland and farmland soils on the Illumina sequencing platform.

Highlights:

- Comparison of three existing primer sets for soil fungal amplicon sequencing.
- ITS region has better performance than 18S rRNA gene in amplicon sequencing.
- ITS3/ITS4 primer set is more suitable for the analysis of soil fungal biodiversity.

KEYWORDS

18S rRNA gene, fungal diversity, fungal ITS, high-throughput sequencing

Fungi are primarily involved in the cycling of soil nutrient elemental cycles due to their large repertoire of catabolic activities (Liu et al., 2019; Yang et al., 2020). It has been demonstrated that soil fungi are more responsible for the degradation of complex compounds in soil (Goyer et al., 2022). A comprehensive understanding of the diversity and ecology of soil fungi is complementary to that of soil bacteria. Beyond traditional morphological identification, the investigation of soil fungi is increasingly relying on molecular phylogenetic methods (Koljalg

et al., 2013). The amplicon high-throughput sequencing is more and more commonly used in comparative microbial ecology to assess the diversity of microbial communities and their responses to changing environments (Cheeke et al., 2021; Chen et al., 2020). However, limitations in DNA polymerase activity and laser technique restrict the length of sequencing reads on the Illumina sequencing platform. Therefore, the selection of primer sets for amplifying soil fungi is still a point of contention.

Both 18S rRNA genes and the internal transcribed spacer (ITS) region were applied to investigate fungal diversity in amplicon sequencing. 18S rRNA gene amplicon sequencing is commonly used in the investigation of fungal diversity in arable soils (Mise & Otsuka, 2020; Sugiyama et al., 2010). For the ITS region, there was some controversy about their role as a practical fungal biomarker. Though it was widely employed in molecular ecology and has been proven to be useful in the investigation of fungal diversity, ITS biomarkers were assumed to not always allow the direct identification of all fungal species since the alignment of ITS sequences across genetically distant taxa was not confident in taxonomic resolution (Asemaninejad et al., 2016; Liu et al., 2015). Some studies demonstrated that amplicon sequencing with ITS biomarkers could provide more taxonomical information than that of the 18S rRNA gene (Bachy et al., 2013; Liu et al., 2015). In contrast, it was also reported that 18S rDNA fragments could discriminate more fungal taxonomies in activated sludge (Evans et al., 2014). Both ITS and 18S rDNA primers have been reported to be useful for identifying fungal diversity in various soil ecosystems (Bachy et al., 2013). However, a comparison of the accuracy between these two ribosomal RNA regions is still unclear during amplicon high-throughput sequencing.

Here, we conducted parallel experiments to assess the performance of three fungal primer sets, that is, FR1/FF390, ITS1/ITS2 and ITS3/ITS4, targeting 18S rRNA gene (Vainio & Hantula, 2000) and ITS region (Asemaninejad et al., 2016; Gardes & Bruns, 1993) in the amplicon sequencing of desert, forest, grassland and farmland soils. A total of 24 samples were collected, with six replicates for each type of soil. Soil total DNA was extracted using the FastDNA® SPIN Kit for soil (MP Biomedicals, CA). Fluorescence melting curve analyses and amplicon high-throughput sequencing were conducted to evaluate the performance of each fungal primer set in all collected soil samples. Details of soil sampling, sequencing and data analysis are described in the Supplementary Information.

Fluorescence melting curve analyses were conducted by quantitative PCR to assess the specificity of the three fungal primer sets, since melting curves can differentiate nonspecific products of PCR amplification (Ririe et al., 1997). The results showed single peak patterns in FR1/FF390 primers, and the melting temperatures of FR1/FF390 PCR products were 86.0°C (Figure 1a). The melting curves of ITS1/ITS2 products showed a double-peak pattern. The melting temperatures of ITS1/ITS2 products ranged from 84.0°C to 87.0°C (Figure 1b). The melting curve of ITS3/ITS4 basically showed a single peak pattern. The melting temperature of ITS3/ITS4 was around 73.5°C (Figure 1c). It indicated that the GC/AT ratio of nucleotide sequences coincided within different soil samples for FR1/FF390 and ITS3/ITS4 primer sets, but the GC/AT ratio of ITS1/ITS2 PCR products varied greatly between different soil samples. Although melting peak analysis has several limitations, such as the absolute position and width of melting curves could be affected by dye concentration (Higuchi et al., 1993) and the rate of temperature (Keohavong & Thilly, 1989; Ririe et al., 1997), it still provides a promising approach for simultaneous amplification, detection and differentiation of PCR products. According to the melting curve analysis, the

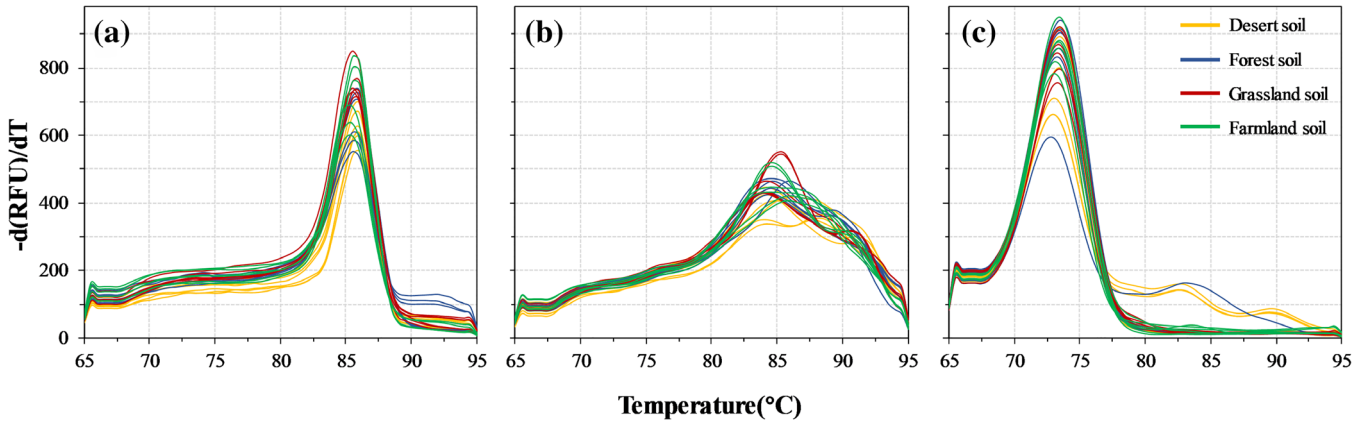

FIGURE 1 Melting curves of PCR assays using different fungal primer sets. (a), FR1/FF390; (b), ITS1/ITS2; (c), ITS3/ITS4

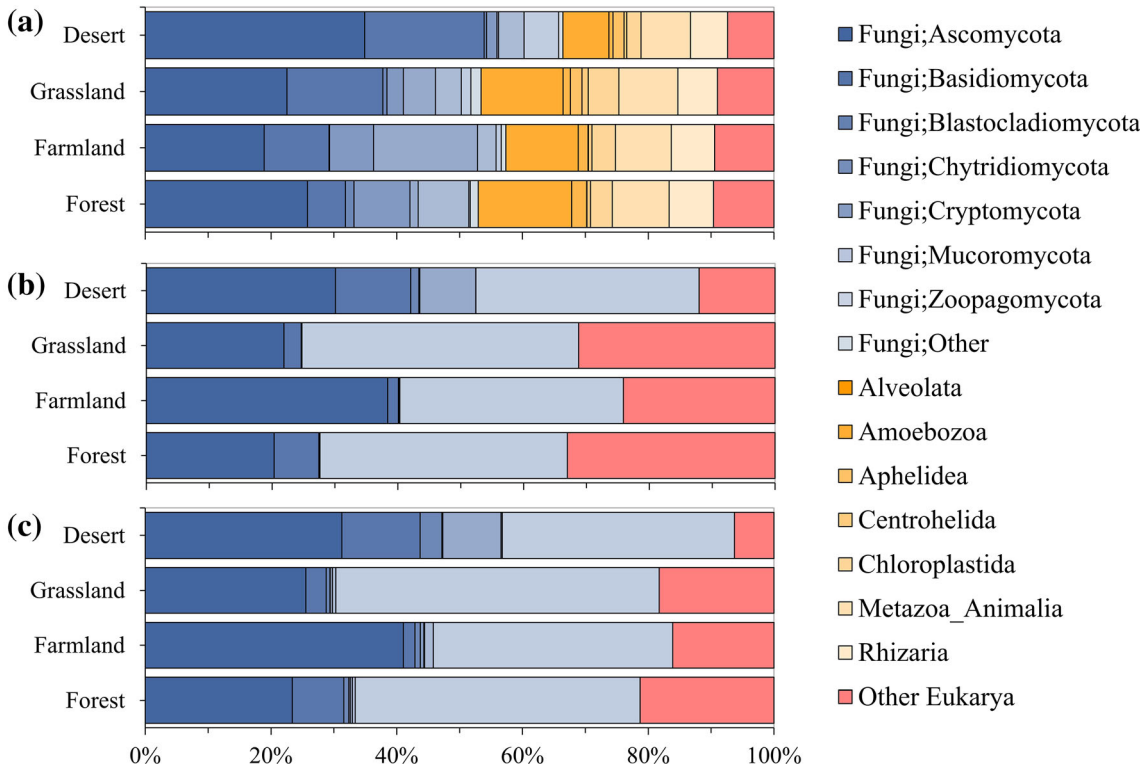

FIGURE 2 Abundance of soil microbial phylotypes using different fungal primer sets. (a), FR1/FF390; (b), ITS1/ITS2; (c), ITS3/ITS4

FR1/FF390 and ITS3/ITS4 primer sets provided better performance than the ITS1/ITS2 primer set in the melting curve analysis of all four types of soils.

The FR1/FF390 amplicon sequencing yielded 601,589 valid sequences, and 10,494–59,872 sequences were obtained for each sample. Alignment with the Silva132 gene database revealed that 57.5% were fungal and the remaining 42.5% sequences were Alveolata, Alveolata, Amoebozoa and Rhizaria, and other eukaryotic phyla (Figure 2a). Sequencing based on ITS1/ITS2 amplicons yielded 814, 576 valid sequences, with 11, 242–60, and 789 sequences obtained for each sample. Alignment with the UNITE database revealed that 74.9% were fungal and 25.1% sequences did not obtain valid comparisons in the Genebank database (Figure 2b). The highest percentage of fungal sequences was found in the desert soil samples, at 87.9%, while the percentage of fungi in the remaining three soil samples ranged from 67% to 75%. Sequencing based on ITS3/ITS4 amplicons yielded 956, 531 valid sequences, with 12, 879–75, and 371 sequences obtained for each sample. Alignment with the UNITE database revealed that 84.7% were fungal and 15.3% of the sequences did not obtain valid comparisons in the database (Figure 2c). The desert soil samples were 93.7% fungal and the other three soil samples were between 79 and 84% fungal. The composition (i.e., assemblage of OTUs) of soil fungal communities changed markedly in the sequencing results of different fungal primer sets. Only half of the obtained FR1/FF390 fragments were aligned as kingdom Fungi, while more than 85% of the valid sequences obtained by ITS1/ITS2 and ITS3/ITS4 were identified as kingdom Fungi in all tested soils. The phenomena may result from the lower specificity level 18S rRNA gene than the ITS region (Lord et al., 2002). The partial region of the 18S rRNA between soil fungi and other Eukarya is similar in the sequence order (Schmidt et al., 2013). Hence, 18S rRNA sequences may not always provide sufficient taxonomic resolution to allow identification of soil fungal communities. As a result, 18S rRNA sequences may not always give enough taxonomic resolution to identify soil fungi composition compared to ITS primers.

By trimming the non-fungal reads in the amplicon sequencing, the rarefaction curve analysis and non-metric multi-dimensional scaling (NMDS) plots of soil fungal communities in different types of soil samples were conducted to assess whether the different fungal primers would produce similar patterns of fungal diversity. The rarefaction curves of all primer sets tended to approach the saturation plateau (Figure 3a–c). The desert soils showed the lowest number of OTUs, the forest soils showed the highest level of OTU richness. The lowest number of OTUs at 10,000 sequences per sample was obtained for the ITS1/ITS2 primer pair in the same soil

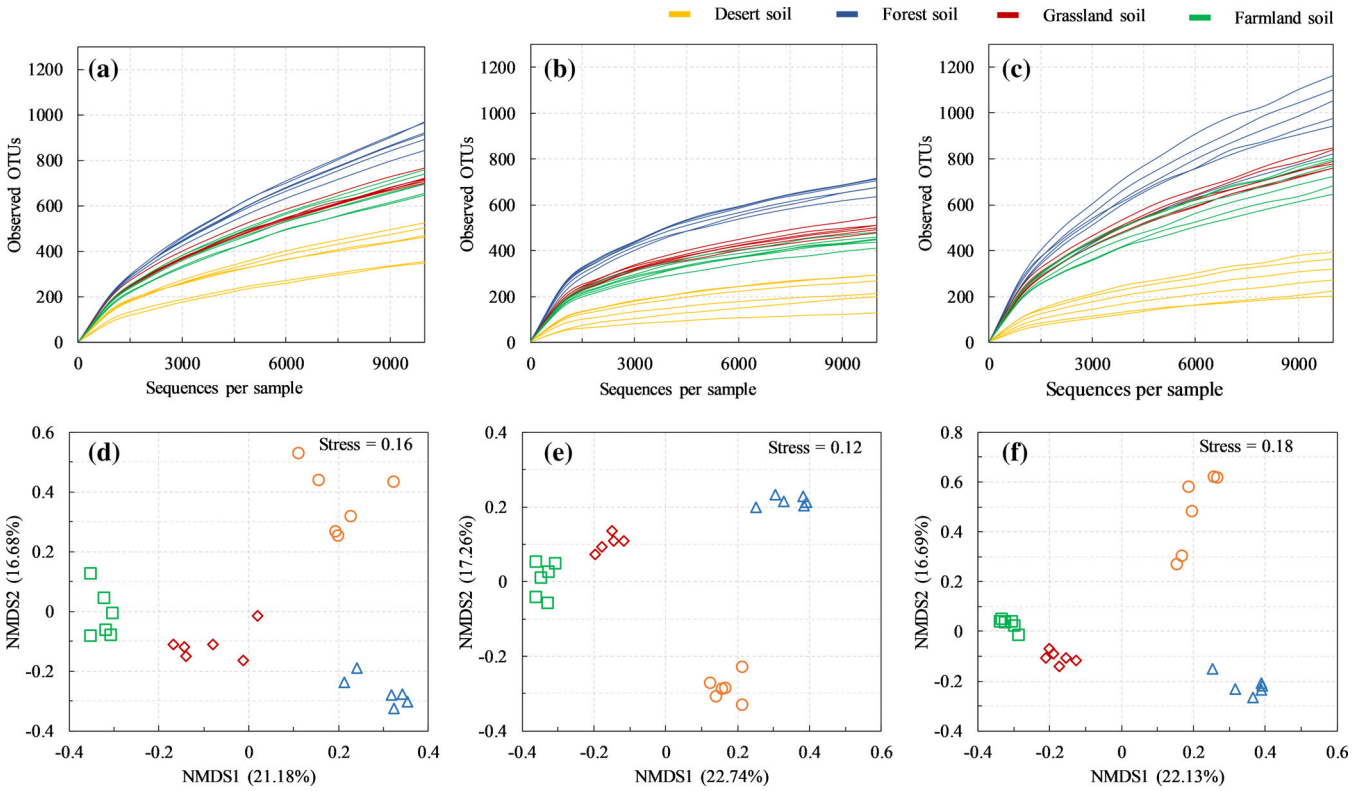

FIGURE 3 Rarefaction curves and non-metric multi-dimensional scaling (NMDS) plots using different fungal primer sets. Colours of lines and spots indicated different types of soils. (a), rarefaction curves of FR1/FF390; (b), rarefaction curves of ITS1/ITS2; (c), rarefaction curves of ITS3/ITS4; (d), NMDS of FR1/FF390; (e), NMDS of ITS1/ITS2; (f), NMDS of ITS3/ITS4

samples (Figure 3b), while the highest level of species richness was obtained for the ITS3/ITS4 primer pair in the same samples (Figure 3c). NMDS analysis showed the fungal community patterns resulted from three primer sets were significantly separated clearly between desert, forest, grassland and farmland soils (Figure 3d–f, Figure S1, $p < 0.05$). These results indicated that the appearance of soil fauna in the 18S sequencing results did not influence the fungal community structure except occupying the depth of sequencing compared to ITS primer sets. Our results consisted of the previous study by Liu et al. (2015), which compared the performances of fungal primer sets targeting 18S rRNA gene and ITS region by using denaturing gradient gel electrophoresis methods in testing the fungal diversity in aquatic and soil samples.

To sum up, though the FR1/FF390, ITS1/ITS2 and ITS3/ITS4 fungal primer sets can all be used for the amplicon sequencing of soil fungi on the Illumina platform, the performance of each fungal primer set showed significant differences between each other. The melting curve analysis showed that both FR1/FF390 and ITS3/ITS4 primer sets have better specificity, but the sequencing results showed that ITS1/ITS2 and ITS3/ITS4 primer sets obtained a higher proportion of fungal sequences. In a comprehensive comparison, it is more conservative to use ITS3/ITS4 primer sets for amplicon sequencing of soil fungi, which not only have better band length consistency but can also ensure the number of effective sequences. In addition, this investigation only evaluated the performance of different fungal primer sets on the Illumina sequencing platform, while their performance on the Pacbio and Oxford nanopore platforms still needs further investigation.

AUTHOR CONTRIBUTIONS

Yongjie Yu: Conceptualization (equal); funding acquisition (equal); investigation (equal); project administration (equal). **Qinyu Yang:** Data curation (equal); formal analysis (equal); investigation (equal). **Evangelos Petropoulos:** Data curation (equal); formal analysis (equal); methodology (equal). **Tongbin Zhu:** Conceptualization (equal); formal analysis (equal); funding acquisition (equal); investigation (equal).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Asemaninejad, A., Weerasuriya, N., Gloor, G. B., Lindo, Z., & Thorn, R. G. (2016). New primers for discovering fungal diversity using nuclear large ribosomal DNA. *PLoS One*, *11*, e0159043.

Bachy, C., Dolan, J. R., Lopez-Garcia, P., Deschamps, P., & Moreira, D. (2013). Accuracy of protist diversity assessments: Morphology compared with cloning and direct pyrosequencing of 18S rRNA genes and ITS regions using the conspicuous tintinnid ciliates as a case study. *ISME Journal*, *7*, 244–255.

Cheeke, T. E., Phillips, R. P., Kuhn, A., Rosling, A., & Fransson, P. (2021). Variation in hyphal production rather than turnover regulates standing fungal biomass in temperate hardwood forests. *Ecology*, *102*, e03260.

Chen, Y., Sun, R., Sun, T., Chen, P., Yu, Z., Ding, L., Jiang, Y., Wang, X., Dai, C., & Sun, B. (2020). Evidence for involvement of keystone fungal taxa in organic phosphorus mineralization in subtropical soil and the impact of labile carbon. *Soil Biology and Biochemistry*, *148*, 107900.

Evans, T. N., Watson, G., Rees, G. N., & Seviour, R. J. (2014). Comparing activated sludge fungal community population diversity using denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism. *Antonie Van Leeuwenhoek*, *105*, 559–569.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology*, *2*, 113–118.

Goyer, C., Neupane, S., Zebarth, B. J., Burton, D. L., Wilson, C., & Sennett, L. (2022). Diverse compost products influence soil bacterial and fungal community diversity in a potato crop production system. *Applied Soil Ecology*, *169*, 104247.

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993). Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. *Bio/Technology*, *11*, 1026–1030.

Keohavong, P., & Thilly, W. G. (1989). Fidelity of DNA polymerases in DNA amplification. *Proceedings of the National Academy of Sciences of the United States of America*, *86*, 9253–9257.

Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., Douglas, B., Drenkhan, T., Eberhardt, U., Duenas, M., Grebenc, T., Griffith, G. W., Hartmann, M., Kirk, P. M., Kohout, P., ... Larsson, K.-H. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology*, *22*, 5271–5277.

Liu, H., Pan, F., Han, X., Song, F., Zhang, Z., Yan, J., & Xu, Y. (2019). Response of soil fungal community structure to long-term continuous soybean cropping. *Frontiers in Microbiology*, *9*, 3316.

Liu, J., Yu, Y. Y., Cai, Z., Bartlam, M., & Wang, Y. Y. (2015). Comparison of ITS and 18S rDNA for estimating fungal diversity using PCR-DGGE. *World Journal of Microbiology & Biotechnology*, *31*, 1387–1395.

Lord, N. S., Kaplan, C. W., Shank, P., Kitts, C. L., & Elrod, S. L. (2002). Assessment of fungal diversity using terminal restriction fragment (TRF) pattern analysis: Comparison of 18S and ITS ribosomal regions. *FEMS Microbiology Ecology*, *42*, 327–337.

Mise, K., & Otsuka, S. (2020). Eukaryotic microbial communities in Japanese arable Andisols investigated by amplicon sequencing of 18S rRNA genes. *Microbiology Resource Announcements*, *9*, e00218-20.

Ririe, K. M., Rasmussen, R. P., & Wittwer, C. T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry*, *245*, 154–160.

Schmidt, P.-A., Balint, M., Greshake, B., Bandow, C., Roembke, J., & Schmitt, I. (2013). Illumina metabarcoding of a soil fungal community. *Soil Biology & Biochemistry*, *65*, 128–132.

Sugiyama, A., Vivanco, J. M., Jayanty, S. S., & Manter, D. K. (2010). Pyrosequencing assessment of soil microbial communities in organic and conventional potato farms. *Plant Disease*, *94*, 1329–1335.

Vainio, E. J., & Hantula, J. (2000). Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycological Research*, *104*, 927–936.

Yang, Y., Cheng, H., Dou, Y., & An, S. (2020). Plant and soil traits driving soil fungal community due to tree plantation on the loess plateau. *Science of the Total Environment*, *708*, 134560.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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