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TECHNICAL REPORT

Residual soil DNA extraction increases the discriminatory power between samples

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Abstract Forensic soil analysis relies on capturing an accurate and reproducible representation of the diversity from limited quantities of soil; however, inefficient DNA extraction can markedly alter the taxonomic abundance. The performance of a standard commercial DNA extraction kit (MOBIO PowerSoil DNA Isolation kit) and three modified protocols of this kit: soil pellet re-extraction (RE); an additional 24-h lysis incubation step at room temperature (RT); and 24-h lysis incubation step at 55 °C (55) were compared using high-throughput sequencing of the internal transcribed spacer I ribosomal DNA. DNA yield was not correlated with fungal diversity and the four DNA extraction methods displayed distinct fungal community profiles for individual samples, with some phyla detected exclusively using the modified methods. Application of a 24 h lysis step will provide a more complete inventory of fungal biodiversity, and re-extraction of the residual soil pellet offers a novel tool for increasing discriminatory power between forensic soil samples.

Keywords Soil · Forensics · Metagenomics · DNA extraction · High-throughput sequencing

Introduction

Soil is a powerful form of contact trace evidence that can link a suspect to a location, object or victim [1], or provide information on the likely origin of an unknown sample [2]. Soil microbial DNA fingerprinting methods, such as T-RFLP, have limited resolution and individual taxa cannot be identified [3]. In contrast, high-throughput sequencing (HTS) can detect a more detailed picture of the soil community, in particular, fungal diversity can provide better discrimination between soil samples than bacterial profiles [4, 5]. However, obtaining an accurate representation of soil communities has proven problematic due to difficulties in recovering DNA from complex soil matrices [6–9]. Studies have shown that portions of the endogenous DNA are not captured using standard extraction kit protocols [10, 11]. For example, previous studies indicate poor DNA recovery rates from commercial kits when soils are spiked with known amounts of DNA [12–14] and successive extraction from the residual soil pellet has shown increased DNA yield and diversity [10, 15]; however, the effect of such approach on discriminatory power remains unknown.

Using HTS of the internal transcribed spacer I (ITS1) ribosomal DNA, DNA yield, fungal diversity and discriminatory power using a standard commercial DNA extraction kit (MOBIO PowerSoil DNA Isolation kit) was compared to three modified methods: soil pellet re-extraction (RE); an additional 24-h lysis incubation step at room temperature (RT) and 24-h lysis incubation step at 55 °C (55). Five soil samples with distinct physical and chemical properties were used. The standard DNA extraction failed to detect some fungal phyla, and a 24 h lysis step is recommended. Furthermore, soil re-extraction isolated discriminative taxa and increased discriminatory power between soils.

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Materials and methods

Sample collection and DNA extraction

Five upper layer (0–20 cm) soil samples with different chemical properties (Table 1) were collected in sterile screw cap containers and stored at 4 °C prior to extraction. 250 mg soil was processed using the PowerSoil DNA Isolation kit (MOBIO, Carlsbad, CA, USA) following the manufacturer's protocol (MB). The residual soil pellet from MB was re-extracted (RE) following the same protocol. In addition, two modified versions of the protocol were performed involving a 24 h lysis incubation step at room temperature (RT) or 55 °C (55). Extraction blank controls were included. DNA yield was quantified using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, USA).

PCR amplification and library preparation

The internal transcribed spacer I (ITS1) was PCR amplified in triplicate [16] using universal fungal primers ITS5 (5′-CCTCTCTATGGGCAGTCGGTGATGGAAGTAAAAGTCGTAACAAGG-3′) and 5.8S_fungi (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG**nnnnnnn**CAAGAGATCCGTTGTTGAAAGTT-3′) [17] modified to include Ion Torrent sequencing adapters (underlined: P1 adapter on the ITS5 primer; An adapter on the 5.8S_fungi primer) and a multiplex identifier (in bold: MID tag) [18]. Each 25 µL reaction mix contained 2.5 mM $MgCl_2$, 0.24 mM dNTPs, 0.24 µm of each primer, 0.4 mg/µL BSA, 0.5 U Amplitaq Gold DNA polymerase in 10× reaction buffer (Applied Biosystems, Melbourne, Australia) and included 9 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 7 min. A no-template control was included for each MID tag. Agarose gel electrophoresis revealed no PCR products in the no-template or extraction blank controls. Purified PCR products (Agencourt AMPure XP PCR Purification kits, Beckman Coulter Genomics, Australia) were quantified (HS dsDNA Qubit Assay (Life Technologies, Carlsbad, CA, USA) and pooled to equimolar concentration. The amplicon library was sequenced on the Ion Torrent Personal Genome Machine™ using the Ion PGM™ 200 Sequencing Kit and an Ion 316™ semiconductor chip (Life Technologies).

Data analysis

Sequence reads were de-multiplexed (fastx_barcode_splitter, FASTX-toolkit v0.0.12; http://hannonlab.cshi.edu/fastx_toolkit), primer sequences were trimmed (Cutadapt v1.1 [19]), short sequences (<100 bp) were excluded, and remaining sequences quality filtered (fastq_quality_filter, FASTX-toolkit v0.0.12). 2,099,417 sequences, ranging from 27,639 to 231,488 sequences per sample remained (Table S1). In QIIME v.1.5.0 [20], all samples were rarefied to 1217 sequences to exclude differences due to sequencing depth and reads were de novo clustered at 97 % identity to create operational taxonomic units (OTUs) using UCLUST [21]. Venn diagrams were generated to visualize OTU overlap between different extraction methods (http://bioinfogp.cnb.csic.es/tools/venny/index.html) and a Bray–Curtis cluster dendrogram was generated in PRIMER6 (*PRIMER-E Ltd,* Luton UK) with default parameters. Discriminatory power using each method was measured as the mean pair-wise Bray–Curtis distance between the soils ($n = 10$), and statistical significance was determined using one-way ANOVA in SPSS Statistics 21 software package (IBM, USA). In addition OTUs detected exclusively upon re-extraction were isolated bio-informatically and the mean pair-wise Bray–Curtis distance was calculated using these OTUs independently.

Results

DNA yield and fungal diversity

Each modified method increased the DNA yield compared to the standard kit extraction (MB) demonstrating that a standard DNA extraction kit protocol fails to recover all DNA

Table 1 The optimal DNA extraction method for each soil type with regards to DNA yield and OTU count

Sample ID	Soil texture classification	Clay content (%)	pH (1:5)	Location	Latitude, Longitude	Highest DNA concentration (ng/mg soil)	Highest number OTUs detected
12092	Clay	60	8.3	Claremont (SA)	−34.58, 138.38	55	RT
12094	Clay loam	45	7.3	McLaren Vale (SA)	−35.15, 138.33	RT	RT
12093	Sandy loam	40	6.4	Urrbrae (SA)	−34.58, 138.38	RT	RT
12096	Sandy loam	33	7.6	Tammin (WA)	−31.49, 117.59	55	RT
12097	Silty loam	8	5.8	Drouin (VIC)	−38.13, 145.82	RT	RE

Extraction modifications: extraction of the residual soil pellet from MB (RE); 24 h lysis step at room temperature (RT); 24 h lysis step at 55 °C (55)

present within a sample (Fig. 1a). The increase in DNA yield afforded by each modification varied across soil types; however, the increase upon re-extraction was most consistent (75 ± 8 %, mean ± SD). The number of fungal OTUs was not correlated with total DNA yield (Fig. 1b). For soils with pH < 8, 24 h incubation showed greatest increased in OTU count, whereas for the soil with pH > 8 (12097) re-extraction was comparable to 24 h incubation. Across all soil types, a core set of OTUs (representing only 18.7 ± 3.1 % of the sample total) was detected for a given sample by all four extraction methods (Fig. S1). At phylum level, Ascomycota, Basidiomycota, and Zygomycota were consistently detected at relatively high abundance (>10 %) by all extraction methods (Table S2), whereas Blastiomycota, Chytridiomycota, Glomeromycota, and Neocallimastigomycota were present in very low abundance (<1 %) and inconsistently detected between different extraction methods. In some instances, these phyla were detected only when a modified method was applied. For example, Blastiomycota, Chytridiomycota, and Glomeromycota were only detected in sample 12097 using modified protocols.

Discriminatory power

Variation in OTU composition due to DNA extraction method did not prevent sample differentiation and discrimination (Fig. 2), indicating that extraction bias altered OTU composition less than the naturally occurring differences between samples. No significant difference in the mean Bray–Curtis distance was observed between the four different methods (one-way ANOVA, $F_{3,36} = 0.190$, $p = 0.856$), indicating that no single DNA extraction method consistently provided better discrimination between the soils (Fig. 2b). However, when the OTUs detected exclusively upon re-extraction were analyzed independently the discriminatory power between samples (i.e. the mean Bray–Curtis distance) was significantly increased.

Discussion

Detection of fungal diversity is complicated by natural distribution of taxa and the fact that individual fungal taxa have varying degrees of resistance to lysis, thus influencing the release of intracellular DNA into solution. Many soil fungi have melanized cell walls which provide resistance to lysis or can form resting structures, such as sclerotia, that allow fungi to survive in extreme conditions [22]. The data presented indicates that taxa remain undetected using only a single standard DNA extraction method and all modified protocols increased DNA yield and fungal diversity. Re-extraction offered the most consistent means of increasing

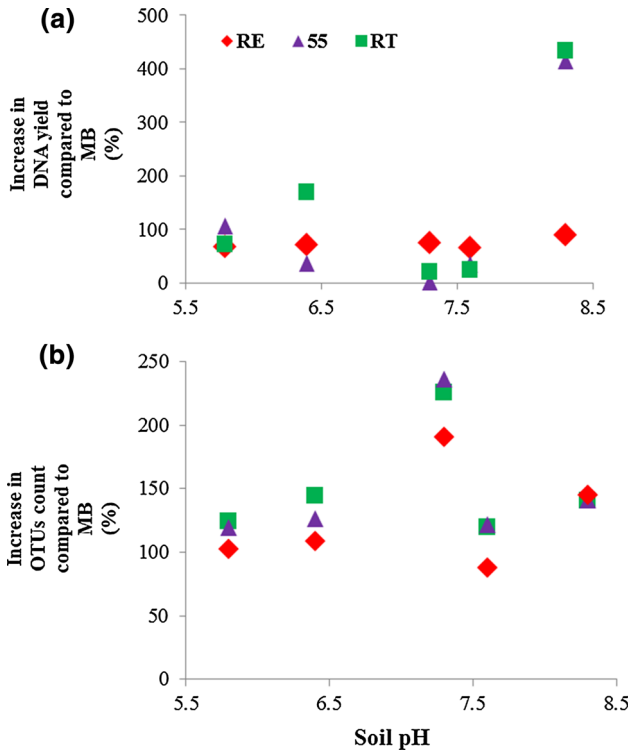

Fig. 1 Percent increase in **a** DNA yield and **b** OTU count using the standard MOBIO commercial kit and three modified DNA extraction methods. See Table 1 legend for abbreviations

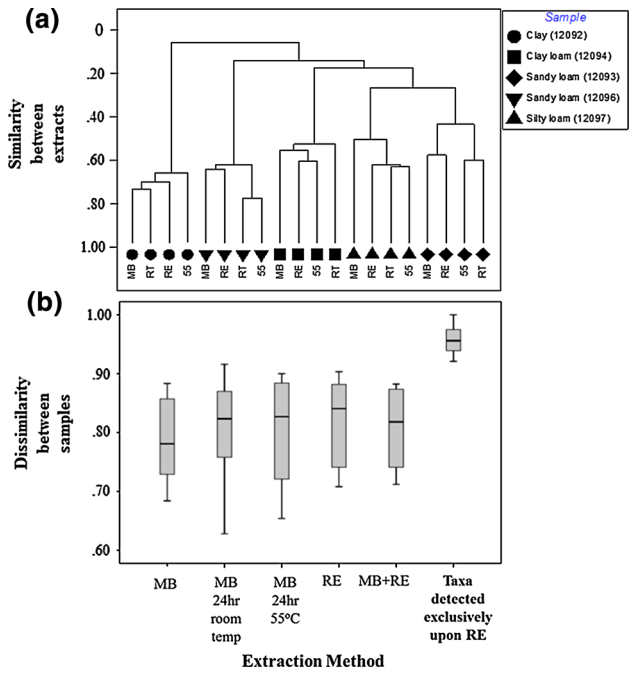

Fig. 2 Effect of DNA extraction modifications on discriminatory power. **a** Bray–Curtis cluster dendrogram illustrating that all five soils could be distinguished regardless of extraction method and **b** increase in discriminatory power when OTUs detected exclusively upon re-extraction were analyzed independently

DNA yield across different sample types; whereas, 24 h incubation varied depending on soil pH. DNA yield was not correlated with fungal diversity or the ability to discriminate between samples. However, 24 h incubation showed the greatest increase in OTU count, particularly for low pH soils. Re-extraction of the soil pellet offers a simple and efficient means to further increase diversity but most notably it offers a novel tool to increase discriminatory power between soils by isolating the least ubiquitous taxa. Further work is required to assess re-extraction between close proximity soils, and those with similar chemical properties and habitat locations. In addition, the effect of soil re-extraction on bacterial 16S rRNA profiles and eukaryote 18S rRNA profiles would be of interest, as these markers have the potential to complement the ITS fungal-specific results [5] and thus increase the strength of soil DNA evidence.

Key Points

1. During forensic soil analysis, maximizing the genetic information recovered and capturing an accurate representation of the diversity from limited quantities of soil is vital to produce robust, reproducible comparisons between forensic samples.
2. Using HTS of the internal transcribed spacer I (ITS1) ribosomal DNA, this study examines the performance of a standard commercial DNA extraction kit (MOBIO PowerSoil DNA Isolation kit) and three modified DNA extraction methods in terms of DNA recovery, fungal diversity, and the ability to discriminate between soil samples.
3. This study confirms that much of the DNA and fungal taxa present in single soil samples is not extracted using a single application of a standard DNA extraction kit, and that the optimal DNA extraction protocol varies depending on soil pH and clay content.
4. For a single soil type, unique OTUs were detected by applying different DNA extraction modifications; however, such variation in fungal diversity did not prevent discrimination between different samples.
5. The application of multiple DNA extraction methods will provide a more complete inventory of fungal biodiversity, and in particular, re-extraction of the residual soil pellet offers a novel tool for forensic soil analysis when only trace quantities are available.

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