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High throughput method of 16S rRNA gene sequencing library preparation for plant root microbial community profiling

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Microbiota are a major component of agroecosystems. Root microbiota, which inhabit the inside and surface of plant roots, play a significant role in plant growth and health. As next-generation sequencing technology allows the capture of microbial profiles without culturing the microbes, profiling of plant microbiota has become a staple tool in plant science and agriculture. Here, we have increased sample handling efficiency in a two-step PCR amplification protocol for 16S rRNA gene sequencing of plant root microbiota, improving DNA extraction using AMPure XP magnetic beads and PCR purification using exonuclease. These modifications reduce sample handling and capture microbial diversity comparable to that obtained by the manual method. We found a buffer with AMPure XP magnetic beads enabled efficient extraction of microbial DNA directly from plant roots. We also demonstrated that purification using exonuclease before the second PCR step enabled the capture of higher degrees of microbial diversity, thus allowing for the detection of minor bacteria compared with the purification using magnetic beads in this step. In addition, our method generated comparable microbiome profile data in plant roots and soils to that of using common commercially available DNA extraction kits, such as DNeasy PowerSoil Pro Kit and FastDNA SPIN Kit for Soil. Our method offers a simple and high-throughput option for maintaining the quality of plant root microbial community profiling.

The plant root is a key underground organ that interacts with soil, in which one of the richest microbial ecosystems on Earth exists[1]. Plant root microbiota, which inhabit the inside and surface of plant roots, improve plant growth by producing phytohormones, supplying nutrients, and protecting plants against pathogens and environmental perturbations, including drought and climate-dependent salinity changes[2–7].

Extensive efforts by the Earth Microbiome Project have led to the characterization of global taxonomic and functional microbial diversity, including the microbiome associated with plants[8]. In addition, many studies targeting plant microbiomes have been carried out, leading to the accumulation of large datasets[1,9–12], which will be utilized in agricultural applications through industry-academic collaborative projects[13]. Given that, along with the microbiome data, multi-omics analysis has been utilized in agricultural studies[14–16], high-throughput methods for the detection and analysis of plant microbiomes have become increasingly necessary.

Next-generation sequencing technology is continuously improving platforms to increase sequencing speed and quality. Even as sequencing capacity increases, sample preparation is still laborious and time-consuming, which leaves much to be desired for upgrading and expanding plant microbiome databases. Currently, many plant microbiome-based studies have used a kit-based or traditional DNA extraction method in a two-step PCR amplification protocol employed in 16S rRNA gene sequencing[12,17–20]. DNA extraction consists of homogenization and lysis, removal of contaminants, DNA purification processes. As a rigorous method, using isopropanol, acetone, RNase treatment, ethanol precipitation for removal of contaminants and DNA purification requires total 19 steps

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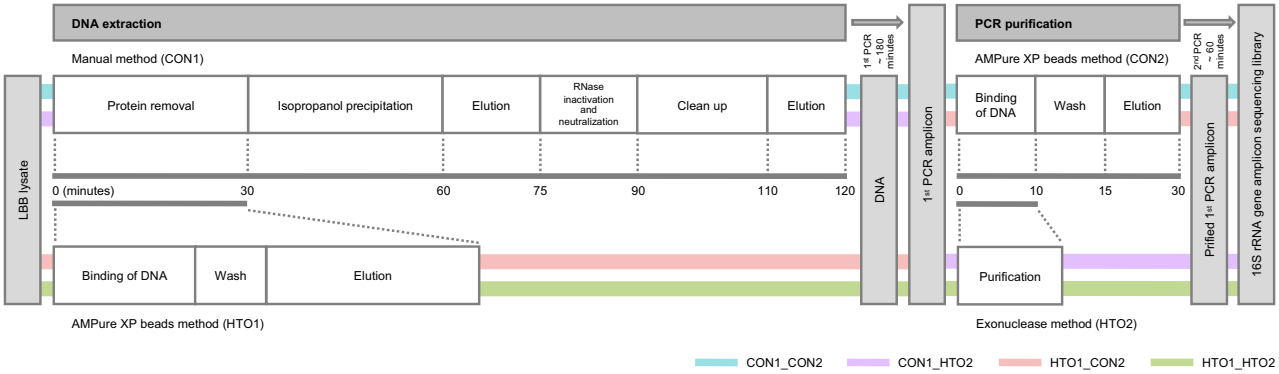

Figure 1. Schematic overview of the methods tested in this study. Experimental procedure for microbiome analysis. For DNA extraction, the AMPure XP bead method (High-throughput option 1, HTO1) and manual method as control (Control 1, CON1) were tested. For the first PCR purification in the library preparation, the exonuclease method (High-throughput option 2, HTO2) and AMPure XP bead method as control (Control 2, CON2) were tested.

and takes approximately 2 h (Fig. 1). Extensive efforts have been made in the improvement on each process, and column-based method, where removal of contaminants and DNA purification processes are performed on the column, are currently used as the standard methods of DNA extraction for microbiome studies[1,10,12,17–19,21–34]. However, these standard methods of DNA extraction were difficult to be applied for a high-throughput procedure. On the other hand, a high-throughput platform requiring the dedicated devices and kits have been used for Earth Microbiome Project[35], but this is not always available for the labs conducting small projects.

Although single-step PCR for library preparation has been previously used for plant microbiome studies[1,10,21,22,24–29,31,34], the single-step PCR required full length primers including adaptors with indices, leading to the potential bias due to the use of different indices in each PCR amplification. To avoid the bias, the two-step amplification method has been developed[30]. The standard two-step PCR amplification method, including the method provided by Illumina, used AMPure XP beads for PCR purification before the second PCR, while a custom protocol used for phyllosphere microbiome study used exonuclease treatment (Fig. 1)[20]. However, no study compared these different methods employed for PCR purification before the second PCR.

In this study, we present several improvements to the protocol of 16S rRNA gene sequencing (Fig. 1). We performed benchmarking experiments comparing our protocol with manual methods as well as several commonly used kits for DNA extraction to demonstrate a simple and high-throughput option for plant root microbial community profiling.

Materials and methods

Sample collection. Soybean (*Glycine max* (L.) Merr., Peking) was sown in a sandy field at the Arid Land Research Center, Tottori University, Japan, in July 2018. Plants were cultivated under two water conditions (well-watered and water-limited drought block) in two replicates. White mulching sheets (Tyvek, Dupont, US) and watering tubes were installed to control the soil conditions. Artificial irrigation from watering tubes was applied for 5 h daily in the well-watered blocks, while no artificial irrigation was used in the water-limited blocks from 14 days after sowing. Sixty-two days after sowing, the plant roots were harvested and washed with tap water. The tips (~ 2 cm in length) of lateral roots developed from the main roots at 0–10 cm from the shoot/root junction were collected and kept at − 80 °C until sample preparation. The sampled root tissues were thought to contain endophytes and may have also contained the bacteria remaining from the rhizoplane. For mock bacterial community experiment, we used soil sample with ZymoBIOMICS Microbial Community Standard (Zymo Research, Cat. #D6300), where we added 5 µL of the mock microbial community standards in 500 mg of gray lowland soil. To compare our method with commonly used DNA extraction kits (KIT1/2, details in the following section), we used 5 plant root samples and 4 soil samples collected from different plant species (soybean roots, rice roots, and *Andropogoneae* sp. roots) and different sites (brown forest soil [36° N, 140° E], gray lowland soil [37° N, 140° E; 43° N, 141° E; 33° N, 130° E], peat soil of rice paddy [43° N, 141° E]).

DNA extraction. The collected root tissues were cooled with liquid nitrogen and immediately were ground to a fine powder at 3000 rpm for 15 s using a Multi-Beads Shocker (Yasui Kikai Co. Osaka, Japan, Cat. #MB2200(S)). For each of the collected tissue samples, 500 mg of the powdered sample was transferred into a 1.5 mL tube cooled by liquid nitrogen. One mL of lysate binding buffer (1 M LiCl Sigma-Aldrich, Cat. #L7026-500ML; 100 mM Tris–HCl, Wako, Cat. #318-90225; 1% SDS, Wako, Cat. #313-90275; 10 mM EDTA pH 8.0, Wako, Cat. #311-90075; Antifoam A, Sigma-Aldrich, Cat. #A5633-25G; 5 mM DTT, Wako, Cat. #048-29224; 11.2 M 3-Mercapto-1,2-propanediol, Wako, Cat. #139-16452; DNase/RNase-free H_2O, Thermo Fisher Scientific, Cat. #10977015)[36] was added to the sample, which was then homogenized by vortexing, followed by incubation at room temperature (~ 22 °C) for 5 min. The tube was centrifuged at 15,000 rpm for 10 min at room temperature, and the supernatant (LBB lysate) was transferred to a new 1.5 mL tube. DNA extraction was performed using the following two methods: our custom protocol involving isopropanol, acetone, RNase treatment, ethanol precipitation method[36] as the control manual method, and extraction using AMPure XP beads (Beckman Coul-

ter, Cat. #A63881). DNA concentration and absorbance were measured with a spectrophotometer (NanoDrop One^{C} Microvolume UV–Vis Spectrophotometer with WiFi, Thermo Fisher Scientific, Cat. #ND-ONEC-W) as well as a fluorometer (Qubit™ 4 Fluorometer, Thermo Fisher Scientific, Cat. #Q33238). To compare our method with commonly used kits, we used the same amount of ground samples to extract DNA with DNeasy PowerSoil Pro Kit (denoted as KIT1, QIAGEN, Cat. #47014) and FastDNA SPIN Kit for Soil (denoted as KIT2, GEN, Cat. #6560-200) following the manufacturer's protocols.

Manual method for DNA extraction (Control 1, CON1). The detailed method has been described in our previous publications[36,37] and was also used for soil and plant root microbial community profiling[14]. Briefly, LBB lysate (200 µL) was added to a 1.5 mL tube, and 5 µL of 10 mg/mL proteinase K was added to it; the mixture was then incubated at 37 °C for 30 min. Next, 200 µL of 100% isopropanol was added to this sample, and this mixture was mixed gently, incubated at room temperature for 5 min, and centrifuged at 15,000 rpm for 5 min. The supernatant was discarded to avoid pellet loss, and 400 µL of 100% acetone was added to the tube. This was mixed gently, incubated at room temperature for 5 min, and centrifuged at 15,000 rpm for 5 min. The supernatant was carefully discarded to avoid pellet loss, and the pellet was dried. After repeated decolorization using acetone, we added to the tube 100 µL of 10 mM Tris–HCl (pH 7.5), incubated at 65 °C for 10 min, and subsequently centrifuged at 15,000 rpm for 1 min. The supernatant was transferred to a new 1.5 mL tube, and 1 µL of 1 µg/µL RNase A was added and incubated at 37 °C for 15 min. The supernatant was added to 10 µL of 3 M ammonium acetate and 250 µL of 100% ethanol, mixed, incubated at room temperature for 5 min, and centrifuged. The supernatant was discarded, and 400 µL of 80% ethanol was added, mixed, incubated at room temperature for 2 min, and centrifuged at 15,000 rpm for 1 min. The supernatant was carefully discarded to avoid pellet loss, and the pellet was dried. DNA was eluted in 50 µL of 10 mM Tris–HCl (pH 7.5).

AMPure XP beads method for DNA extraction (High-throughput option 1, HTO1). Fifty µL of LBB lysate was put into 1.5 mL tubes, and an equal amount of AMPure XP beads was added, followed by incubation at room temperature for 5 min after vortexing. The mixture was placed on a magnetic station for 5 min, and the supernatant was removed. The magnetic beads were washed twice with 200 µL of 80% ethanol. Finally, DNA was eluted with 20 µL of 10 mM Tris–HCl (pH 7.5).

16S rRNA gene sequencing. Library preparation using a two-step PCR amplification protocol has been reported in our previous publication[14]. In this study, we compared two purification methods: magnetic beads-based purification and exonuclease after the first PCR step. Briefly, the V4 region of bacterial 16S rRNA gene was amplified with 515f and 806rB primers (forward primer: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG- [3–6-mer Ns]—GTG YCA GCM GCC GCG GTA A-3′; reverse primer: 5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G [3–6-mer Ns]—GGA CTA CNV GGG TWT CTA AT-3′)[21,38] Each sample (1 µL of tenfold diluted DNA) was amplified in a 10 µL reaction volume containing 0.2 U KOD FX Neo DNA polymerase (TOYOBO Co., Ltd., Osaka, Japan), 2 × PCR buffer (TOYOBO), 0.4 mM dNTPs (TOYOBO), 0.2 µM forward and reverse primers, and 1 µM blocking primers (mPNA and pPNA, PNA BIO, Inc., Newbury Park, CA). PCR was performed using the following specifications: 94 °C for 2 min followed by 35 cycles at 98 °C for 10 s, 78 °C for 10 s, 55 °C for 30 s, 68 °C for 50 s, and a final extension at 68 °C for 5 min (ramp rate = 1 °C/s). The PCR products were then purified by two separate methods (See 3–1 and 3–2). The second PCR was carried out with the following primers: forward primer: 5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC AC—[8-mer index]—TCG TCG GCA GCG TC -3′, and reverse primer: 5′- CAA GCA GAA GAC GGC ATA CGA GAT—[8-mer index]—GTC TCG TGG GCT CGG-3′[39]. Each sample (0.8 µL of purified product from the first PCR) was amplified in a 10 µL reaction volume containing 0.2 U KOD FX Neo DNA polymerase (TOYOBO), 2 × PCR buffer (TOYOBO), 0.4 mM dNTPs (TOYOBO), 0.3 µM forward and reverse primers, and 1 µM blocking primers (mPNA and pPNA). PCR was performed as follows: 94 °C for 2 min, followed by 8 cycles at 98 °C for 10 s, 78 °C for 10 s, 55 °C for 30 s, 68 °C for 50 s, and a final extension at 68 °C for 5 min (ramp rate = 1 °C/s). Following amplification, PCR products for each sample were cleaned and size-selected using AMPure XP beads and washed twice with 80% ethanol. The libraries were eluted from the pellet with 10 µL of 10 mM Tris–HCl pH 7.5, quantified with a microplate photometer (Infinite 200 PRO M Nano^{+}, TECAN Japan Co., Ltd.), and pooled into a single library in equal molar quantities. The pooled library was sequenced on an Illumina MiSeq platform using a MiSeq Reagent Kit v3 (600-cycles) and MiSeq Reagent Nano Kit v2 (500-cycles) (Illumina, CA, USA).

AMPure XP beads method for PCR purification (Control 2, CON2). A solution containing AMPure XP beads (10 µL) was added to 10 µL of product obtained from the first PCR, and the mixture was incubated at room temperature for 5 min after mixing by vortexing. The mixture was then placed on a magnetic station for 5 min, and the supernatant was subsequently removed. The magnetic beads were washed twice with 200 µL of 80% ethanol. The purified sample was eluted from the beads by incubation with 10 µL of 10 mM Tris–HCl (pH 7.5).

Exonuclease method for PCR purification (High-throughput option 2, HTO2). Two µL of ExoSAP-IT Express (Thermo Fisher Scientific, Cat #75001.1.EA) was added to 5 µL of the product obtained from the first PCR, and the mixture was incubated at 37 °C for 4 min, followed by 80 °C for 1 min.

Bioinformatics. Bioinformatics and statistical analyses were carried out using the Quantitative Insights Into Microbial Ecology 2 program (QIIME 2, ver. 2020.6.0, https://qiime2.org/) installed through a docker[40].

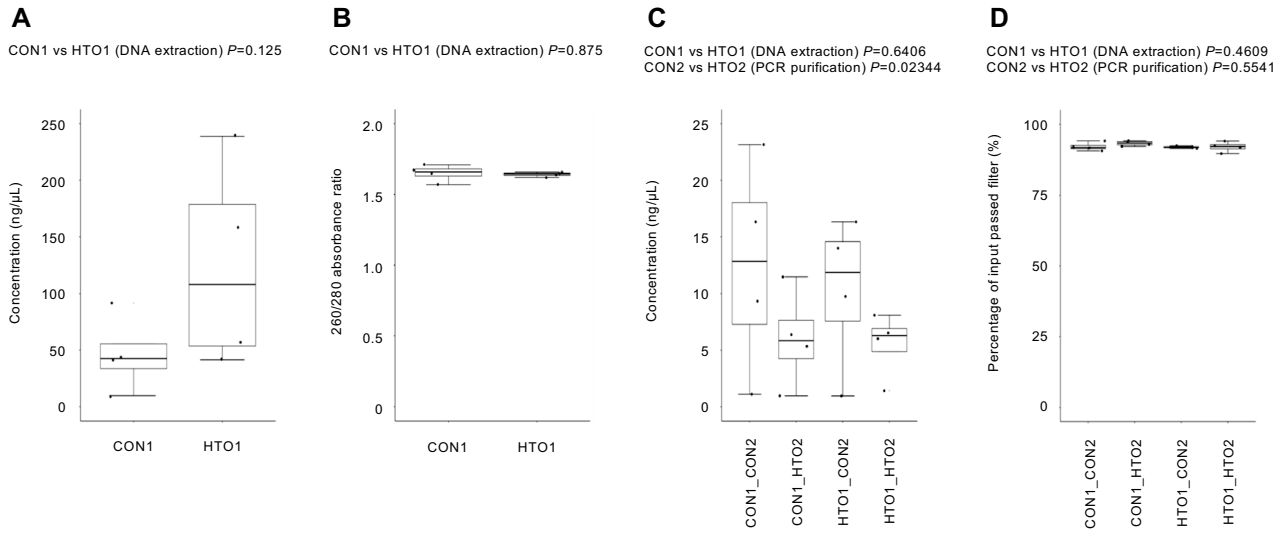

Figure 2. Yield and quality of DNA extraction and library preparation among different methods. The yield (ng/μL) (**A**) and quality (260/280 absorbance ratio) of nucleic acid (**B**) are shown for the AMPure XP bead and isopropanol methods. The concentration of the library (ng/μL) (**C**) and reads passing set quality filter (%) (**D**) are shown for all methods described in Fig. 1.

The raw paired-end FASTQ files were imported into the QIIME2 program and demultiplexed using a native plugin. Thereafter, the Cutadapt plugin was processed primer trimmed. The Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin in QIIME2 was used for quality filtering. The demultiplexed FASTQ file was trimmed, de-noised, the chimera was removed, and the data were merged[41]. We applied the parameter with truncation length of 220 for both forward and reverse reads. Taxonomic groups were assigned identity with the Naive Bayes q2-feature-classifier trained using the 515F/806R region from 99% operational taxonomic units (OTUs) from the SILVA 138 rRNA database[42,43]. Contaminating archaeal, eukaryotic, mitochondrial, and chloroplast sequences were filtered out of the resulting feature table. After taxonomic assignment of amplicon sequence variants (ASVs), the remaining representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in IQ-TREE multicore version 2.0.3[44]. The sampling depth parameter were set to 1,181 (HTO1/2 vs. CON1/2, Supplementary Dataset 1) and 2,988 (HTO1_HTO2 vs. KIT1/2_CON2, Supplementary Dataset 2), which were chosen based on the number of sequences in the sample containing lowest number of sequences (Supplementary Fig. S1). Finally, diversity indices such as Shannon diversity, Faith phylogenetic diversity, Bray–Curtis distance, and weighted UniFrac distance were calculated using the QIIME2 diversity plugin. The resulting data were exported as a BIOM table and imported to the LDA Effect Size (LEfSe) algorithm to determine the differences in biomarkers[45]. The LEfSe was performed with the following parameters: non-parametric factorial Kruskal–Wallis test, pairwise Wilcoxon test ($P < 0.05$), and LDA > 2.0. Rank-Abundance Dominance (RAD) analysis was performed using the R package *RADanalysis* ver. 0.5.5[46]. These RAD curves display logarithmic species abundances against rank order using the minimum richness ($R = 40$) to normalized data.

Ethics declarations. All experiments using plant materials were in accordance with local and national regulations.

Results

Yield and quality of DNA extraction among different methods. We initially searched for commercial DNA extraction methods for plant tissue and soil samples to establish a high-throughput experimental procedure; however, we ultimately found that utilizing AMPure XP beads combined with the proper buffer enabled the extraction of DNA from plant tissue and soil samples (HTO1, Supplementary Fig. S2). When we compared the use of Tris–EDTA (TE) buffer and Lysate Binding Buffer (LBB) in the process of magnetic bead binding, DNA was successfully extracted from plant roots and soil samples when using LBB rather than TE (Supplementary Fig. S3). In addition, the AMPure XP beads method using LBB (HTO1) showed compatible yields of extracted nucleic acids with those obtained with a rigorous manual method using multiple procedures in removal of contaminants and DNA purification (CON1)[36,37] (Fig. 2A). This is supported by the result using a fluorometer that CON1 shows 8.176 (2.943) ng/μL, mean (SE), and HTO1 shows 6.409 (2.434) ng/μL ($P = 0.66$). Notably, unlike CON1, HTO1 does not include RNase treatment, which could be a reason for the resultant higher yield of extracted nucleic acids. On the other hand, both methods showed a 260/280 absorbance ratio of ~ 1.8, suggesting that the extracted nucleic acids were relatively pure DNA from the plant tissue sample (Fig. 2B). Like CON1, HTO1 showed compatible molecular weight of DNA bands in agarose gel electrophoresis analysis (Supplementary Fig. S3). Given that HTO1 achieved a 75% reduction in the sample handling time compared to CON1 (Fig. 1), the DNA extraction using AMPure XP beads could prove to be a high-throughput option that maintains a compatible yield and quality with that of the rigorous manual method for plant roots and soil samples.

Yield and quality of library preparation among different methods. The 2-step PCR amplification protocol is a common method used for 16S rRNA gene sequencing. During library preparation, PCR purification with AMPure XP beads before the second PCR is commonly used in the standard methods including the Illumina kit (CON2), but several cases used exonuclease treatment in the PCR purification (HTO2)[14,39]. To assess the effect of HTO2 combined with our DNA extraction protocol (HTO1), we compared the methods with all combinations of extraction and purification protocols, that is, the manual method for DNA extraction and AMPure XP beads method for PCR purification (CON1_CON2), the manual method for DNA extraction and exonuclease method for PCR purification (CON1_HTO2), AMPure XP beads method for DNA extraction and AMPure XP beads method for PCR purification (HTO1_CON2), and AMPure XP beads method for DNA extraction and exonuclease method for PCR purification (HTO1_HTO2) (Fig. 1). Since we used DNA solutions with equal concentrations for 16S rRNA gene sequencing library preparation, the concentration of the library was a result of the differences in the yield of nucleic acids among different DNA extraction methods (Fig. 2C). HTO2 showed a lower yield compared to CON2 (Fig. 2C). Using AMPure XP beads method in CON2 also functions size selection, such that the size selection before the second PCR (prior to final size selection) might enrich the first PCR product with the target size, leading to a high yield of library products. In contrast, size selection before the second PCR is associated with a risk of biased generation of specific PCR amplicons; the exonuclease treatment in HTO2 could reduce this risk and rescue the minor PCR amplicons. Alternatively, HTO2 might inhibit the second PCR. Sequencing of the V4 region of bacterial 16S rRNA gene was carried out using 16 samples, which included all combinations of the methods that we tested in this study. A total of 120,849 reads with a mean read count of 7,553 reads per sample and a range of 1,510–24,782 reads were obtained. A total of 89.58–94.15% of reads passed the set quality filter (Fig. 2D). To evaluate the entire process of HTO1_HTO2 method in 16S rRNA gene sequencing, we performed an independent experiment using the soil sample including mock bacterial community standard and confirmed to detect 75% of mock bacterial species in HTO1_HTO2 method (Supplementary Fig. S4). Notably, HTO2, in addition to maintaining the sequencing quality, achieved greater than 60% reduction in the sample handling time compared to that required for CON2 (Fig. 1).

Comparison of the diversity of plant microbial community observed among different methods. The alpha diversity based on the number of observed species, such as number of observed ASV, Shannon index, and Faith phylogenetic diversity, were compared among the methods. HTO2 detected ~ 96% more ASVs ($P < 0.05$, Fig. 3A) and significantly increased the alpha diversity relative to CON2 ($P < 0.05$, Fig. 3B,C). These data support the idea that the exonuclease treatment in HTO2 could rescue the minor PCR amplicons, suggesting that this method can capture higher degrees of microbial diversity. To evaluate these methods based on the profiling efficiency of the microbial communities, principal coordinate analysis (PCoA) based on Bray–Curtis distances and weighted UniFrac distances[47] was performed. As a result, samples with two water conditions and two biological replicates were separated from each other (PerMANOVA $P < 0.05$; Dispersion $P > 0.05$), while samples processed using different methods were clustered together in the PCoA space considering both Bray–Curtis distances and weighted UniFrac distances (PerMANOVA $P > 0.05$, Fig. 3D,E). This indicates that the effect of differences among methods was much smaller for the microbial community profile than that of the biological sample differences, suggesting that our modified method (HTO1_HTO2) has a similar ability to capture the overall profile of the microbial community as that obtained using the manual method.

Comparison of the taxonomic profile of plant root microbiome obtained using different methods. Our data showed that *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *and Bacteroidetes* were the most abundant bacterial phyla, while *Streptomycetaceae*, *Oxalobacteraceae*, *Burkholderiaceae*, *Bacillaceae*, *Chitinophagaceae*, *Paenibacillaceae*, and *Xanthobacteraceae* were the most abundant bacterial families in our samples (Fig. 4A,B). The taxonomic profile detected order *Rhizobiales*, which includes *rhizobia*[48] and is similar to that obtained from soybean rhizosphere soil samples reported in previous studies[49,50]. In addition, our data showed that the phyla *Actinobacteria* and *Firmicutes* were enriched, and *Proteobacteria* and *Bacteroidetes* were depleted in the root microbiota of soybeans cultivated under water-limited drought conditions (Fig. 4A,B); this is consistent with the results of a previous study that investigated the rhizosphere communities of several plant species[51–53], confirming that our method can generate comparable microbiome profile data. Furthermore, we detected no significant differences in the abundance of the gram-positive *Bacillus* sp. among the different methods ($P > 0.1$, Fig. 4C), showing that HTO1 does not interfere the downstream processes from lysis of gram-positive bacteria with a thick peptidoglycan layer of the cell wall, which is comparable to that of CON1. Hierarchical clustering heat map based on the 20 most abundant ASVs showed that samples clustered into four groups according to the different biological samples and not according to the different methods (Fig. 4D). Furthermore, our LEfSe analysis discriminating four different methods showed no significant differences ($P > 0.05$); nonetheless, the following varied minor bacteria were identified based on different DNA extraction and PCR purification methods ($P < 0.05$): *Enterobacteriaceae* (average 2.46% relative abundance, enriched in CON1) and *Planctomycetales* (0.07%, enriched in HTO1) for the tested DNA extraction method; *Xanthobacteraceae* (0.25%, enriched in HTO2), *Sphingobacteriaceae* (0.21%, enriched in HTO2), *KD4_96* (0.08%, enriched in HTO2), *Noviherbaspirillum* (0.07%, enriched in HTO2), *Acetobacterales* (0.06%, enriched in HTO2), *Nitrospirota* (0.05%, enriched in HTO2), and *Bacteriovoracales* (0.03%, enriched in HTO2) for the tested PCR purification method. Thus, our modified method (HTO1_HTO2) can capture similarly abundant taxonomic profiles of plant root microbiome when compared to those obtained using manual methods, as well as show the potential to detect minor bacteria (less than 0.3% relative abundance, Supplementary Fig. S5).

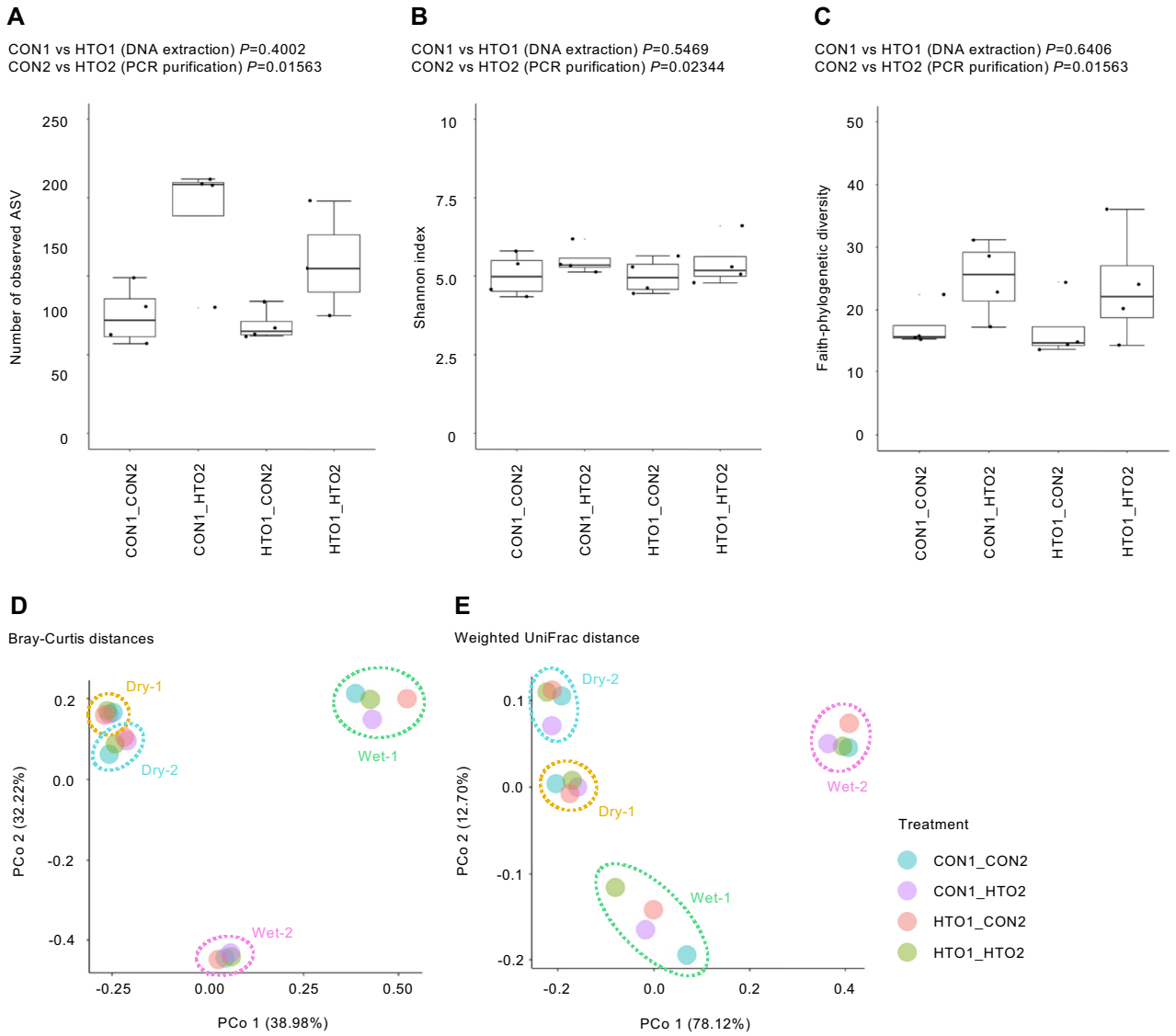

Figure 3. Plant microbial community diversity among different methods. Alpha diversity metrics of the number of observed ASVs (**A**), Shannon diversity (**B**), and Faith phylogenetic diversity (**C**). Principal coordinate analysis based on Bray–Curtis distances (**D**) and weighted UniFrac distances (**E**). The circles with different colors indicate biological samples with different methods.

Comparison of new high-throughput method with common commercially available kits in plant root and soil samples. To assess our new high-throughput method (HTO1_HTO2) as an alternative to the procedures using common commercially available DNA extraction kits adopted by many previous studies[1,10,12,17–19,21–34], we used 5 plant root samples and 4 soil samples collected from different plant species and sites to compare between the HTO1_HTO2 method and the protocols using DNeasy PowerSoil Pro Kit, and FastDNA SPIN Kit for Soil as DNA extraction (KIT1 and KIT2, respectively) combined with CON2 as PCR purification in the library preparation. The PCoA based on Bray–Curtis distances and weighted UniFrac distances showed the clear difference between plant roots and soils, and root samples of different plant species were separated from each other, while samples processed using different methods were clustered together (Fig. 5). Although alpha diversity is different among methods in several sample (Supplementary Fig. S6), PerMANOVA for beta diversity did not detect any significant differences among methods for each sample ($P > 0.05$), supported that the overall taxonomic profiles are consistent among different methods for each sample (Supplementary Fig. S7). Taken together, these results of diversity analyses and taxonomic profiling showed that our new method generated comparable microbiome profile data to that of using standard DNA extraction kits in all plant roots and soils tested in this study.

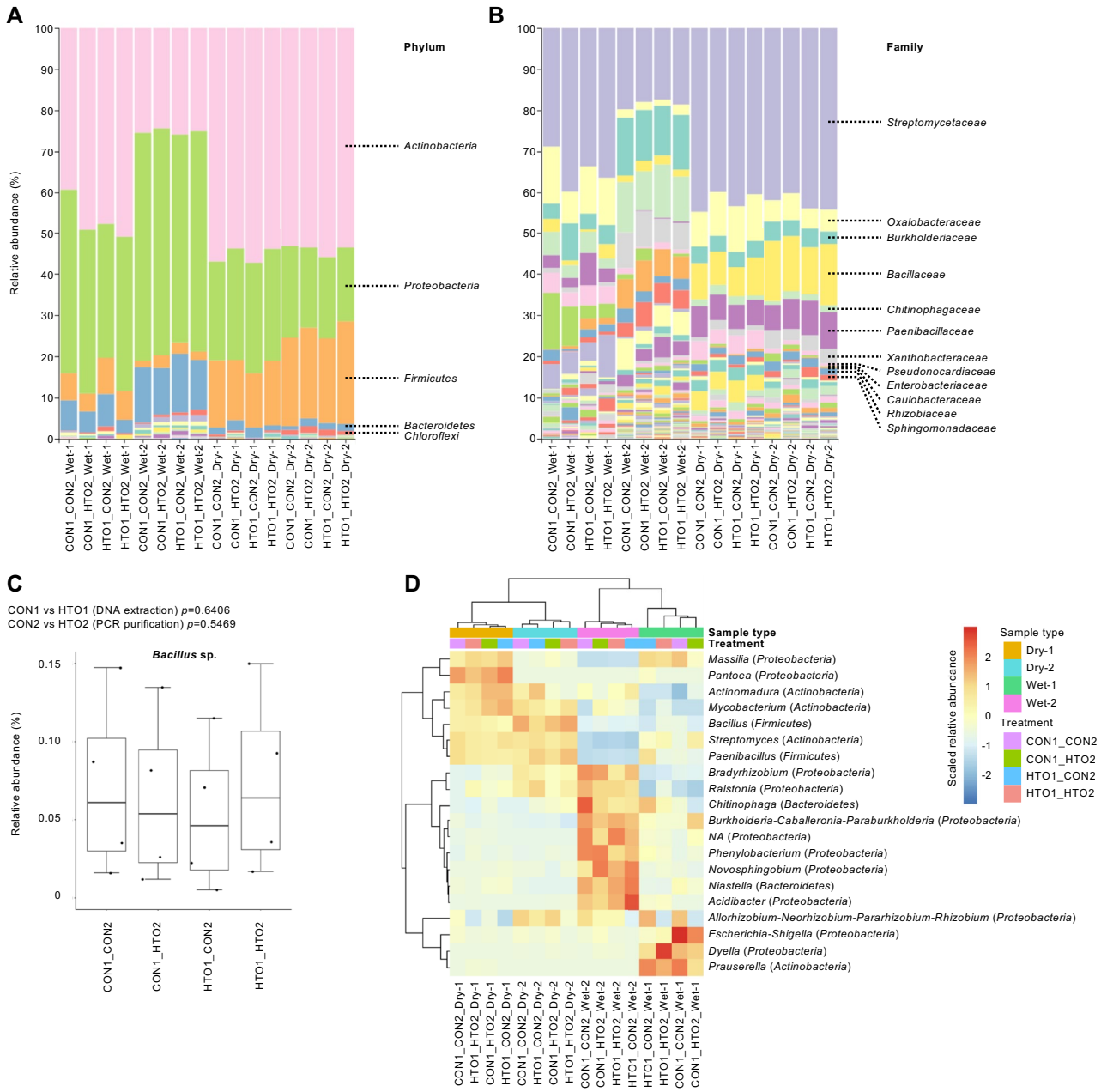

Figure 4. Taxonomic profile of plant root microbiome among different methods. The relative abundances of major phyla (**A**), family (**B**), and gram-positive *Bacillus* sp. (**C**) are shown. (**D**) Hierarchical clustering heat map based on the relative abundance of the 20 most abundant ASVs. The heat map was visualized using pheatmap version 1.0.12 (https://cran.r-project.org/web/packages/pheatmap/index.html).

Discussion

We have developed a new, simple, and high throughput protocol of 16S rRNA gene sequencing library preparation method for plant root microbial community profiling, providing benchmarking data comparing between the newly developed method and other manual methods (Figs. 1, 2, 3, 4) as well as methods using commonly used DNA extraction kits (Fig. 5). In contrast to the manual protocols, our method generated high-quality plant root microbiome data with a marked improvement in the ability to detect minor bacteria (Fig. 3 and Supplementary Fig. S5). The data showed good agreement with that showing the taxonomic profile of the soybean rhizosphere microbiome, as well as successfully detected the changes in the taxonomic profile of the rhizosphere in response to drought treatment, a phenomenon also reported in previous studies[51–53] (Fig. 4). In addition, our method generated comparable microbiome data in plant root and soils to that of methods using commonly used DNA extraction kits (Fig. 5). Our method uses AMPure XP magnetic beads for DNA extraction and exonuclease treatment for PCR purification, both of which are compatible with an automated process, enabling the simultaneous

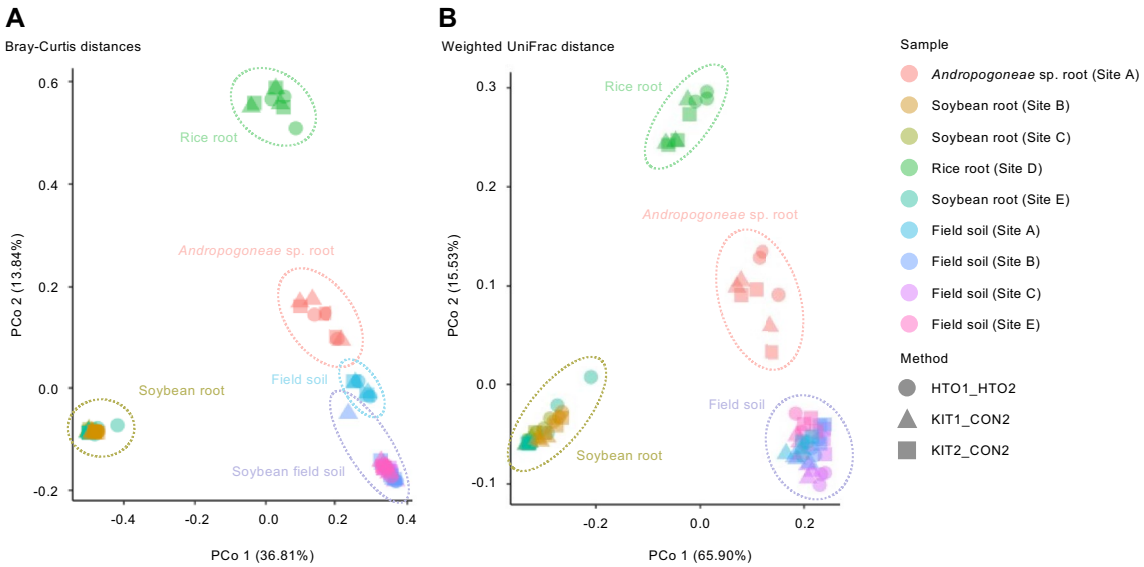

Figure 5. Plant root and soil microbial community diversity among new method and commonly used kits. Principal coordinate analysis based on Bray–Curtis distances (**A**) and weighted UniFrac distances (**B**). The circle with different biological samples. Cultivated or growing naturally plant roots and various types of soils were sampled from different geographical sites: Site A, brown forest soil (36°N, 140°E); Site B, gray lowland soil (37°N, 140°E); Site C, gray lowland soil (43°N, 141°E); Site D, peat soil of rice paddy (43°N, 141°E); Site E, gley lowland soil (33°N, 130°E).

sequencing of thousands of samples. In addition, our method can be applied for more variable regions in 16S rRNA gene to improve the resolution in microbial community profiling[54], which necessary for further studies.

Previous studies regarding plant microbiomes have commonly used column-based methods for DNA extraction, combined with bead-beating to lyse bacterial cells in plant tissues[1,10,12,17–19,21–28] and soils[29–34]. The column-based DNA extraction method performed removal of contaminants and DNA purification processes on the column, but still requires laborious and time-consuming handlings like rigorous manual method. Also, the column-based method requires a large amount of sample (> 100 mg). Our method using AMPure XP magnetic beads, HTO1, has successfully minimized the number of procedures to enable the extraction of DNA from low amounts of sample (~ 20 mg), which is reflected in the fact that the reaction is completed in a single tube (Fig. 1). One of the achievements of this study is finding a buffer that can be utilized for DNA extraction using AMPure XP magnetic beads (Supplementary Figs. S2, S3). The buffer LBB was originally developed for RNA-seq library preparation in our previous study and can be used for DNA extraction with the manual method[36,37]. Since we have applied our DNA extraction method not only for plant root samples, but also for soil samples (Fig. 5 and Supplementary Fig. S3), this method can be further applied for microbiome analysis of environmental samples. In addition, recent studies associated to plant microbiomes have been focused on the functional aspects of microbiota at the gene level using metagenome sequencing in addition to taxonomic community profiling[11]; the HTO1 can be applied to fulfill the demand of high-throughput options for metagenome sequencing.

Another significant finding of this study is that the exonuclease treatment for PCR purification showed a high ability to capture higher degrees of microbial diversity, especially minor bacteria (Figs. 3 and Supplementary Fig. S5). Rare bacterial species are increasingly recognized as crucial components of Earth's ecosystems[55]. Several studies have shown that low-abundance plant-associated microbes enhance crop productivity and defense[56–58]. Given that our method HTO2 can detect minor bacteria and capture the abundant taxonomic profile (Figs. 3 and 4, Supplementary Fig. S5), this methodology would certainly contribute to the systematic accumulation of high-quality microbiome data.

Conclusions

We have successfully developed a simple and high throughput protocol of 16S rRNA gene sequencing library preparation method for plant root microbial community profiling. Using this method, we have produced libraries not only from soybean and *Oryza* sp., but also from *Arabidopsis thaliana*, *Brachypodium distachyon*, and *Brassica* sp., in addition to producing more than 3,200 libraries from plant roots cultivated in different agricultural fields from gray lowland soil to andosol (Ichihashi lab, unpublished results). Our method with reduced sample handling and compatibility with automated processes can be instrumental in future microbiome research with large-scale data.

Data availability

The reported DNA sequence data are available in the DDBJ Sequence Read Archive under the accession number DRA011499 and DRA013843.

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Author contributions

Y.I. designed the project; K.K. and E.U. performed most of the experiments and data analyses; K.S. and S.K. participate in bioinformatics analyses; T.S., Y.T., H.T., S.S., M.N., A.T., K.M., Y.Y., H.T., and H.I. performed filed experiment and collected samples; K.K., E.U., and Y.I. wrote the manuscript with inputs from all other authors. The authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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