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Composition and diversity of root-inhabiting bacterial microbiota in the perennial sweet sorghum cultivar at the maturing stage

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Abstract

The bacterial microbiota inhabiting the endosphere and rhizoplane regulate plant growth. The mutualistic interaction between sweet sorghum and soil bacteria has drawn increasing research attention. Nevertheless, the root-inhabiting bacterial microbiota of sweet sorghum's perennial analog have rarely been characterized. Here, the root-inhabiting bacterial microbiota of the perennial sweet sorghum cultivar NaPBS778 (N778 simply) and its control TP60 were discovered at the flowering and maturing stages under field growth by high-throughput amplicon sequencing of the 16S rRNA gene via Illumina MiSeq. Nearly all alpha diversity indices of aerial and primary root samples of N778 were not significantly distinct from those of TP60 at the maturing stage, except for the observed species (Sobs) and phylogenetic diversity indices. The beta diversity of aerial and primary root samples showed no significant differences between N778 and its control TP60 at the maturing stage. Moreover, the bacterial microbiota in N778 aerial and primary roots was not only predominated by Proteobacteria, Actinobacteria, and Bacteroidetes at the phylum level but also strikingly distinct from the bacterial microbiota in rhizosphere soil at the genus level. Additionally, the root samples of N778 at the maturing stage were considerably enriched with OTU1262 being a potential cold-adapted bacterium belonging to *Pseudarthrobacter*, OTU434 plus OTU1304 belonging to *Streptomyces* and associated with crop nitrogen stress-tolerance, and OTU836 belonging to the family Oxalobacteraceae and potentially promoting crop growth. Our findings suggest that the perennial sweet sorghum cultivar N778 may recruit potentially cold-tolerant, plant growth-promoting, and nitrogen stress-tolerant bacterial taxa into roots at the maturing stage.

Keywords *Sorghum bicolor* (L.) · Perennial sweet sorghum cultivar · Root · Bacterial microbiota · High-throughput sequencing · 16S rRNA gene (16S rDNA).

Introduction

Since time immemorial, plants have evolved various mechanisms to cope with stresses and help their growth and development (Lamers et al. 2020). One mechanism involves forming a mutualistic relationship with soil bacteria, where plant roots act as 'gatekeepers' screening soil bacteria from the outer rhizosphere to the inner endosphere (Liu et al. 2017). Since they inhabit inside plant tissues, including roots, stem leaves, fruits, and seeds, these microbes are called endophytes (White et al. 2018).

Despite originating from rhizosphere bacteria, comparative genome studies of nine bacterial species belonging to 7 genera reveal that bacterial endophytes are versatile and may comprise genes for novel traits benefiting the host plant (Ali et al. 2014). Bacterial endophytes stimulate the host

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plant to produce metabolites that protect the plant against abiotic and biotic stresses and aid in improving growth and yield (Liu et al. 2017; Lata et al. 2018). For instance, the endophytic *Bacillus amyloliquefaciens* causes an increases in root architecture in cotton seedlings under salt stress conditions (Irizarry and White 2017). Elsewhere, inoculation with the endophytic *Burkholderia phytofirmans* strain PsJN improves drought resistance of tropical wheat seedlings (Naveed et al. 2014).

Moreover, *Klebsiella pneumonae*, an endophytic bacterium, activates the ethylene (ET) signaling pathway after colonizing *Medicago truncatula* roots (Iniguez et al. 2005). Plant growth-promoting *Enterobacter* improves the root system architecture of rice seedlings by modulating endogenous auxin levels (Verma et al. 2017). The growth and development of cotton seedlings were significantly promoted after inoculation with the endophytic bacterium *Bacillus amyloliquefaciens* (Irizarry and White 2018). Recently, *Herbaspirillum seropedicae*, a bacterial endophyte, was reported to significantly increase plant height, chlorophyll content, root volume and root length in different maize genotypes (Schultz et al. 2022).

Sweet sorghum (*Sorghum bicolor* (L.) Moench) is a multipurpose crop belonging to the Poaceae family (Mareque et al. 2018). It is an important feedstock crop besides being a source of bioenergy and alcoholic beverages (Appiah-Nkansah et al. 2019). As a multipurpose crop, different genotypes of the genus sorghum (*Sorghum bicolor*) have been introduced into the market, including annual as well as tropical perennial species (Cox et al. 2018; Habyarimana et al. 2018).Since *Sorghum bicolor* originated from Africa and was domesticated from a tropical ancestor, it is susceptible to cold stress (Zheng et al. 2011; Casto et al. 2021). Chilling or cold stress (above freezing) not only causes delays or abortions of sorghum seed germination and seedling emergence at the heterotrophic stage but also reduces vigor and slows growth at the vegetative stage, as well as reduced yield at the maturing stage (Casto et al. 2021). Although many genotypes and regions of the sorghum genome have been linked to chilling or cold tolerance during the past two decades (Yu and Tuinstra 2001; Yu et al. 2004; Balota et al. 2010; Maulana and Tesso 2013; Bekele et al. 2014; Zegada-Lizarazu et al. 2016; Chopra et al. 2017; Marla et al. 2017, 2019; Ortiz et al. 2017; Woldesemayat et al. 2018; Menamo et al. 2021; Rutayisire et al. 2021), little progress has been made in significantly improving the cold tolerance of sorghum and extending its growing range and season (Reddy and Gomashe 2017; Burke et al. 2019; Casto et al. 2021). In addition to sorghum genetic sources of cold tolerance, identification of sorghum genes responding to chilling tolerance, translating known functional genes from maize to sorghum, and breeding new sorghum germplasm with a shorter growing season (Casto et al. 2021), inoculation with psychrotolerant plant-growth-promoting (PGP) bacteria could be another option to improve the cold tolerance of sorghum since previous studies documented that psychrotolerant PGP bacteria or rhizobacteria improve the cold tolerance of several plants, including wheat (Mishra et al. 2011; Abd El-Daim et al. 2019), grape (Fernandez et al. 2012; Theocharis et al. 2012), tomato (Subramanian et al. 2015; Tapia-Vazquez et al. 2020), rice (Kakar et al. 2016), bean (Tiryaki et al. 2019), quinoa (Chumpitaz-Segovia et al. 2020), *Elymus nutans* seedlings (Li et al. 2021), and maize (Beirinckx et al. 2020).

Recent research has shown that the bacterial microbiota composition of maize roots is modified slightly but significantly under chilling temperature (Beirinckx et al. 2020). Besides, microbial taxa potentially enhancing soybean cold tolerance have been identified through soybean rhizosphere microbiome analysis (Bandara et al. 2021). A recent study discovered that cold stress considerably affects microbial populations of sorghum, suggesting that plant–microbe interactions could increase frost tolerance potential in sorghum (Cloutier et al. 2021). Of note, the mutualistic relationship between sweet sorghum and soil bacteria has drawn increasing research attention (Mareque et al. 2018; Heijo et al. 2021; Lopes et al. 2021).

The perennial analogs produce competitive grain yield and aboveground biomass, but their interactions with microorganisms such as root endophytes have been inadequately characterized (Habyarimana et al. 2018). Although bacterial inoculations are effective in promoting growth under adverse conditions (da Silva et al. 2018; Heijo et al. 2021), microorganisms with specific functional traits allowing a perennial plant to survive the cold winter seasons are largely understudied.

We recently reported that the perennial sweet sorghum cultivar NaPBS778 (N778 simply) recruits potential psychrotolerant dominant bacterial taxa belonging to *Pseudarthrobacter* and *Pseudomonas* into its rhizosphere at the maturing stage (Lu et al. 2022). Therefore, there is a need to add more insights into the composition and diversity of the bacterial microbiota inhabiting the roots of the perennial sorghum cultivar N778 at the maturing stage under cold stress.

Materials and methods

Plant materials

The perennial sweet sorghum cultivar NaPBS778 (N778 simply) was described in a previous report (Lu et al. 2022). This study used two control sorghum lines, i.e., P607 and

the F11 recombinant inbred line (RIL) TP60, developed through hybridizing the cultivars T70 × P607 of sorghum (Dong et al. 2013).

Sampling locations and soil types

Sampling was performed in three locations. The first sampling location was the experimental field base (N 25° 6′ 29″, E 102° 38′ 36″) of Yunnan Eco-Agriculture Research Institute (YEARI), Kunming, China; the local soil was a mixture of udic ferrisols (60%) and perudic luvisols (30%) based on China soil taxonomy (CST) (http://www.soilinfo.cn/map/index.aspx). The second sampling location was the experimental field base (N 31° 32′ 38″, E 118° 37′ 48″) in Guanma village, Gushu town, Dangtu County, Anhui Province, China; based on CST, the soil was a mixture of 60% stagnic anthrosols and 25% alluvic entisols. Lastly, the third sampling site was the experimental field at Huaiyin Normal University (HNU) campus, and the local soil was described in our previous report (Lu et al. 2022).

Field design and management

The design and management of the experimental field on the HNU campus for the perennial sweet sorghum cultivar N778 and the control line TP60 were described in our previous report (Lu et al. 2022). Since 2014, over 20 plants of the perennial sorghum cultivar N778 have been growing in the experimental field of YEARI at Kunming. Moreover, 36 plants, with 12 plants of sorghum P607 in each row, were cultivated in the experimental field in Dangtu County in 2019.

Harvesting and sampling

Sorghum plants of different genotypes were dug out, then samples from different compartments were carefully harvested as described previously (Lu et al. 2018a) with some modifications. Bulk soil, rhizosphere soil, and roots of five independent P607 plants and three independent N778 plants were collected on Dec. 5, 2019, in Dangtu County and on Dec. 6, 2019, in Kunming, respectively, at the maturation stage. Additionally, roots together with the rhizosphere soil of the perennial sorghum cultivar N778 at the flowering stage were sampled on Oct. 18, 2020, at the HNU campus. Furthermore, roots together with the rhizosphere soil of sorghum cultivar N778 and line TP60 at the maturation stage were sampled from Dec. 11 to 12, 2020, at the HNU campus (Lu et al. 2022) under cold temperatures with a daily range from − 1 to 10 °C for more than a week (Fig. S1a-b); Actually, sorghum plants suffered below freezing temperatures from Dec. 3 to 5, 2020, before sampling (Fig. S1a). All stay-green leaves of the control line TP60 wilted and faded in green color under cold stress during sampling on Dec. 11, 2020 (Fig. S1c). However, nearly all stay-green leaves of N778 only faded in green color but did not wilt during sampling on Dec. 12, 2020 (Fig. S1d). After most rhizospheric soils were brushed with clean toothbrushes, aerial or primary sorghum roots were cut into segments approximately 5 cm in length. Then, root segments were vigorously shaken and washed three or more times with pH 7.3 phosphate-buffered saline (PBS) in sterile 50 ml tubes until the last supernatant became completely clear, and no pellet existed in the bottom of 50 ml tubes after root segments with PBS were centrifuged at 10,000 × g for 2 min at 4 °C. Moreover, clean root segments in sterile 50 ml tubes were centrifuged again at 10,000 × g for 2 min at 4 °C, whereas the remaining tiny PBS was removed from the tube bottom using a micropipette. Finally, clean and centrifugally dried root segments were stored at − 65 °C before being ground by a mortar and pestle in liquid nitrogen for metagenomic DNA extraction.

DNA extraction from root and soil samples

Before DNA extraction, clean root segments were ground into extremely tiny powders using a mortar and pestle in liquid nitrogen. Subsequently, DNeasy PowerSoil Kit (Qiagen, CA, USA) was used to extract total metagenomic DNA from approximately 0.30 g per sample following the manufacturer's instructions with modification, in which homogenization of soil and root samples was performed with FastPrep-24 (MP Biomedicals, CA, USA). All DNA samples were stored at − 65 °C before the DNA integrity was further examined through 1% agarose gel electrophoresis.

16S rDNA amplicon high-throughput sequencing via Illumina MiSeq

Regarding the bulk soil, rhizosphere soil, and root samples of N778 and P607 sampled from the first and second locations, the fusion primers containing the P5 or P7 Illumina adapter, an 8 nucleotide (nt) barcode, and the gene-specific primer pair 338F and 806R (Peiffer et al. 2013; Xu et al. 2016) were used for amplifying the bacterial 16S rDNA V3-V4 region. Additionally, the gene-specific primer pair 799F (5'-AACMGGATTAGATACCCKG-3') and 1392R (5'-ACGGGCGGTGTGTRC-3') (Bulgarelli et al. 2012; Lundberg et al. 2012) was used to amplify the long fragment of the 16S rDNA V5-V7 region of bacterial communities in sorghum N778 and TP60 root samples collected from the third location during the first round of PCR with 27 cycles. aFusion primers containing P5 or P7 Illumina adapter, an 8 nt barcode, and the gene-specific primer pair of 799 F and 1193R (5'-ACGTCATCCCCACCTTCC-3') (Bulgarelli

et al. 2015) were used in the next round of PCR for 13 cycles with an annealing temperature of 55 °C to amplify the short fragment of the 16S rDNA V5-V7 region. Triplicate PCR amplification, PCR product purification, library quality determination, library quantification, and paired-end 300 bp high-throughput sequencing were conducted on the Illumina MiSeq platform (Illumina, CA, USA) as described byHu et al. (2019). The raw data of 36 samples have been deposited into the China National Gene Bank Database (CNGBdb) (https://db.cngb.org/), and the accession number of the CNGB Sequence Archive (CNSA) is CNP0003148.

Analysis of amplicon data

Amplicon data analysis was carried out as previously reported (Fazal et al. 2020) with a few modifications (Lu et al. 2022). Briefly, raw reads were first filtered to remove reads with base quality scores of less than 20 on average, ambiguous N bases, and lengths less than 50 nt via Fastp (v0.19.6) (Chen et al. 2018). Next, clean tags were generated by merging the paired-end reads with high-quality using FLASH (v1.2.11) (Magoc and Salzberg 2011) and removing the chimeric sequence and singletons. Then, operational taxonomic units (OTUs) at 97% sequence similarity were clustered from effective tags using UPARSE (v7.0.1090) (Edgar 2013). Furthermore, the resulting representative OTUs were taxonomically classified using the Ribosomal Database Project (RDP) classifier (v2.11) (Wang et al. 2007) based on the SILVA 16S rDNA database (v138) (Quast et al. 2013). Analysis of amplicon data was further carried out using the online Majorbio Cloud Platform (www.majorbio.com).

Phylogenetic analysis

The phylogenetic analysis via the online Majorbio Cloud Platform was performed by FastTree (v2.1.3) (Price et al. 2010). The phylogenetic tree was drawn by the R language (v3.3.1) according to the maximum likelihood (ML) method when nucleotide sequences of 16S rDNA of 12 Pseudarthrobacter species were downloaded from the NCBI nucleotide database.

Statistical analysis

Analysis of variance (ANOVA) was carried out using SPSS (v16.0) software, followed by Tukey's honestly significant difference (HSD) post hoc test. For pairwise group comparisons, either Welch's t- test (for unknown variance) or Student's t-test (equal variance) was performed. The Kruskal–Wallis H test was performed for multiple group comparisons, followed by a post hoc Tukey–Kramer test via the online Majorbio Cloud Platform (www.majorbio.com). Adonis (permutational multivariate analysis of variance) and analysis of similarities (ANOSIM) based on Bray–Curtis or weighted UniFrac distance metrics were conducted using the software R (v3.3.1) vegan package.

Results

This study examined the composition and diversity of the bacterial microbiota inhabiting perennial sorghum N778 roots at the maturing stage under cold stress conditions.

Coamplification of chloroplast DNA and amplification of bacterial rDNA from sorghum root samples via the primer pair 338 F and 806R

In the winter of 2019, root samples, bulk and rhizosphere soil of the perennial sweet sorghum cultivar N778 and a control sorghum line P607 were collected. After total metagenomic DNA was extracted, amplicon deep sequencing of the 16S rDNA V3-V4 region was carried out via Illumina MiSeq. Consequently, 818,955 clean tags were generated, averaging 45,497 clean tags per sample (Table S1). The average effective taxonomic tags at 97% similarity, including or excluding chloroplast DNA (CpDNA) and mitochondrial DNA (MtDNA), were 35,484 or 28,422 per sample, respectively (Table S1). Unlike bulk soil or rhizosphere soil samples, the average effective tags for bacterial 16S rDNA (V3-V4 region) per root sample of N778 and P607 were only 15,783.3 and 14,481.7, respectively (Table 1).

In contrast, the average effective tags for CpDNA per root sample of N778 and P607 were 20,887.3 and 20,375, respectively, which were noticeably higher than those of bulk soil or rhizosphere soil samples (Table 1). Additionally, the average effective tags for bacterial rDNA (V3-V4 region) per root sample of N778 or P607 were less than those for CpDNA (Table 1).

Even though the total effective tags at 97% similarity per sample were greater than 30,984 (Table S1 and S3), only 7,371 tags of each sample were retained for alpha and beta diversity analyses, and the normalized OTU table was obtained (Table S2).

Specific amplification of bacterial rDNA from sorghum root samples via the primer pair 799 F and 1193R

Since the primer pairs 338 F and 806R coamplified significantly more chloroplast DNA from sorghum root samples, the primer pairs 799 F-1392R and 799 F-1193R were chosen for nested PCR amplification of the 16S rDNA V5-V7

Table 1 Amplification of bacterial rDNA and coamplification of non-target DNA from bulk soil, rhizosphere soil, and roots of N778 and P607 via primer pair 338 F-806R of the 16S rDNA

Effective tags	N778BS (mean ± SD)	N778RS (mean ± SD)	N778RT (mean ± SD)	P607BS (mean ± SD)	P607RS (mean ± SD)	P607RT (mean ± SD)
Total Effective tags	33076.3 ± 1875.20	35873.7 ± 3373.66	37023.7 ± 2263.21	33082.7 ± 1022.21	38566.0 ± 176.77	35283.0 ± 4150.47
CpDNA#	**54.3 ± 45.98^{a}**	**18.3 ± 8.08^{a}**	**20887.3 ± 11928.94^{b}**	**156.3 ± 89.23^{a}**	**88.0 ± 23.26^{a}**	**20375.0 ± 6852.12^{b}**
MtDNA	ND	ND	353.0 ± 159.15	5.7 ± 4.04	6.7 ± 2.89	426.3 ± 350.28
Bacterial rDNA#	**33022.0 ± 1870.14^{a}**	**35855.3 ± 3380.22^{a}**	**15783.3 ± 11610.61^{b}**	**32920.7 ± 1112.74^{a}**	**38471.3 ± 162.97^{a}**	**14481.7 ± 6340.25^{b}**

Note: SD indicates the standard deviation (n = 3). N778 and P607 indicate the perennial sweet sorghum cultivar NaPSB778 and the control sorghum line P607, respectively. BS, RS, and RT represent the bulk soil, rhizosphere soil, and root samples, respectively. CpDNA, MtDNA and bacterial rDNA refer to effective tags of chloroplast DNA, mitochondrial DNA, and bacterial 16S rRNA gene (16S rDNA), respectively. One-way ANOVA followed by Tukey's HSD post hoc test was the statistical method used here. # indicates that critical values were not monotonic, but substitutions ensured monotonicity; hence, the type I error was smaller. The values in bold letters refer to a significant difference ($P < 0.05$) among different groups at the maturation stage. ND refers to "not detected"

Table 2 Amplification of bacterial rDNA and coamplification of non-target DNA from root samples of N778 and the control line TP60 via primer pairs 799 F-1193R of the 16S rDNA

Effective tags	NaFARt (mean ± SD)	NaFPRt (mean ± SD)	NaMARt (mean ± SD)	NaMPRt (mean ± SD)	TPMARt (mean ± SD)	TPMPRt (mean ± SD)
Total Effective tags	42732.7 ± 3035.51	47152.0 ± 5903.35	40927.7 ± 3531.01	46132.0 ± 393.78	36216.3 ± 12976.92	42495.7 ± 11839.02
CpDNA	ND	ND	ND	ND	ND	ND
MtDNA	**364.0 ± 100.86^{ab}**	**980.7 ± 540.73^{b}**	**511.0 ± 282.09^{ab}**	**382.0 ± 81.51^{ab}**	**148.7 ± 53.59^{a}**	**250.0±8.54^{a}**
Bacterial rDNA	42368.7 ± 2944.20	46171.3 ± 5632.94	40416.7 ± 3265.59	45750.0 ± 443.2	36067.7 ± 12923.50	42245.7 ± 11847.29

Note: SD indicates the standard deviation (n = 3). Na and TP indicate the perennial sweet sorghum cultivar NaPSB778 (N778) and control sorghum line TP60, respectively. F and M indicate the flowering and maturing stages, respectively. ARt and PRt refer to the latest aerial root and the primary root (taproot) samples, respectively. CpDNA, MtDNA and bacterial rDNA present effective tags of chloroplast DNA, mitochondrial DNA, and bacterial 16S rDNA, respectively. Statistical analyses were performed using one-way ANOVA followed by Tukey's HSD post hoc test. The values in bold letters indicate a significant difference ($P < 0.05$) among different groups at the maturation stage. ND refers to "not detected"

region of bacterial communities in root samples of sorghum N778 and TP60 grown at the HNU campus.

A total of 1,189,466 clean tags were generated with an average number of 66,081 tags per root sample (Table S4); the average number of effective tags at 97% similarity, including or excluding MtDNA and CpDNA, was 42,609 or 42,170 per root sample, respectively (Table S4). Coamplification of CpDNA was removed in the root samples (Table 2), although a few tags of MtDNA were coamplified per root sample by the primer pairs 799 F-1392R and 799 F-1193R (Table 2). Additionally, the average length of clean tags was 377 nt, whereas the average Q30 value was 96.17% (Table S4). In total, 18 root samples of N778 and TP60 yielded 2,015 OTUs belonging to 991 species, 554 genera, and 34 phyla (Table S5, S6).

Alpha diversity analysis of bacterial microbiota between N778 and TP60

After excluding tags of MtDNA, effective tags of bacterial rDNA from all root samples were normalized based on the minimal number of 21,145 tags from TP_1MARt (Table S4 and S7). Six alpha diversity indices, including Observed Species (Sobs), Chao1, Shannon's diversity, phylogenetic diversity, Simpsoneven, and Good's coverage at the OTU level, were selected for pairwise group comparisons. Welch's t-test was used to determine the significant differences between pairwise groups (Fig. 1). The Good's coverage index of all samples exceeded 98.5% (Fig. 1f); rarefaction curves of Good's coverage and the other five indices nearly reached the saturation plateau (Fig. S2). This indicates that the sequencing depth had sufficient detectable species and captured the diversity of bacterial endophyte communities.

Among the different genotypes, the alpha diversity indices of N778 during the maturing stage did not differ significantly from those of TP60, except for the Sobs and phylogenetic diversity indices of the N778 aerial root sample (NaMARt), which were markedly lower than those of TP60 (TPMARt) (Fig. 1a, d). At different growth stages, the Simpsoneven index of N778 aerial root samples at the flowering stage (NaFARt) was strikingly lower than that of N778 aerial root samples (NaMARt) and N778 primary root samples (NaMPRt) at the maturing stage (Fig. 1e).

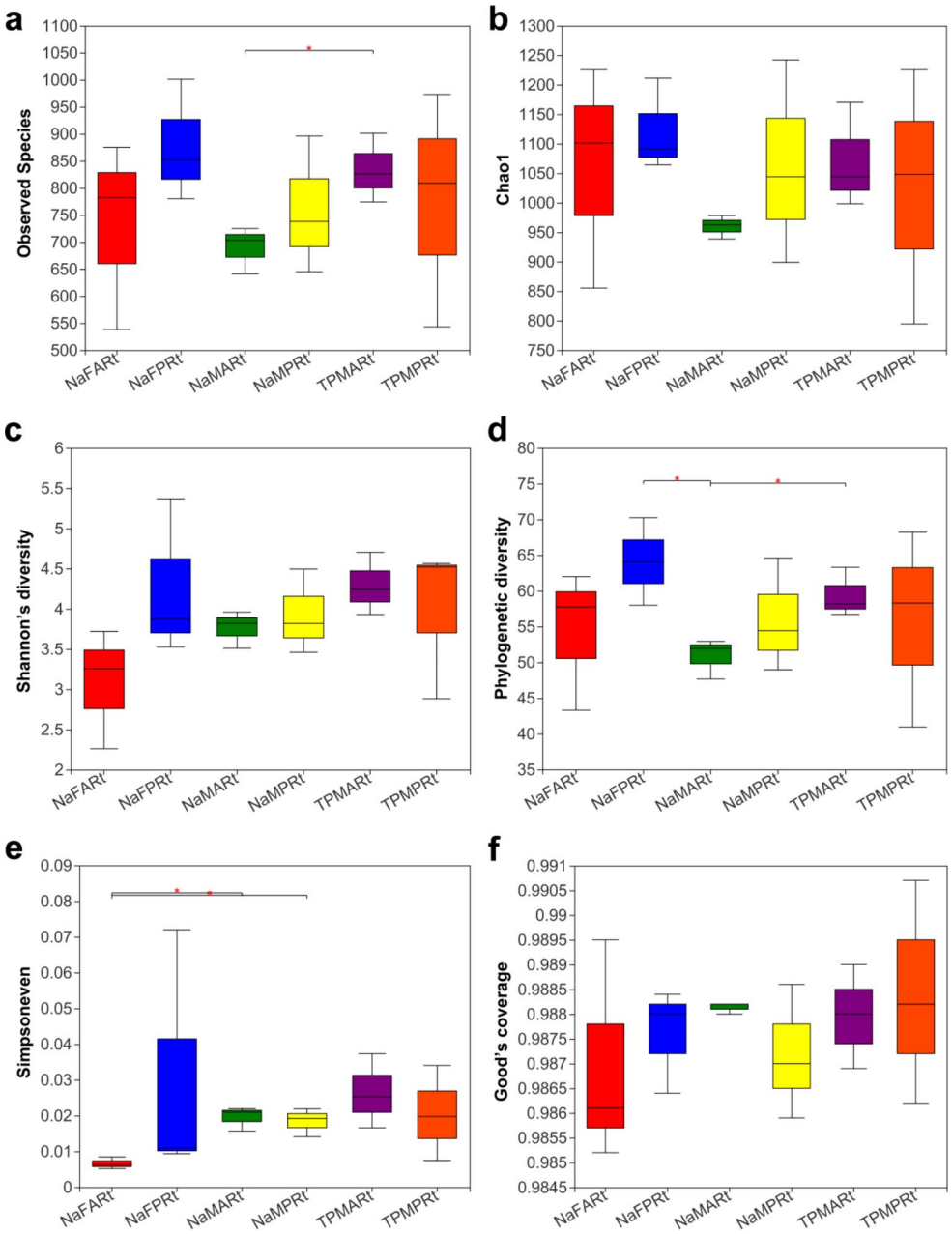

Fig. 1 Alpha diversity analysis of root samples at the OTU level between N778 and TP60. (a) to (f) refer to Observed Species (Sobs), Chao1, Shannon's diversity, Phylogenetic diversity, Simpsoneven, and Good's coverage, respectively. Table 2 shows the treatment details. Welch's t-test was applied to determine significant differences between pairwise groups. * indicates $P < 0.05$.

Additionally, the aerial root samples of N778 at the maturing stage (NaMARt) had a considerably lower phylogenetic diversity index than the primary root samples of N778 during the flowering stage (NaFPRt) (Fig. 1d). The remaining four indices of samples of N778 aerial roots or primary roots at the flowering stage did not substantially differ from those of N778 at the maturation stage (Fig. 1a-c, f).

Beta diversity analysis of bacterial microbiota between N778 and TP60

The variations in root samples between N778 and TP60 were initially evaluated at the OTU level using principal coordinate analysis (PCoA). Adonis and ANOSIM statistical analysis of PCoA results based on Bray–Curtis or weighted UniFrac (WUF) distance metrics (Table S8, S9) showed that there were no significant differences in the beta diversity of bacterial microbiota among various root samples between N778 and TP60 (Fig. 2a and b). In addition, no significant alterations were noted in the beta diversity of the N778 root microbiota at different growth stages (Fig. 2a and b). However, there was a significant difference between sample groups compared to that within the group (Fig. 2a and b). The results of nonmetric multidimensional scaling (NMDS) analysis, followed by Adonis and ANOSIM statistical analysis, revealed no striking differences in the beta

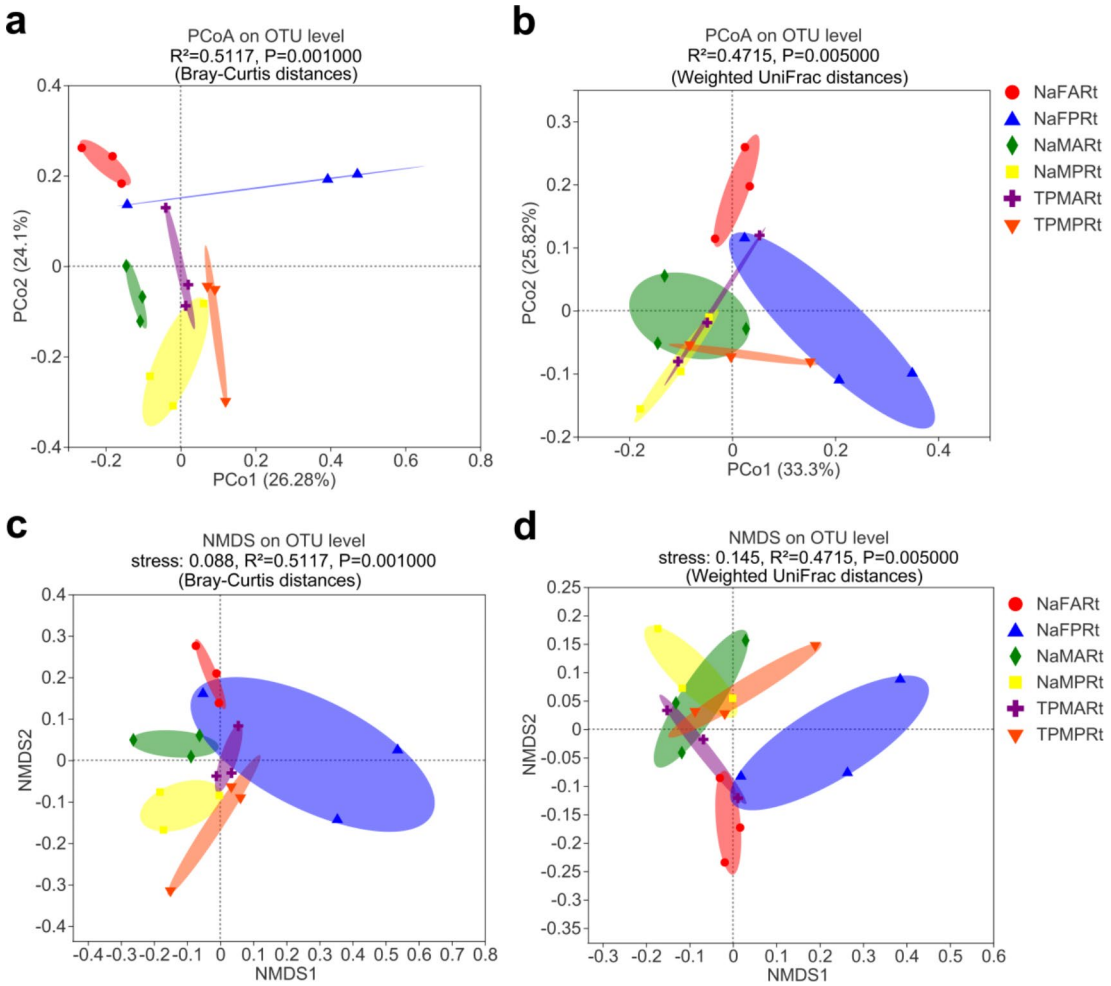

Fig. 2 Beta diversity of bacterial microbiota at the OTU level between N778 and TP60 analyzed by nonmetric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) with statistical results from Adonis. (a) PCoA is based on Bray–Curtis distance, and the statistical results from ANOSIM are R = 0.4160 and *P* = 0.001; (b) PCoA is based on weighted UniFrac (WUF) distance, and statistical results from ANOSIM are shown as R = 0.2329 and *P* = 0.011; (c) NMDS based on Bray–Curtis distance, the stress value was 0.088; (d) NMDS based on WUF distance, and the stress value was 0.145. Table 2 shows the treatment details.

diversity of bacterial microbiota between N778 and TP60 root samples (Table S9). Both stress values (Fig. 2c and d) were less than 0.2, indicating the validity of the NMDS analysis results.

Dominant bacterial OTUs and genera in root samples of the perennial sweet sorghum N778

A total of 594 of the 2015 OTUs (Tables S5 and S6) were shared by all 6 groups, whereas 28 and 32 OTUs were unique to the aerial and primary roots of N778, respectively, at the maturing stage (Fig. S3a). The relative abundances of the top nine OTUs exceeded 50% (Fig. S3b). Moreover, 670 OTUs were shared between the aerial and primary roots of N778 at the flowering and maturing stages (Fig. 3a), whereas 688 OTUs were shared between the aerial and primary roots of N778 and TP60 at the maturing stage (Fig. 3b). At the maturation stage of N778, the top 5 OTUs in the aerial roots included OTU1902, OTU989, OTU1207, OTU1726, and OTU414 (Fig. 3c), accounting for 14.43%, 12.29%, 8.69%, 8.22% and 4.88% of the relative abundances (Fig. S3c), respectively.

On the other hand, the top 5 OTUs in the primary roots were OTU1726, OTU1902, OTU989, OTU901, and OTU1262 (Fig. 3c), which accounted for 17.29%, 15.06%, 4.41%, 4.23%, and 3.74% of the relative abundances (Fig. S3d), respectively. OTU1902, OTU1726, and OTU988, the three most prevalent OTUs, were shared by both the aerial and primary roots of N778 at the maturing stage (Fig. S3c, d), which belonged to the genera *Streptomyces*, *Pseudomonas* and an unidentified genus of the family Comamonadaceae, respectively (Table S7).

Among 554 genera, the top ten genera were *Streptomyces*, *unclassified*_f__Comamonadaceae, *Pseudomonas*,

Lechevalieria, *Allorhizobium* (*Neorhizobium-Pararhizobium-Rhizobium*), *unclassified*_f__Oxalobacteraceae, *unclassified*_f__Enterobacteriaceae, *Steroidobacter*, *Klebsiella*, and *Pantoea*, accounting for 19.788%, 18.872%, 9.162%, 4.317%, 2.744%, 2.143%, 2.046%, 1.860%, 1.556% and 1.496% of all bacterial taxa with absolute abundances in all 18 root samples, respectively (Table S6, sheet of genus).

Based on the normalized OTU taxonomic table (Table S7), the top ten genera with high relative abundances were similar to the top ten genera with absolute abundances, except for *Klebsiella* being replaced by *Flavobacterium* (Fig. 3d). Furthermore, at the maturing stage of N778, the top ten abundant genera in the aerial roots were *Streptomyces*, *unclassified*_f__Comamonadaceae, *Pseudomonas*, *Pantoea*, *Lechevalieria*, *unclassified*_f__Oxalobacteraceae, *Massilia*, *Steroidobacter*, *Pseudarthrobacter* (1.06%) and *Ideonella* (Fig. 3d and S4c), while the top ten abundant genera in the primary roots were *Streptomyces*, *Pseudomonas*, *unclassified*_f__Comamonadaceae, *unclassified*_f__Oxalobacteraceae, *Pseudarthrobacter* (3.74%), *Flavobacterium*, *Lechevalieria*, *Myceligenerans*, *Variovorax*, and *Rhizobacter* (Fig. 3d and S4d).

Bacterial taxa enriched in root samples of the perennial sweet sorghum N778 at the maturing stage

At the maturing stage, the proportions of OTU434, OTU220, OTU1589, OTU1304, and OTU80, among the top 18 distinct OTUs, were markedly higher in the aerial root sample of N778 (NaMARt) than in TP60 (TPMARt) (Fig. S5a). Conversely, the proportions of OTU901, OTU1589, OTU1304, OTU499, OTU475, OTU1101, and OTU1175, among the other top 18 OTUs, were markedly higher in the primary root sample of N778 at the maturing stage (NaMPRt) than in TPMPRt (Fig. S5b). Among these OTUs, only the relative abundance of OTU1304, belonging to an unclassified species of *Streptomyces*, was significantly higher in both aerial root and primary root samples of N778 compared to TP60 at the maturing stage (Fig. S5).

Ternary plot analysis was used to compare the bacterial composition of aerial root samples between N778 and TP60 at both the flowering and maturation stages (Fig. 4a). With a relative abundance of 2.91%, OTU1207 from the genus *Pantoea* was enriched in NaMARt (Fig. 4a). The proportions of OTU803, OTU836, and OTU1170 in NaMARt were 86.9%, 72.3%, and 62.5%, respectively, which were significantly greater than those in NaFARt and TPMARt (Fig. 4a). OTU803, OTU1170, and OTU836 belong to *Massilia*, *Pseudomonas*, and an unclassified genus of the family Oxalobacteraceae, respectively, and accounted for 0.505%, 0.705%, and 1.31%, on average, of the relative abundance per group in the 3 aerial root sample groups.

Notably, OTU1262 and OTU836 were higher in NaMPRt than in NaFPRt and TPMPRt, with proportions of 83.0% and 87.0%, respectively (Fig. 4b), which belong to the genus *Pseudarthrobacter* and an unclassified genus of the family Oxalobacteraceae, respectively, accounting for 1.50% and 0.885% of the average relative abundance in the three primary root sample groups, respectively.

Further analysis showed that the relative abundance of OTU836 was markedly higher in the primary root sample of N778 at the maturing stage (NaMPRt) than that in NaFPRt and TPMPRt (Fig. S6b). However, OTU803, OTU1170, OTU1207, and OTU1262 were not significantly different in the three group comparisons (Fig. S6). Additionally, the proportions of OTU434 and OTU1304 were substantially higher in the aerial root sample of N778 at the maturing stage (NaMARt) than those in NaFARt and TPMARt (Fig. S6a).

(a) Dominant OTUs (≥ 0.5% relative abundance in all samples) in the three groups of aerial root samples; (b) Dominant OTUs (≥ 0.5% relative abundance in all samples) in the three groups of primary root samples. Table 2 shows the treatment details.

Furthermore, the proportions of OTU1262, OTU836, and OTU1304 were considerably higher in the primary root sample of N778 at the maturing stage (NaMPRt) than those in the other five groups (Fig. 5). Unlike in the other four groups, the proportion of OTU434 was markedly higher in the aerial and primary root samples of N778 at the maturing stage (NaMARt, NaMPRt) (Fig. 5).

Overall, the root sample of N778 was significantly enriched with OTU1262, OTU836, OTU434, and OTU1304 at the maturing stage.

Phylogenetic analysis of OTU1262 enriched in the primary root sample of the perennial sweet sorghum N778 at the maturing stage

Based on a normalized OTU table, OTU1262 was an unidentified species of the genus *Pseudarthrobacter* with a relative abundance of approximately 1.11% in all root samples (Table S7). To predict the potential role of OTU1262, FastTree was used for phylogenetic analysis. OTU1262 was strongly associated with *Pseudarthrobacter psychrotolerans* YJ56, a known psychrotolerant bacterial species (Fig. 6).

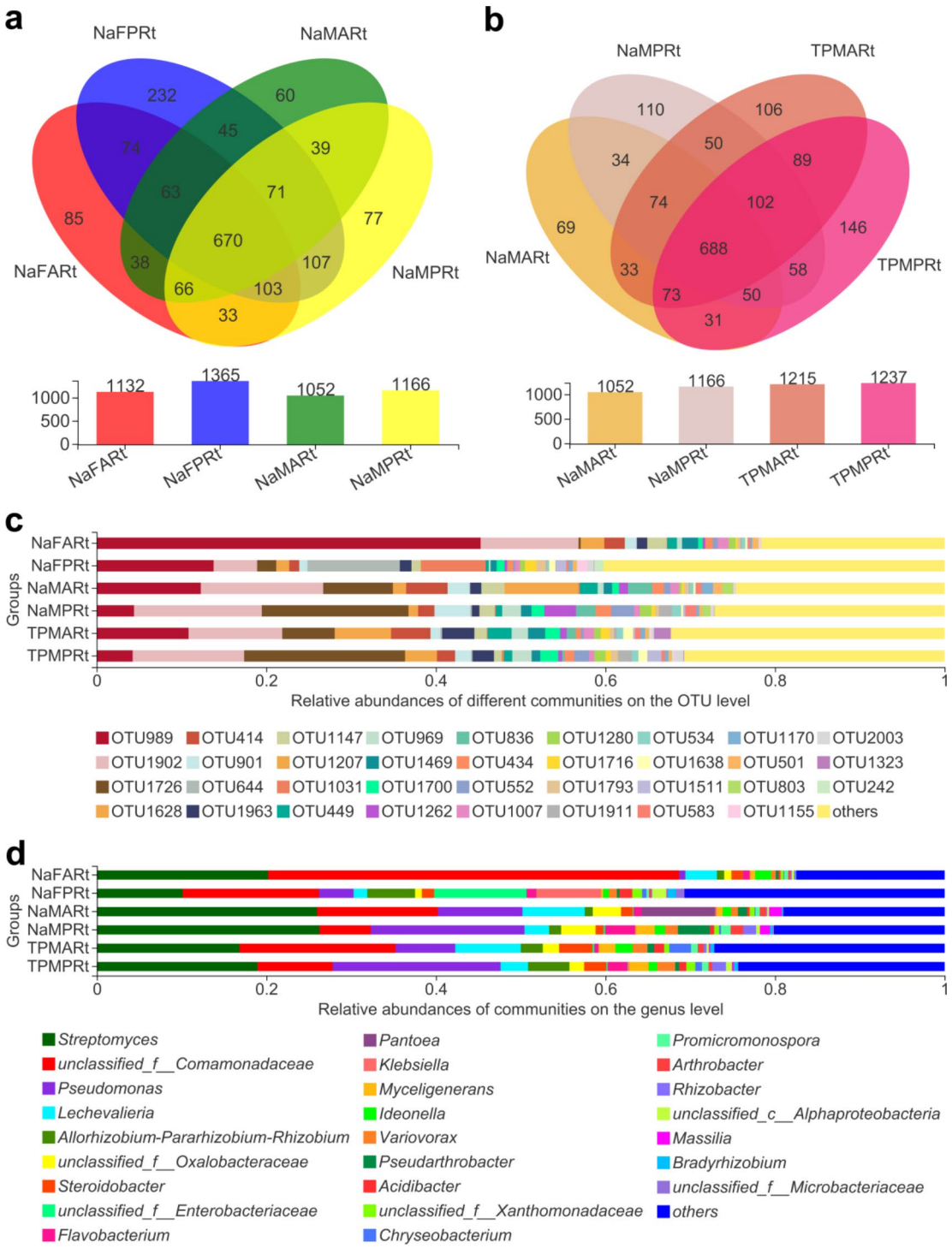

Fig. 3 Composition of bacterial microbiota at the OTU and genus levels between N778 and TP60. (a) Shared OTUs between the aerial and primary roots of N778 at different stages; (b) shared OTUs between the aerial and primary roots of N778 and TP60 at the maturing stage; (c) community composition at the OTU the level; (d) community composition at the genus level. Table 2 shows the treatment details. The full name of *Allorhizobium-Pararhizobium-Rhizobium* was *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*; *Neorhizobium* was removed because the total abundance of the species *Neorhizobium* was only 8 effective tags in all 18 root samples after normalization (Table S7).

Discussion

The specificity of amplifying bacterial 16S rDNA from sorghum root microbiota is significantly

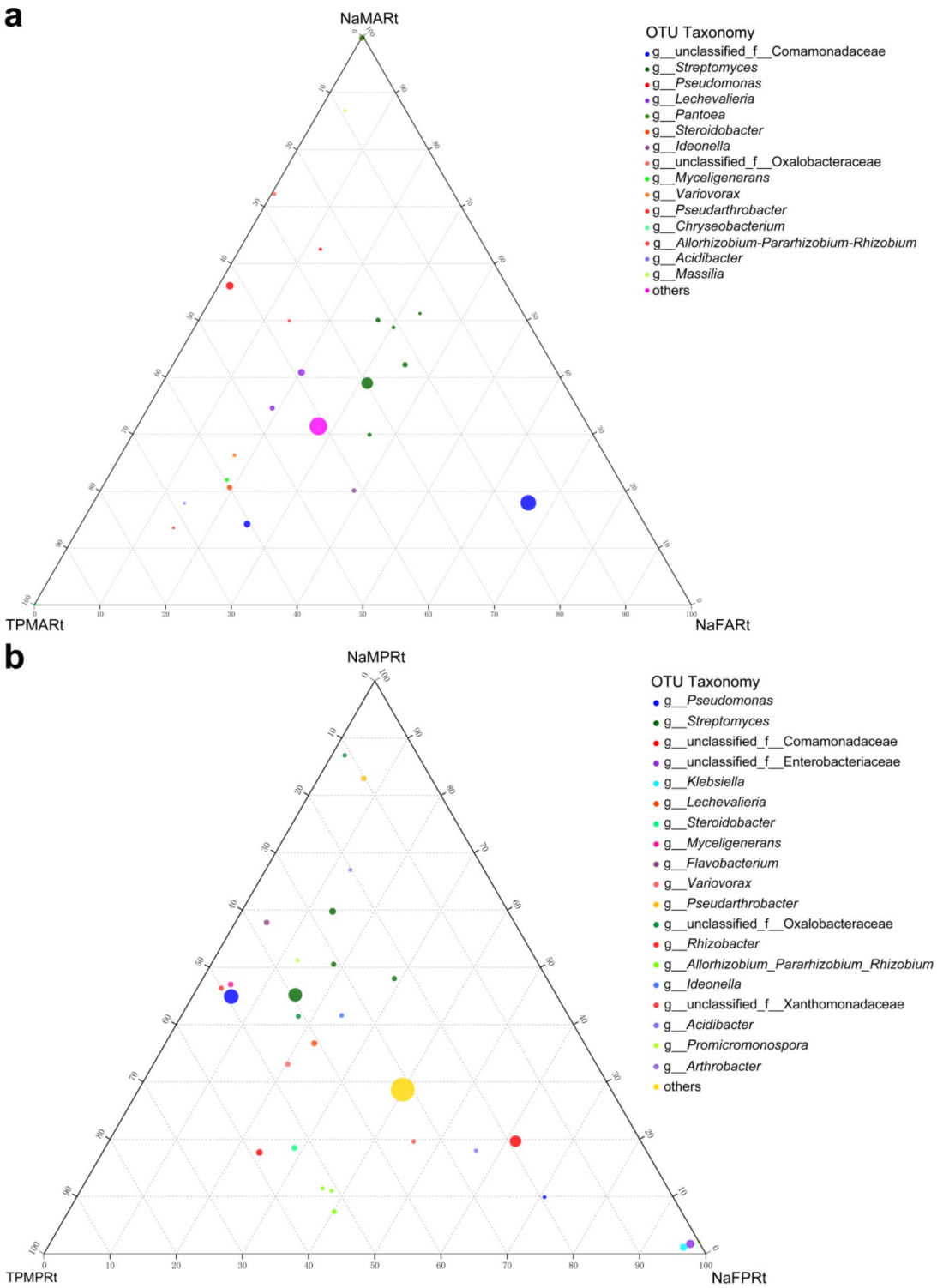

Fig. 4 Ternary plot analysis of dominant OTUs in different root samples between N778 and TP60.

higher under the primer pair 799 F-1193R

Previous studies presented an overall framework of root-associated microbiota (Lu et al. 2018a).Herein, root segments were scissored from the intact roots of sorghum plants, then washed with PBS but were not surface sterilized.Thus, clean sorghum root segments included the endosphere and rhizoplane (Edwards et al. 2015; Lu et al. 2018a).

Since chloroplast DNA (CpDNA) fragments can be amplified from rice root samples using the degenerate primer set

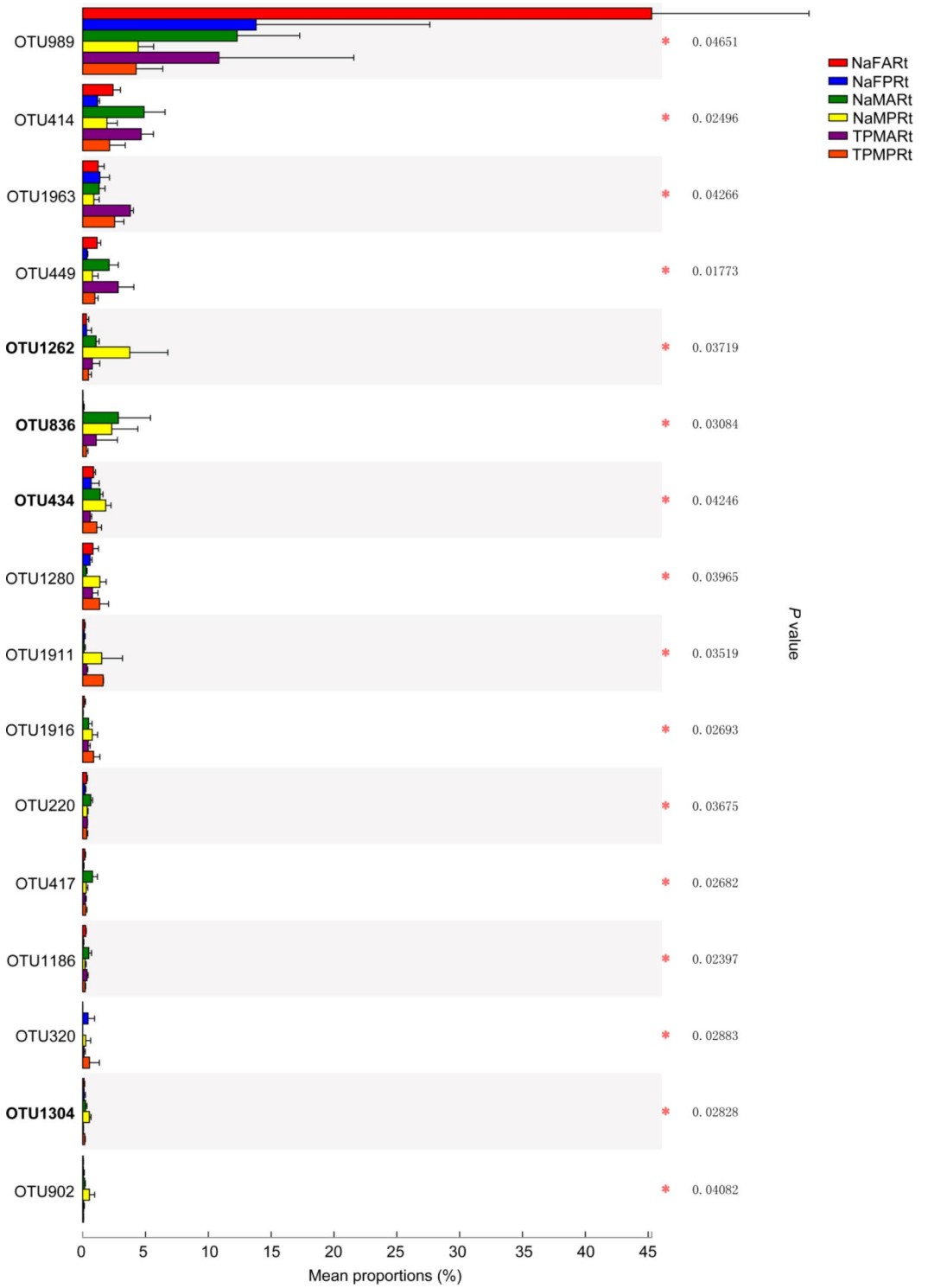

Fig. 5 Top 16 OTUs among multiple group comparisons via Kruskal–Wallis H test followed by Tukey–Kramer post hoc test. The mean proportion (%) is the average relative abundance of taxa in different groups, and the standard deviation (SD) is displayed. Table 2 shows the treatment details. * indicates $P < 0.05$.

of 515F and 806R (Edwards et al. 2015), this study selected another forward primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') (Huse et al. 2008) and degenerate reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al. 2011; Peiffer et al. 2013; Xu et al. 2016). However, there was still significant coamplification of

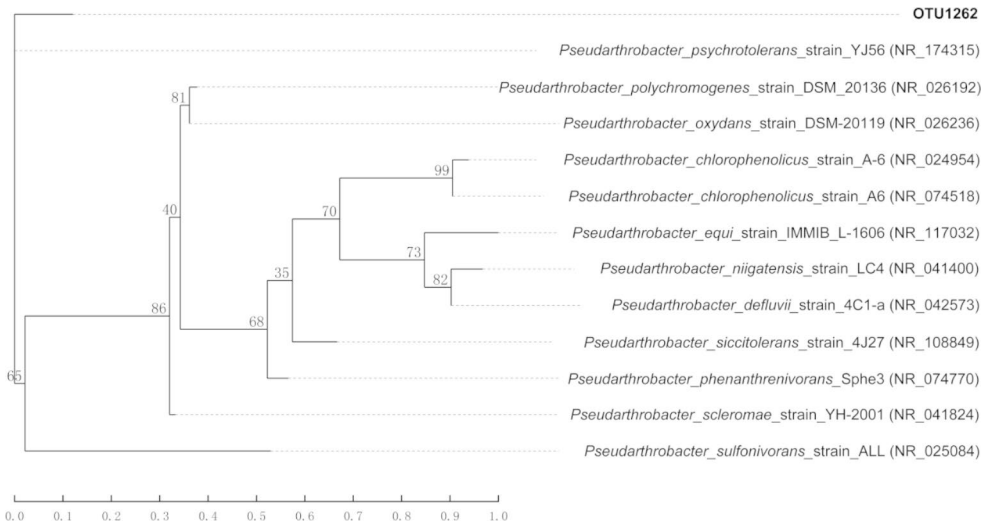

Fig. 6 Maximum-likelihood (ML) phylogenetic tree of OTU1262 based on 16S rDNA nucleotide sequences of 12 species of the genus *Pseudarthrobacter*. The GenBank accession number of 16S rDNA sequence is displayed in parentheses. Bootstrap values are shown at nodes of the phylogenetic tree.

CpDNA from root samples of sorghum N778 and P607 via the primer set 338 F and 806R (Table 1) although it is also a good candidate for amplifying bacterial DNA from prokaryotic microbes, together with CpDNA from plastids or chloroplasts, and MtDNA from mitochondria according to the viewpoint of microbiome acquisition by plant tissue during development (Edwards et al. 2015).

The primer pairs 799 F-1391R and 799 F-1193R were then chosen for amplifying the 16S rDNA V5-V7 region from root samples of sorghum N778 and TP60 by nested PCR because other primer pairs, 341 F-785R, 341 F-783Rabc, 68 F-783Rabc, 68 F-518R, and 967 F-1391R, have significant coverage of CpDNA from root samples of poplar trees (Beckers et al. 2016). As shown in Table 2, the coamplification of CpDNA was fully prevented in sorghum root samples.

Bacterial community compositions of sorghum roots strikingly different from those of rhizosphere soil

Based on our published results (Lu et al. 2022), Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Bacteroidetes, and Gemmatimonadetes were the six most common phyla in the rhizosphere soils of the perennial sweet sorghum N778 and the control line TP60 (Fig. S7a). In this study, Proteobacteria, Actinobacteriota, and Bacteroidota predominated the bacterial microbiota in root samples (Fig. S7b), accounting for 57.236%, 35.501%, and 2.765%, respectively, of all bacterial taxa with absolute abundances in 18 root samples (Table S6, sheet of Phylum). The bacterial community compositions of the rhizosphere and root microbiota at the phylum level in this study are consistent with a recently published study (Sun et al. 2021), while the relative abundances of the top 3 most abundant phyla in root samples of N778 and TP60 exceeded 95%. This can be attributed to different amplicon deep sequencing of 16S rDNA and different sorghum genotypes.

Proteobacteria, the most prevalent phylum, was further classified into Alphaproteobacteria, Gammaproteobacteria, and an unclassified class (Fig. S7c-d). Gammaproteobacteria was enriched in root microbiota (Fig. S7d, S7c). These findings corroborate with a recently published study (Sun et al. 2021). The relative abundance of Acidobacteriota was reduced to less than 0.5% in the root samples of the perennial sweet sorghum N778 and the control line TP60 (Fig. S7b).

At the genus level, the bacterial endophyte compositions in both sorghum N778 and TP60 root samples were strikingly different from those in rhizosphere soil and bulk soil, except for four shared genera, *Pseudomonas*, *Allorhizobium-Pararhizobium-Rhizobium*, *Pseudarthrobacter* and an unclassified genus, with more than 1% relative abundance per taxon (Fig. S8).

Taken together, our results show that the model of host plant genotype-dependent selection of root endophytes works (Bulgarelli et al. 2013).

The putative function of bacterial taxa particularly enriched in roots of the perennial sweet sorghum N778 at the maturing stage

Amplicon deep sequencing of the 16S rDNA V3-V4 region revealed that an unclassified species of the genus *Pseudarthrobacter* with approximately 14% relative abundance is potentially psychrotolerant and markedly enriched in the rhizosphere soil of the perennial sweet sorghum N778 aerial and primary root at the maturing stage (Lu et al. 2022) under below freezing temperature conditions (Fig. S1a-b). In this study, OTU1262 was particularly abundant in the root sample of perennial sweet sorghum N778 during the maturing stage, associated with a popular cold-adapted

bacterium *Pseudarthrobacter psychrotolerans* YJ56 isolated from Antarctic soil (Shin et al. 2020). According to the combined ML phylogenetic tree of OTU1262 (16S rDNA V5-V7 region) and OTU2574 (16S rDNA V3-V4 region) (Fig. S9), the root-inhabiting OTU1262 and rhizospheric OTU2574 can be categorized into the unclassified species of the genus *Pseudarthrobacter*.

A recent study reported that the genus *Streptomyces* is enriched in the root endosphere of a nitrogen stress-tolerant sweet sorghum line under low-nitrogen field growth (Lopes et al. 2021). In the present study, not only the genus *Streptomyces* predominated the bacterial microbiota in the maturing roots of N778, but OTU434 and OTU1304 were also particularly enriched in the maturing root sample of N778 compared to the control sorghum line TP60 (Table S7). Our findings suggest that the perennial sweet sorghum N778 might also be nitrogen stress tolerant, recruiting numerous *Streptomyces* into its roots in a nitrogen-deficient environment.

Another recent study demonstrated that maize recruits the Oxalobacteraceae family including *Collimonas*, *Duganella*, *Massilia*, and *Pseudoduganella*, in the rhizosphere under nitrogen deprivation; these taxa in turn promote nitrogen capture and improve maize growth under nitrogen deprivation (Yu et al. 2021). In this study, OTU836, an unclassified genus of the family Oxalobacteraceae, was enriched in the maturing root sample of N778. Additionally, *Massilia*, the genus of the family Oxalobacteraceae, was enriched in the maturing aerial and primary root samples of N778 (Fig. S8). Our findings suggest that the aforementioned Oxalobacteraceae taxa may also improve the growth of the perennial sweet sorghum N778 under nitrogen stress. It is worth identifying these taxa and further characterizing their functions.

Conclusion

In conclusion, nearly all alpha diversity indices of aerial and primary root samples of N778 were not significantly different from those of TP60 at the maturing stage, except for the Sobs and phylogenetic diversity indices. Most alpha diversity indices of aerial and primary root samples of N778 also demonstrated no significant difference between the two growth stages, except for Simpsoneven and phylogenetic diversity indices. Additionally, no discernible variations were noted in the beta diversity of aerial and primary root samples between N778 and its control TP60 or between the flowering and the maturing stages of N778 itself. The bacterial microbiota in N778 aerial and primary roots was not only dominated by Proteobacteria, Actinobacteria, and Bacteroidetes at the phylum level but also markedly different from the bacterial microbiota in rhizosphere soil at the genus level. Furthermore, OTU1262, OTU434 plus OTU1304, and OTU836 were significantly enriched in the root samples of N778 at the maturing stage and belonged to the genera Pseudarthrobacter, Streptomyces, and an unidentified genus of the family Oxalobacteraceae, respectively. Our findings imply that the perennial sweet sorghum cultivar N778 may recruit potential cold-tolerant, plant growth-promoting, and nitrogen stress-tolerant bacterial taxa into its aerial or primary roots at the maturing stage.

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Author contributions Gui-Hua Lu and Zhong-Yuan Na conceived and designed the experiments; Gui-Hua Lu, Rui Cao, and Zhiye Na performed the experiments; Gui-Hua Lu and Kezhi Zheng analyzed the data; Yonghua Yang, Bo Sun, and Hongjun Yang provided resources; and Gui-Hua Lu and Aliya Fazal wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare no conflicts of interest. The funding sponsors had no impact on the design of the experiments, the study, the data interpretation, the manuscript's writing, or the decision to publish the results.

Ethical approval This article does not contain any research on human participants or animals performed by any authors.

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