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THE PERENNIAL SWEET SORGHUM CULTIVAR AND ITS RECRUITING RHIZOSPHERE DOMINANT BACTERIAL TAXA UNDER FIELD GROWTH

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Abstract

Breeding new perennial sorghum germplasms has been ongoing for decades since perennial crops provide significantly greater environmental benefits in terms of resource utilization, soil erosion, and soil conservation. However, perennial sweet sorghum cultivars or varieties have rarely been reported to date. Additionally, little is known about the potential consequences of perennial sorghum on belowground soil microbial communities. In this study, we reported the perennial sweet sorghum cultivar NaPBS778 (simply N778) with prominent panicle length, plant height, stalk weight, and Brix, which was generated from an annual sweet sorghum cultivar via the Gene Phenotype Induction Technique (GPIT). Further, we designed an experiment to detect the dominant rhizosphere bacterial taxa of the perennial sweet sorghum cultivar N778 and its control sorghum line TP60 at the flowering and maturation stages under natural field growth conditions. The Illumina MiSeq platform was utilized to high-throughput sequence amplicons of the V3–V4 hypervariable region of the 16S rRNA gene (16S rDNA). According to our findings, almost all six alpha diversity indices of N778 rhizosphere soil from either aerial roots and primary roots showed no significant difference when compared to those of TP60 at the same growth stage. There were also no significant differences in the beta diversity of rhizosphere soil between N778 and its control TP60 at the flowering stage. at the maturation stage; however, significant differences in taxonomic and phylogenetic beta diversity of rhizosphere soil were found between N778 and its control TP60. Furthermore, at the maturation stage, the relative abundances of two genera, *Pseudarthrobacter* and *Pseudomonas*, were significantly higher in the rhizosphere soil of N778 than in all other groups. Our results indicate that two dominant OTUs belonging to *Pseudarthrobacter* and *Pseudomonas* with stress tolerant phenotypes are significantly enriched in the rhizosphere of N778 at the maturation stage, implying that the perennial sweet sorghum cultivar N778 could recruit potential psychrotolerant bacterial taxa into its rhizosphere and assist it in surviving the winter below freezing temperature.

Key words: *Sorghum bicolor* (L.); Perennial sweet sorghum cultivar; Rhizosphere bacteriome; Dominant taxa; 16S rRNA gene (16S rDNA); High-throughput sequencing; Illumina MiSeq.

Introduction

Sorghum bicolor (L.) is the fifth most important cereal crop in the world, providing food, fiber, fodder and fuel, especially in the arid, semiarid areas and other vulnerable situations, such as salinity and alkalinity (Zheng *et al.*, 2011; Cox *et al.*, 2018; Ananda *et al.*, 2020). *Sorghum bicolor* responds to stress by experiencing complicated biological processes that include changes in sorghum's physiology and biochemistry, genome, proteome, and metabolome (Borrell *et al.*, 2014; Laursen *et al.*, 2016; Abreha *et al.*, 2022).

Sorghum bicolor (L.) Moench, commonly known as sweet sorghum (Dewet, 1978) or broomcorn (Sikora *et al.*, 2018), is a popular feedstock crop for new-age biofuels due to its high sugar content and ease of extraction (Zheng *et al.*, 2011; Mehmood *et al.*, 2014) and therefore serves as an important source of bioethanol and alcoholic beverages (Mathur *et al.*, 2017; Appiah-Nkansah *et al.*, 2019). The US Department of Agriculture estimates the energy invested in energy obtained during sweet sorghum biofuel extraction at 1:8 (Mathur *et al.*, 2017). Additionally, sweet sorghum-derived ethanol is eco-friendly due to its low sulfur content, low biological and chemical oxygen demand, and high octane rating (Clauser *et al.*, 2021). In addition to being used in traditional agriculture and biofuel generation, sweet sorghum is frequently employed in the remediation of heavy-metal-contaminated soils (Jia *et al.*, 2016).

Although the majority of species of the genus *Sorghum* are annual, a few tropical perennial species exist, such as the tetraploid perennial *Sorghum halepense*, commonly known as Johnson grass and an invasive species, and the perennial diploid *Sorghum propinquum*, which have been used to hybridize with *Sorghum bicolor* to breed new perennial germplasms (Piper & Kulakow, 1994; Paterson *et al.*, 1995; Washburn *et al.*, 2013; Kong *et al.*, 2015; Nabukalu & Cox, 2016; Sezen *et al.*, 2016; Cox *et al.*, 2018), because perennial crops, compared to annualanalogs, provide a variety of environmental benefits in terms of resource utilization, soil erosion, and soil conservation (Rasche *et al.*, 2017; Cox *et al.*, 2018). Recently, Habyarimana et al. identified cross lines of *S. bicolor* × *S. halepense* genotypes producing competitive

grain yield and aboveground biomass, the majority of which showed desirable overwintering rates in northern Italy together with a high-level fiber mass fraction (Habyarimana *et al.,* 2018). Perennial sweet sorghum cultivars or lines, on the other hand, have rarely been documented (Habyarimana *et al.,* 2020).

It is well known that soil microorganisms play a significant role in crop production and agricultural sustainability (Santos *et al.,* 2019; Rouphael & Colla, 2020); on the other hand, soil conditions, crop selection, and plant genotype all play a significant role in determining the composition of soil microbial communities (Stagnari *et al.,* 2014; Lopez-Angulo *et al.,* 2020). In the root zone, plants exude a variety of chemical substances depending on the species, developmental stage, and root traits, and those exuded compounds, exudates, have been shown to influence the structure and diversity of the rhizosphere microbiome (Bulgarelli *et al.,* 2013; Zhalnina *et al.,* 2018).

Due to exceptional tolerance to various abiotic stresses including drought, high salinity, alkalinity, nutrient-poor conditions, high temperature and heavy metal toxicity, sorghum and microbiome interactions have recently made significant progress. The development of the sorghum root microbiome is delayed by drought, and monoderm bacteria enriched in the root can positively impact sorghum plant growth during drought stress (Xu *et al.,* 2018). Under salt stress, distinct bacterial species were enriched in the root zone of sorghum at various salt concentrations (Gao *et al.,* 2021); similarly, under nitrogen stress, different sorghum genotypes were shown to recruit unique bacterial populations in their rhizosphere and root zones (Lopes *et al.,* 2021). Sorgoleone, the primary exudate of sorghum roots, has been demonstrated to influence the structure and function of the sorghum-associated bacterial microbiome (Wang *et al.,* 2021). However, little is known about the possible effects of perennial sorghum on above- and belowground soil microbial communities (Rasche *et al.,* 2017).

The main objective of this study was to characterize the rhizosphere bacteriome (bacterial communities) of the perennial sweet sorghum. We hypothesized that the rhizosphere bacteriome of the perennial sweet sorghum cultivar at the maturation stage would be significantly different from that of the annual sorghum cultivar due to the perennial plant's ability to live through the winter. This work will enable researchers to manipulate key functional characteristics of perennial sweet sorghum-associated microbes to increase the crop's cold tolerance and productivity.

Materials and Methods

Plant materials: The perennial sweet sorghum cultivar NaPBS778 (simply N778) was generated from an annual sweet sorghum cultivar via the Gene Phenotype Induction Technique (GPIT) (http://www.gpit.org/h-nd-277. html#_np=103_1194). The control sorghum line was TP60, which was an F_{11} recombinant inbred line (RIL) derived from the hybridization of sorghum cultivars T70 × P607 (Dong *et al.,* 2013).

Soil type field design and management: The sampling location was the experimental field (N 33°39′ 00″–02″, E 119° 00′02″–13″) on the campus of Huaiyin Normal University (HNU) at the Changjiang west road, Huai'an, Jiangsu Province, China. The soil in this experimental field belongs to the Aquic Cambisols based on CST, and contains approximately 133 g/kg organic matter, 0.95 g/kg total nitrogen, 0.61 g/kg total phosphorus, 6 mg/kg available phosphorus, and 21 g/kg total potassium plus 217 mg/kg available potassium (http://vdb3.soil.csdb.cn). The water content of bulk soil was measured via the drying method, and the pH (H_2O) value was detected by a pH meter (Five easy, Mettler-Toledo, Shanghai, China).

The experimental field on the HNU campus was divided into 9 plots (5 m × 6 m per plot) at the end of June 2020. Every three plots were used for a sorghum cultivar or line. Seeds of the sweet sorghum N778 and control line TP60 were sown in the field with single grain sowing on July 5 and July 7, respectively, in 2020. Due to the low germination rate, sorghum seeds were sown in a seedling-raising plate (16 × 8 pores) with prewetted nutrient medium (total content of nitrogen, phosphorus and potassium ≥ 2%; total amount of organic matter ≥ 40%) under natural conditions and appropriate watering on July 14 2020. Sorghum seedlings with 4 or 5 true leaves were then transplanted to the field after two weeks.

Manual weed control was performed for all 6 plots. However, one the pesticide mixtures with pymetrozine (37.5 $g{\cdot}ha^{-1}$) and lufenuron (30 $g{\cdot}ha^{-1}$) and the other pesticide mixtures with pymetrozine (37.5 $g{\cdot}ha^{-1}$) and pyraclostrobine (75 $g{\cdot}ha^{-1}$) were sprayed on Aug. 14, and Aug. 23, respectively, in 2020.

Harvesting and sampling: Sorghum plants were dug out and harvested carefully as described by Wen *et al*., (Wen *et al.,* 2019) with some modification. In brief, bulk soil was collected 15-20 cm from the base of each of five plant stems in each plot, and mixed thoroughly as one replicate. Next, five sorghum plants were excavated from five sampling points in each plot. Then, five roots dug out from one plot were collected as one replicate after the surrounding soil was discarded. Finally, the rhizosphere soil from primary roots or aerial roots was collected by brushing with clean tooth brushes, and root samples were centrifuged and collected after being washed thoroughly with PBS buffer in the laboratory. All of the rhizosphere soil and clean root samples were stored at −65°C for metagenomic DNA extraction. Plants of sorghum lines TP60 and N778 at the maturing stage were collected on Dec.11 and 12, 2020, respectively, while plants of sorghums line TP60 and N778 at the flowering stage were collected on Sept. 27, and Oct. 18, 2020, respectively, due to variable flowering times.

Plant trait and Brix assay: Plant height (cm), main panicle length (cm) and node number were measured at the flowering and maturation stages. Stalk weight (g) was measured at the maturation stage. The Brix (total soluble solids, 1% means 1 g sucrose in 100 g solution) was measured via a refractometer (LYT-390, BOJI YIQIYIBIAO Co., Ltd., Shanghai, China) from three crushed nodes near the aerial root at the maturation stage.

DNA extraction from soil and root samples: Total metagenomic DNA was extracted from approximately 0.30 g of each soil sample, which had been sieved through a 200 mesh stainless steel sieve, by using the DNeasy PowerSoil Kit (Qiagen, CA, USA) in accordance with the manufacturer's instructions with modification, in which FastPrep-24 (MP Biomedicals, CA, USA) was used to homogenize the soil samples. Additionally, total metagenomic DNA was extracted from each root sample as previously described by Lu et al.(Lu *et al.*, 2018a) with modification as mentioned above. The integrity of all DNA samples was further examined via 1% agarose gel electrophoresis before they were stored at −65°C.

High-throughput sequencing of 16S rDNA amplicons: The fusion primers, including the P5 or P7 Illumina adapter, an 8 nucleotide (nt) barcode sequence, and gene-specific primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Mori *et al.*, 2014; Xu *et al.*, 2016), were used for amplifying the 16S rDNA V3-V4 region of bacterial communities in bulk soil and rhizosphere soil samples. PCR amplification in triplicate, purification of the PCR product, determination of library quality, library quantification, and paired-end 300 high-throughput sequencing on the Illumina MiSeq platform (Illumina, CA, USA) were performed as previously described by Hu *et al*., (Hu *et al.*, 2019).

Data availability: Those deep sequencing data of 33 samples have been deposited into CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb), and the accession number was CNP0002630.

Amplicon data analysis: Amplicon data analysis was performed as previously reported (Lu *et al.*, 2017; Fazal *et al.*, 2020) with some modifications. Briefly, raw reads were filtered to eliminate those with ambiguous N bases, average base quality scores of less than 20, and lengths of less than 50 nt via Fastp (v0.19.6) (Chen *et al.*, 2018).Second, tags were obtained by merging the high quality paired-end reads via FLASH (v1.2.11) (Magoc & Salzberg, 2011).Third, effective tags were clustered into operational taxonomic units (OTUs) at 97% sequence similarity using UPARSE (v7.0.1090) (Edgar, 2013) after the chimeric sequence and singleton were removed. Then, the resulting representative sequences were classified using the Ribosomal Database Project (RDP) classifier (v2.11) (Wang *et al.*, 2007) based on the SILVA 16S rRNA database (v138) (Quast *et al.*, 2013) with a confidence threshold of 70%. Furthermore, taxonomic abundance of each OTU and beta diversity distance metrics were carried out via QIIME (v1.9.1) (Caporaso *et al.*, 2010), while alpha diversity was analyzed via Mothur (v1.30.2) (Schloss *et al.*, 2009). Further analysis of amplicon data was performed using the online Majorbio Cloud Platform (www.majorbio.com).

Phenotypic analysis via BugBase: BugBase is a microbiome analysis tool that determines high-level phenotypes present in microbiome samples (https://bugbase.cs.umn.edu/index.html) (Ward *et al.*, 2017). The phenotypes described below can be predicted by BugBase, which are gram-negative, gram-positive, biofilm forming, potential pathogenic, mobile element-containing, oxygen-utilizing (including aerobic, anaerobic, and facultatively anaerobic), and oxidative stress-tolerant types.

Phylogenetic analysis: The phylogenetic analysis was carried out by FastTree (v2.1.3) (Price *et al.*, 2010) based on alignments of nucleotide sequences to study the evolutionary relationships of representative OTUs according to the maximum likelihood (ML) method, and the phylogenetic tree was drawn by R language (v3.3.1). The 16S rDNA nucleotide sequences of 12 *Pseudarthrobacter* species, 6 *Pseudomonas* species, and 4 other psychrotolerant bacterial strains were downloaded from the NCBI nucleotide database to construct the phylogenetic tree.

Statistical analysis: Analysis of variance (ANOVA) was performed via SPSS (v16.0), after which a post hoc Tukey HSD or Scheffe test was applied to examine the pairwise group differences. The Wilcoxon rank-sum test, Student's t-test (equal variance) or Welch's t-test (unknown variance) was carried out for pairwise group comparisons, and the Kruskal–Wallis H test followed by a post hoc Tukey–Kramer test was carried out for multiple group comparisons using the online Majorbio Cloud Platform (www.majorbio.com).

Results

The phenotype of the perennial sweet sorghum cultivar N778: We used GPIT to generate a perennial sweet sorghum cultivar N778 from an annual sweet sorghum (*S. bicolor*) cultivar, which was sown in the experimental field base of Yunnan Eco-Agriculture Research Institute (YEARI) in Kunming, China, and has been living through multiple winters since 2014 with temperatures not falling below − 4°C (Fig. 1A). The plant height and panicle length of the N778 main stem were approximately 220 cm (Fig. 1A) and approximately 30 cm (Fig. 1B), respectively. New tillers were germinated normally from the primary root in November of each year (Fig. 1C). After the mature panicles of the main stem were harvested, new branches from the main stem of N778 normally developed, bloomed, and matured. Due to its specific habitat with a high altitude of approximately 2000 m, only a few *S. bicolor* varieties or cultivars can live in Kunming. To discover the impact of the perennial sweet sorghum N778 on belowground soil microbial communities, we designed and performed experiments at the experimental field of the HNU campus.

Analysis of bulk soil and plant traits: As shown in Table 1, the water content of bulk soil at the experimental field of the HNU campus was significantly higher at sorghum maturation than at the flowering stage, while the pH value of bulk soil did not change significantly. Regarding plant traits, N778 had significantly higher plant height and node number than TP60 at both the flowering and maturation stages, while the plant height and node number of N778 or TP60 themselves did not show significant differences between the flowering and maturation stages (Table 1). The panicle length of both N778 and TP60 showed no significant difference between genotypes or growth stages (Table 1). Furthermore, at the maturation stage, the stalk weight and Brix of N778 were much higher than those of TP60 (Table 1).

Fig. 1. Phenotype of the perennial sweet sorghum cultivar N778 at Kunming. (A) The plants of N778 were growing on the experimental field base of YEARI in Kunming in Nov. 2019; (B) its mature panicle (B); (C) its old stalk, new tillers and living roots.

Table 1. Bulk soil analysis and sorghum plant traits at the flowering and maturation stages in the experimental field located on the HNU campus.

	Trait	Flowering stage (mean ± SD)		Maturation stage (mean ± SD)	
		N778	TP60	N778	TP60
Bulk soil	Water content (%)	8.69 ± 0.81 [a]	8.10 ± 0.39 [a]	15.4 ± 0.31 [b]	14.5 ± 0.06 [b]
	pH value	7.91 ± 0.02	7.92 ± 0.01	7.91 ± 0.06	7.85 ± 0.04
Sorghum plants	Plant height (cm)	425.9 ± 29.49 [b]	391.2 ± 25.32 [a]	442.4 ± 21.34 [b]	371.4 ± 34.24 [a]
	Node number	19.87 ± 1.407 [b]	15.60 ± 1.352 [a]	20.87 ± 1.246 [b]	15.80 ± 2.077 [a]
	Panicle length (cm)	31.53 ± 2.232	32.60 ± 3.439	30.73 ± 1.387	30.63 ± 4.514
	Stalk weight (g)	ND	ND	932.7 ± 141.21 **	372.0 ± 169.91
	Brix (%)	ND	ND	13.5 ± 1.25 **	9.7 ± 2.21

Note: SD refers to the standard deviation (bulk soil samples, n=3; plant samples, n=15). N778 and TP60 refers to the perennial sweet sorghum cultivar NaPSB778 and control sorghum line TP60, respectively. The statistical method was one-way ANOVA followed by Tukey's HSD post-hoc test. The values in bold letters show a significant difference (p<0.05) among N778 and TP60 between the flowering stage and/or the maturation stage. ** indicates p<0.01. ND refers to "not detected"

Table 2. Summary of clean reads, effectivetags, OTU number of bulk and rhizosphere soils of the perennial sweet sorghum N778 and the control line TP60.

Sample name	Effective tags number	Tags mean length (nt)	Q30 of effective tags (%)	Taxonomic tags number at 97% similarity	OTU number
Na1FBS	50282	419.80	93.8742	33290	3458
Na2FBS	54353	420.06	94.0558	34954	3411
Na3FBS	51331	420.62	94.3909	34237	3424
Na1FARS	54713	418.12	94.0926	31642	3555
Na2FARS	51035	418.76	94.4440	33372	3494
Na3FARS	57592	418.40	94.2758	36265	3494
Na1FPRS	50777	419.12	94.5109	31318	3731
Na2FPRS	49367	418.80	94.3183	**27279**	3761
Na3FPRS	57863	418.25	94.6446	34540	3494
Na1MBS	47475	421.22	94.1869	33150	3425
Na2MBS	62416	420.21	94.2918	40671	3505
Na3MBS	62963	420.73	94.3525	40761	3320
Na1MARS	56618	418.58	94.4501	36923	3253
Na2MARS	58799	418.67	94.5122	35915	3321
Na3MARS	70723	418.15	94.7616	43658	2960
Na1MPRS	45758	418.49	94.1835	31492	3261
Na2MPRS	56945	418.04	94.4252	34294	3320
Na3MPRS	60806	417.83	94.5162	37839	2898
TP_1FBS	65055	419.53	94.3420	43272	3289
TP_2FBS	67638	420.93	94.1695	48136	3254
TP_3FBS	70208	420.39	94.3838	48872	3304
TP_1FPRS	55445	418.38	94.3102	38669	3759
TP_2FPRS	64179	419.12	94.6457	42149	3592
TP_3FPRS	58546	417.96	93.8711	41040	3594
TP_1MBS	44273	420.14	93.8503	32268	3492
TP_2MBS	74672	420.14	94.0317	47094	3356
TP_3MBS	70546	419.74	93.7912	48309	3343
TP_1MARS	54994	420.29	93.8939	36971	3107
TP_2MARS	54157	420.25	94.3879	36470	2972
TP_3MARS	57050	419.69	94.2267	43564	3001
TP_1MPRS	65107	419.57	94.5317	42750	3056
TP_2MPRS	72796	421.20	94.5204	48467	2657
TP_3MPRS	56058	417.91	94.6733	36733	2824

Note: Na and TP refer to the perennial sweet sorghum cultivar NaPSB778 and the control sorghum line TP60, respectively. F and M refer to the flowering and maturation stages, respectively. BS refers to bulk soil. ARS and PRS refer to rhizosphere soil collected from the aerial roots and the primary roots, respectively

Overall analysis of bacterial 16S rDNA amplicon sequencing data: Bacterial 16S rDNA (V3-V4) amplicon (338F to 806R) deep sequencing via Illumina MiSeq generated a total of 1,930,540 effective tags with an average number of 58501 per sample (Table 2). The mean length and Q30 average value of effective Tags were 419 nt and 94.30%, respectively (Table 2). The average number of taxonomic tags at 97% similarity and OTUs was 38374 and 3324 per sample, respectively (Table 2).

The total number of OTUs obtained from 33 samples was 8538, which belonged to 2195 species, 999 genera, or 42 phyla. Among those phyla, the top 5 dominant taxa were Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, andBacteroidetes.

Alpha diversity analysis of bacterial communities between N778 and TP60: To assess the alpha diversity across various groups, effective Tags of all samples were normalized according to the minimal number 27279 taxonomic Tags of Na2FPRS (Table 2), and Tags of Order Chloroplast and Family Mitochondria were eliminated during normalization. Six common alpha diversity indices, including Sobs, Chao, Shannon, Simpson, Simpsoneven, and coverage, were selected to make pairwise group comparisons at the OTU level via Welch's t-test due to nonassumed variance (Fig. 2). The coverage index of all samples was higher than 94.9% (Fig. 2F), and the result reflected that the sequencing depth in this study represented the real situation of a sample.

For comparison between bacterial communities in different compartments, the majority of indices of pairwise bulk soil comparisons of either plant genotypes and different stages did not show significant differences, with the exception of Sobs of bulk soil of N778 at the flowering stage (NaFBS) being significantly higher than that of bulk soil of TP60 at the flowering stage (TPFBS) (Fig. 2A), indicating that bulk soil samples as a control were workable for alpha diversity analysis. With regard to the bacterial community in rhizosphere soil collected from aerial roots and primary roots, all indices of pairwise comparison of the same genotype at the same growth stage did not show significant differences (Fig. 2), suggesting that rhizosphere soil samples collected from either aerial roots and primary roots were practicable for alpha diversity analysis.

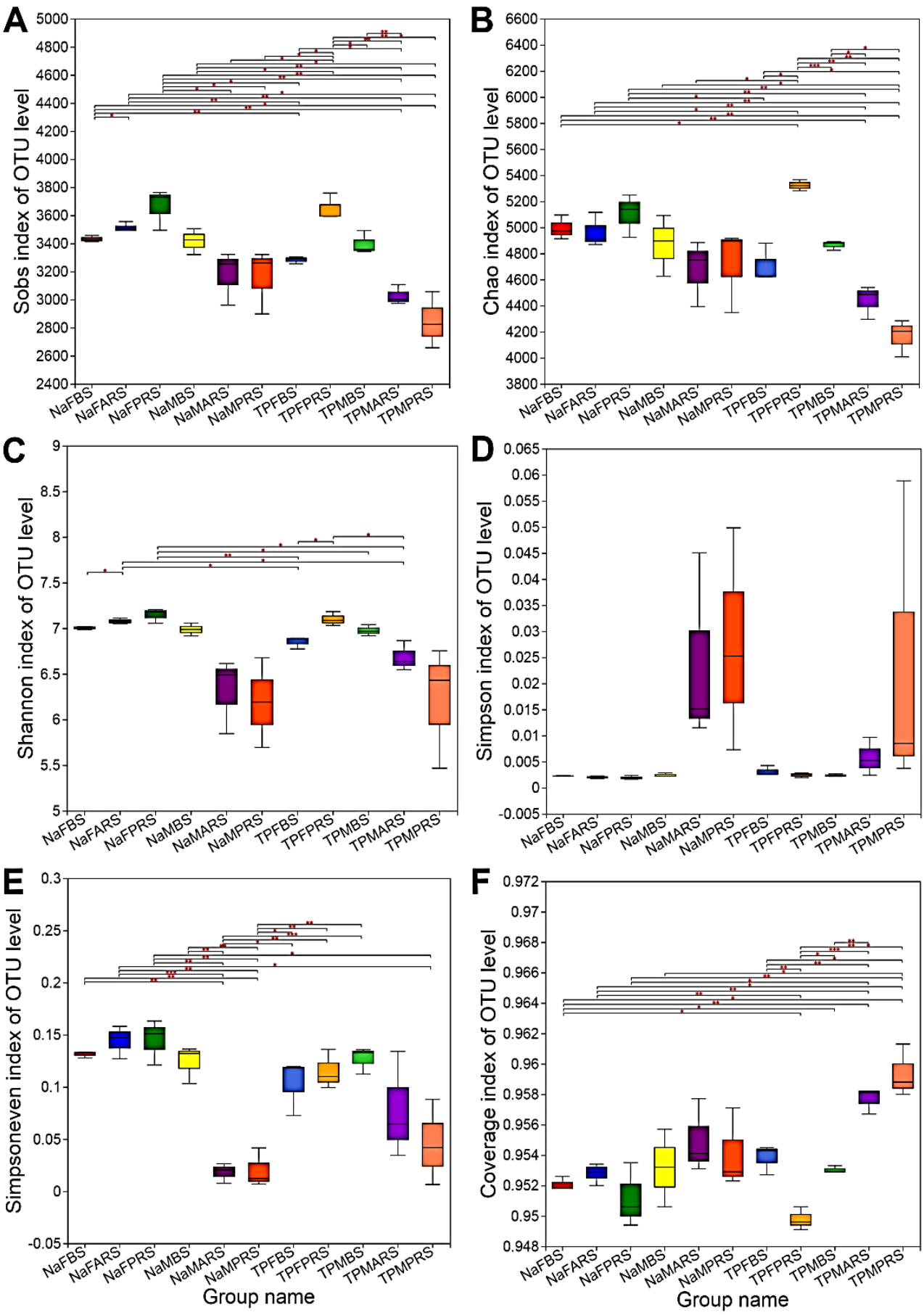

Fig. 2. Alpha diversity based on OTU abundance via boxplot analysis. The treatments detailare shown in Table 2. Significant differences between pairwise groups were determined via Welch's t-test. * means 0.01< p≤ 0.05; ** indicates 0.001< *p*≤0.01; *** indicates p≤0.001.

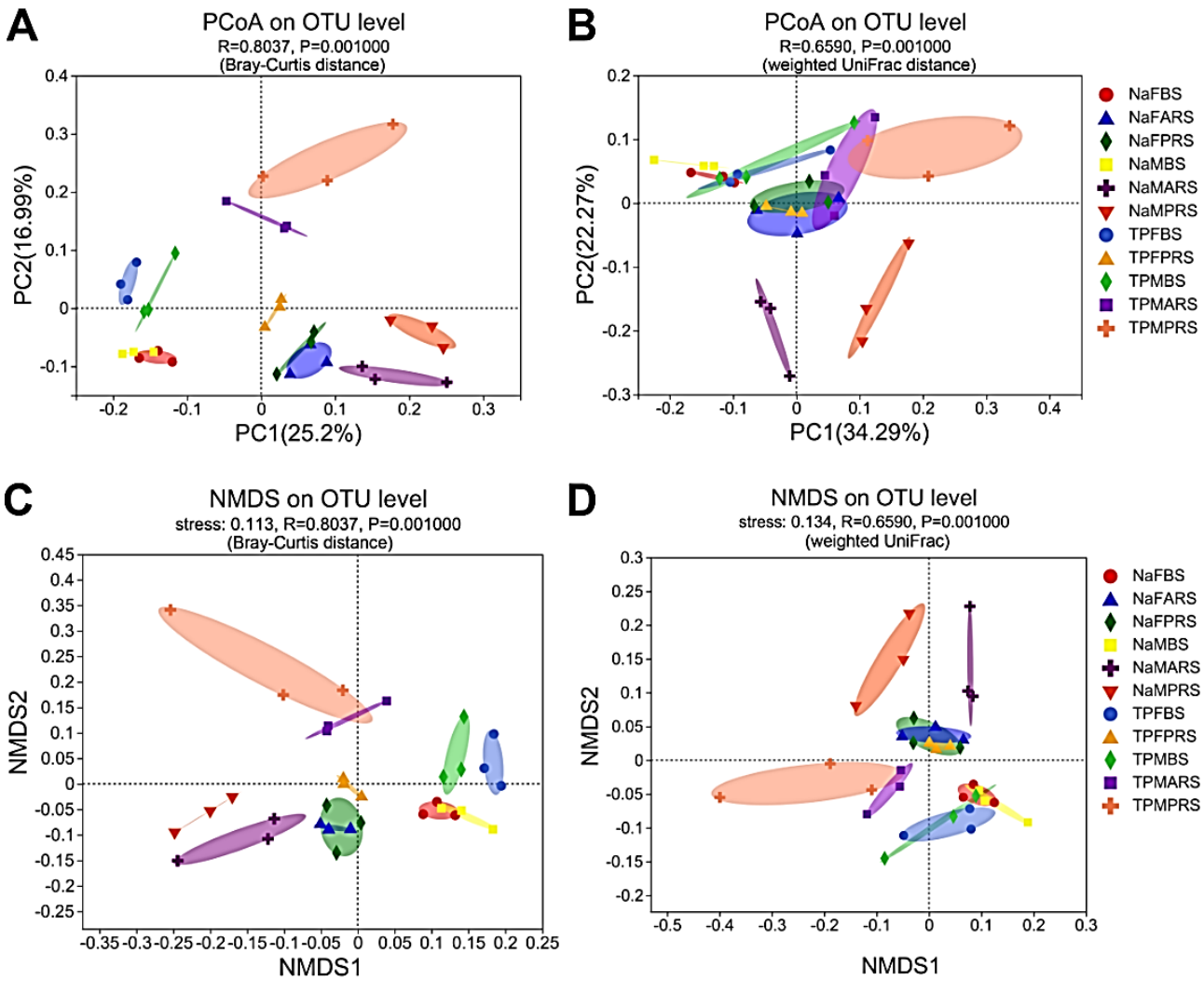

Fig. 3. Beta diversity of bacterial communities between N778 and TP60 by principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) at the OTU level. (A) PCoA based on Bray–Curtis distance, ANOSIM (R=0.8037, P=0.001), Adonis (R^2=0.6506, P=0.001). (B) Based on weighted UniFrac (WUF) distance, ANOSIM (R=0.6590, P=0.001). (C) NMDS based on Bray–Curtis distance; the stress value was 0.113, ANOSIM (R=0.8037, P=0.001), Adonis (R^2=0.6506, P=0.001). (D) NMDS based on WUF distance, the stress value was 0.134, ANOSIM (R=0.6590, P=0.001). The treatments detail are shown in Table 2.

For comparison between bacterial communities in different growth stages, Sobs and Simpsoneven indices of N778 rhizosphere soil at the flowering stage were significantly higher than those at the maturation stage (Fig. 2A, E), indicating that both richness and evenness of the bacterial community of N778 rhizosphere soil were higher at the flowering stage. Similarly, the Sobs, Chao and Shannon indices of TP60 rhizosphere soil at the flowering stage were significantly higher than those at the maturation stage (Fig. 2A-C), while the Simpson and Simpsoneven indices showed no significant difference (Fig. 2D, E), indicating that richness but not evenness of the bacterial community of TP60 rhizosphere soil was higher at the flowering stage.

For the comparison between different genotypes, almost all six indices of N778 rhizosphere soil from either aerial roots and primary roots did not differ significantly from those of TP60 at the same growth stage, with the exception of the Chao index of N778 rhizosphere soil from aerial roots at the flowering stage, which was significantly lower than that of TP60 rhizosphere soil from primary roots (Fig. 2B), and the coverage index of N778 rhizosphere soil from aerial roots at the flowering stage, which was significantly higher than that of TP60 rhizosphere soil from primary roots (Fig. 2F).

Beta diversity analysis of bacterial communities between N778 and TP60: First, principal coordinate analysis (PCoA) at the OTU level was used to examine the differences in bulk, rhizosphere soil samples between N778 and TP60. On the one hand, based on Bray–Curtis distance, PCoA results showed that the taxonomic beta diversity of the bacterial community in the rhizosphere soil of N778 from both aerial and primary root samples was significantly distinct from that of TP60 at the maturation stage according to ANOSIM and Adonis statistical analysis (Fig. 3A). On the other hand, based on weighted UniFrac (WUF) distance, the PCoA results showed that the phylogenetic beta diversity of bacterial communities in the rhizosphere soil of N778 from both aerial and primary root samples was also significantly distinct from that of TP60 at the maturation stage according to ANOSIM statistical analysis (Fig. 3B).

There were no significant differences between the rhizosphere soils of N778 and TP60 at the flowering stage (Fig. 3B). In addition, significant differences in both taxonomic and phylogenetic beta diversity of rhizosphere soil of N778 were found between the flowering stage and the maturation stage (Fig. 3A, B).

Second, nonmetric multidimensional scaling (NMDS) analysis was performed to compare the differences in bulk and rhizosphere soil samples between N778 and TP60. Similar to the PCoA results, the rhizosphere soil samples of N778 aerial and primary roots were significantly separated from those of TP60 at the maturation stage based on Bray–Curtis distance (Fig. 3C) and WUF distance (Fig. 3D), even though there was no significant difference between the rhizosphere soil of N778 and TP60 at the flowering stage (Fig. 3C, D). In addition, rhizosphere soil replicates of N778 at the maturation stage were also separated from those of N778 at the flowering stage (Fig. 3C, D). Moreover, the stress values in Fig. 3C and D were less than 0.2, which reflected that the NMDS analysis results seem to be reliable.

Furthermore, hierarchical clustering based on Bray–Curtis distance at the genus level was carried out to detect the differences in bulk and rhizosphere soil samples between N778 and TP60. Six rhizosphere soil samples of N778 aerial and primary roots at the maturation stage were clearly clustered together and separated from those TP60 aerial and primary root samples (Fig. 4A). All rhizosphere soil samples of N778 and TP60 aerial and primary roots at the flowering stage were clustered together and then clustered together with all bulk soil samples (Fig. 4A). Only the second rhizosphere soil sample of TP60 primary root at the maturation stage was quite specific in the hierarchical clustering tree (Fig. 4A).

Dominant bacterial taxa enriched in the rhizosphere of sweet sorghum N778 at the maturation stage: Among the top 5 dominant genera from the bulk soil and rhizosphere soil of N778 and TP60, the top first, second, and fifth genera were unclassified but belonged to the order Vicinamibacterales, the family Vicinamibacteracea, and the family Gemmatimonadaceae, respectively. *Pseudarthrobacter* and *Pseudomonas* were the top third and fourthgenera, respectively (Fig. 4B, C), which were likewise the top 2 dominant genera in the rhizosphere soil of the N788 root at the maturation stage (Fig. 4B).

Furthermore, OTU2574, an unclassified species of *Pseudarthrobacter*, was discovered to be the top first OTU, and its mean proportion, near 14%, was found to be significantly higher in the rhizosphere soil of N778 aerial and primary roots at the maturation stage than all other sample groups through multiple group comparisons using the Kruskal–Wallis H test (Fig. 5). Likewise, the mean proportion of OTU772 (the top fourth OTU), which was categorized as an unclassified *Pseudomonas* species, was also considerably greater in the rhizosphere soil of N778 aerial and primary roots at the maturation stage than all other groups (Fig. 5).

Then, BugBase was carried out through multiple group comparisons with the Kruskal–Wallis H test to discover the potential phenotypes of OTU2574 and OTU772. At the maturation stage, the mean proportions of gram-positive, aerobic, and stress-tolerant phenotypes were significantly higher, but the mean proportions of potentially pathogenic and gram-negative phenotypes were significantly lower in the rhizosphere soil of N778 aerial and primary roots than those of all other groups (Fig. 6).

These results suggested that at the maturation stage, the mean proportions of gram-positive bacteria, aerobic bacteria, and stress-tolerant bacteria were significantly higher but those of potentially pathogenic and gram-negative bacteria were significantly lower in the rhizosphere soil of N778 aerial and primary roots than those of all other groups.

Phylogenetic analysis of dominant OTUs enriched in the rhizosphere of the perennial sweet sorghum N778 at the maturation stage: To explore the potential function of OTU2574 and OTU772, phylogenetic analysis was performed by FastTree according to the maximum-likelihood (ML) method. As shown in Fig. 7A, OTU2574, a *Pseudarthrobacter* unclassified species, was closely related to *Pseudarthrobacter psychrotolerans* YJ56 that is a psychrotolerant bacterial strain. Additionally, OTU772, an unclassified *Pseudomonas* species, was closely related to *Pseudomonas* sp. TmR5a, which shows ice recrystallization inhibition (IRI) activity, and *Pseudomonas* sp. TmR1b (Fig. 7B).

Discussion

One of our original objectives was to test whether the perennial sweet sorghum cultivar N778 could live through the winter in Huai'an, China, in which the lowest temperature was −7 to 8°C in winter. However, two strong cold waves passed through Huai'an, China and resulted in local extreme low temperatures of −11 to 12°C from late Dec. 2020 to early Jan. 2021, and all sorghum plants were frozen to death. The other objective is to characterize the rhizosphere bacteriome of the perennial sweet sorghum cultivar N778.

The plant height of the perennial sweet sorghum cultivar N778 has very high plasticity: In this study, the plant height of the perennial sweet cultivar N778 has very high plasticity due to different soil types and growth environments, in which the average plant height was 425.9 cm and 442.4 cm at the flowering and maturation stage, respectively, when growing in the experimental field of the HNU campus, while its plant height was approximately 220 cm at the maturation stage, when growing in the experimental field base of YEARI in Kunming, China.

Plant height, the product of node number and stem internode length, has the highest positive correlation with aboveground dry mass yield, and is used as a critical factor of biomass yield with low heritability (Upadhyaya

et al., 2013; Habyarimana *et al.*, 2020). A previous meta-analysis of 24 sorghum trials indicated that plant height and cellulosic content mostly account for sorghum biomass yield advantage (Habyarimana *et al.*, 2016). Traditionally, sorghum plant height is reported to depend on four dwarfing (Dw) genes including *Dw 1* (Sobic.009G230800), *Dw2* (Sobic.006G067600), *Dw3* (Sobic.007G163800), and *Dw4* (not cloned yet) (Habyarimana *et al.*, 2020). Recently, *Dw5*, the fifth *Dw* gene, has been reported (Chen *et al.*, 2019). Furthermore, many others genes have been identified that can affect plant height directly and are involved in brassinosteroid and gibberellin metabolism (Ordonio *et al.*, 2016). latest study reports that sorghum plant height has recorded 42 associated SNPs (single nucleotide polymorphism) spanning 7 chromosomes (Habyarimana *et al.*, 2020).

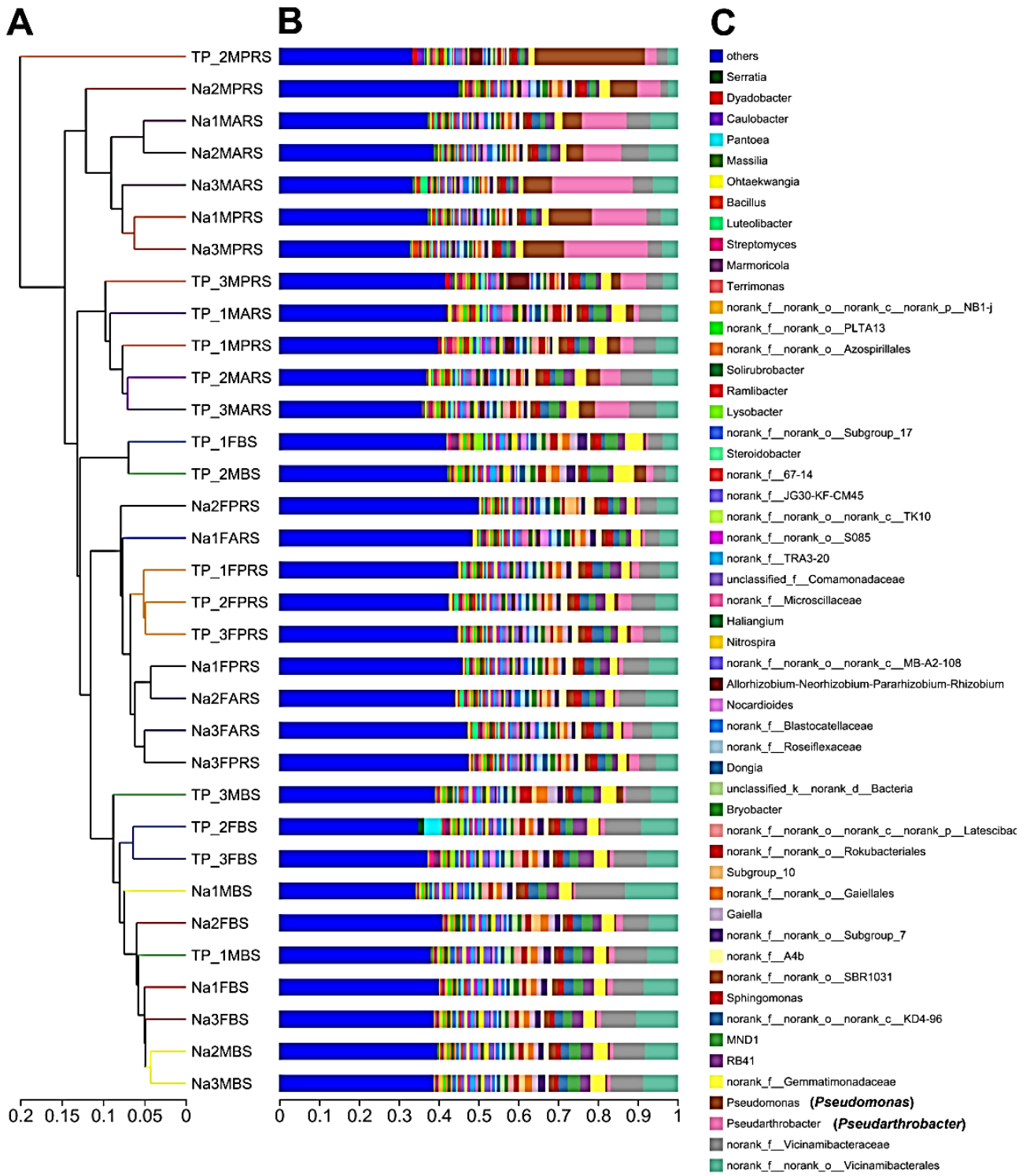

Fig. 4. Composition at the genus level and hierarchical clustering analysis based on Bray–Curtis distance. (A) Hierarchical clustering tree of 33 samples belonging to 11 groups that are presented in different colors, and the length between branches represents the distance between samples. (B) The bacterial composition of 33 samples displayed via stacked column charts at the genus level. (C) Dominant genera in each sample displayed on the stacked column. The treatments detail are shown in Table 2.

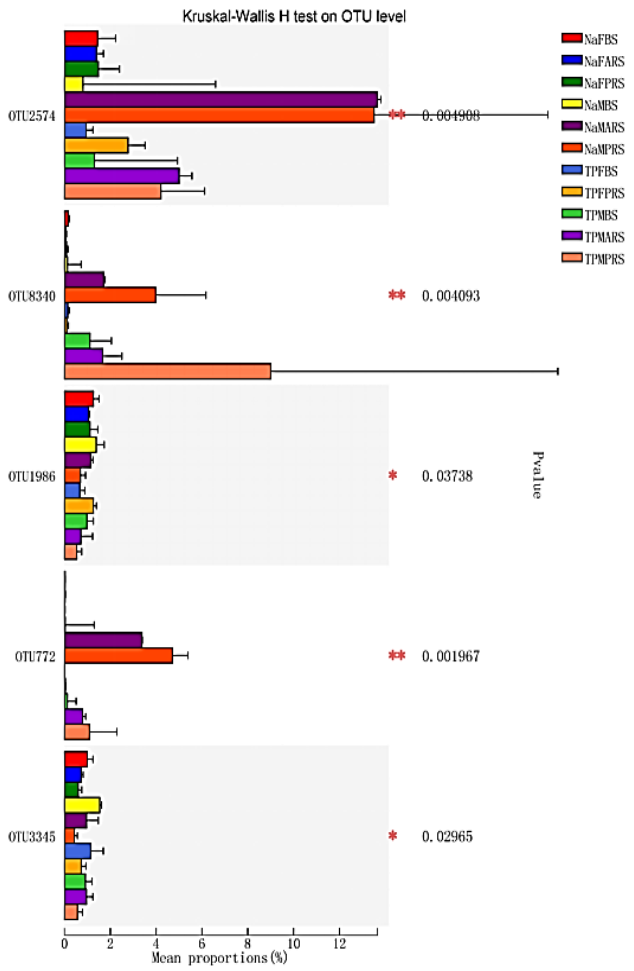

Fig. 5. Top 5 OTUs via multiple group comparisons. Kruskal–Wallis H test followed by Tukey–Kramer post-hoc test was applied to check for significant differences. * indicates $0.01 < p \leq 0.05$; ** indicates $0.001 < p \leq 0.01$. The treatments detail are shown in Table 2.

Recently, seven quantitative trait loci (QTLs) have been reported for sorghum plant height, fresh weight of stem and leaf, and juice weight and could have been identified across four environments repeatedly (Wang *et al.*, 2016). In addition, the panicle yield of sweet sorghum has been reported for the most part to be dependent on the genotype × environment interaction (47.5%) (Sikora *et al.*, 2018). The results of the above two previous studies suggest that sorghum plant height can be significantly affected by the environment.

The actual Brix in the stalk of the perennial sweet sorghum N778 should be higher than 13.5%: Typically, a Brix shows approximately 85% sugar and 15% soluble starch in sweet sorghum sap, and the stalk Brix value is a critical significant trait for bioenergy and ethanol production, although its diversity among different genotypes ranges from 13–24% (Regassa & Wortmann, 2014). However, up to 12–30% of fermentable sugars in sweet sorghum sap may be lost in 3 days, and as much as 40–50% may be lost in one week at room temperature (Wu *et al.*, 2010). In our study, the Brix in the sorghum stem was not measured until one week after harvesting, therefore, the actual Brix in the stalk of sweet sorghum N778 should be much higher than 13.5%.

Theoretical fresh stalk weight of the perennial sweet sorghum N778: In our study, every three plots (5 m × 6 m per plot) were used for the perennial sweet sorghum cultivar N778 or its control line TP60, and there were 12 × 14 seedlings growing in a plot. At the maturation stage, approximately 12 × 13 plants were harvested from each plot. The average stalk weight of the sweet sorghum cultivar N778 was 0.932 kg except for the tillers in this study, so the theoretical fresh stalk yield could be 48,415 kg ha^{-1}. The tillers were recorded, and there were approximately 0.48 tiller per plant of the sweet sorghum cultivar N778; therefore, the final theoretical fresh stalk yield should be more than 50,000 kg ha^{-1} when the tiller yield was included.

Dominant bacterial taxa with potential cold tolerant phenotype enriched in the rhizosphere of the perennial sweet sorghum at the maturation stage: Using high-throughput sequencing amplicons of the V3–V4 hypervariable region of 16S rDNA, OTU2574 and OTU772, two dominant OTUs, were discovered to be unclassified species of *Pseudarthrobacter* and *Pseudomonas*, respectively. Both were found to be significantly enriched in the rhizosphere of N778 at the maturation stage via multiple group comparison. The predicted results by BugBase suggested that OTU2574 and OTU772 might be associated with gram positive bacteria, aerobic bacteria, and stress tolerant bacteria. Furthermore, through phylogenetic analysis, OTU2574 was found to be closely related to *Pseudarthrobacter psychrotolerans* YJ56, which is a psychrotolerant bacterial strain isolated from Antarctic soil (Shin *et al.*, 2020). In addition, OTU772 was found to be closely related not only to *Pseudomonas* sp. TmR5a, which shows IRI activity and can promote the growth of tomato plants under cold stress (Vega-Celedon *et al.*, 2021), but also to *Pseudomonas* sp. TmR1b, a psychrotolerant bacteriumthat has high phosphate solubilization activity at 4°C, contains the *nifH* gene, and shows antimicrobial activity against phytopathogenic *michiganensis* OP3 and *Pseudomonas syringae* pv. Syringae (Vega-Celedon *et al.*, 2021). Taken together, the functions of OTU2574 and OTU772 could most likely be associated with cold tolerance.

Conclusion

In this study, we report that the perennial sweet sorghum cultivar N778 has prominent panicle length, stalk weight, Brix, and plant height with high plasticity. Furthermore, our results indicate that two dominant OTUs belonging to *Pseudarthrobacter* and *Pseudomonas* with stress-tolerant phenotype are significantly enriched in the rhizosphere of the perennial sweet sorghum N778 at the maturation stage, implying that the perennial sweet sorghum N778 can recruit potential cold-tolerant bacterial taxa into its rhizosphere and assist it in surviving the winter below freezing temperature.

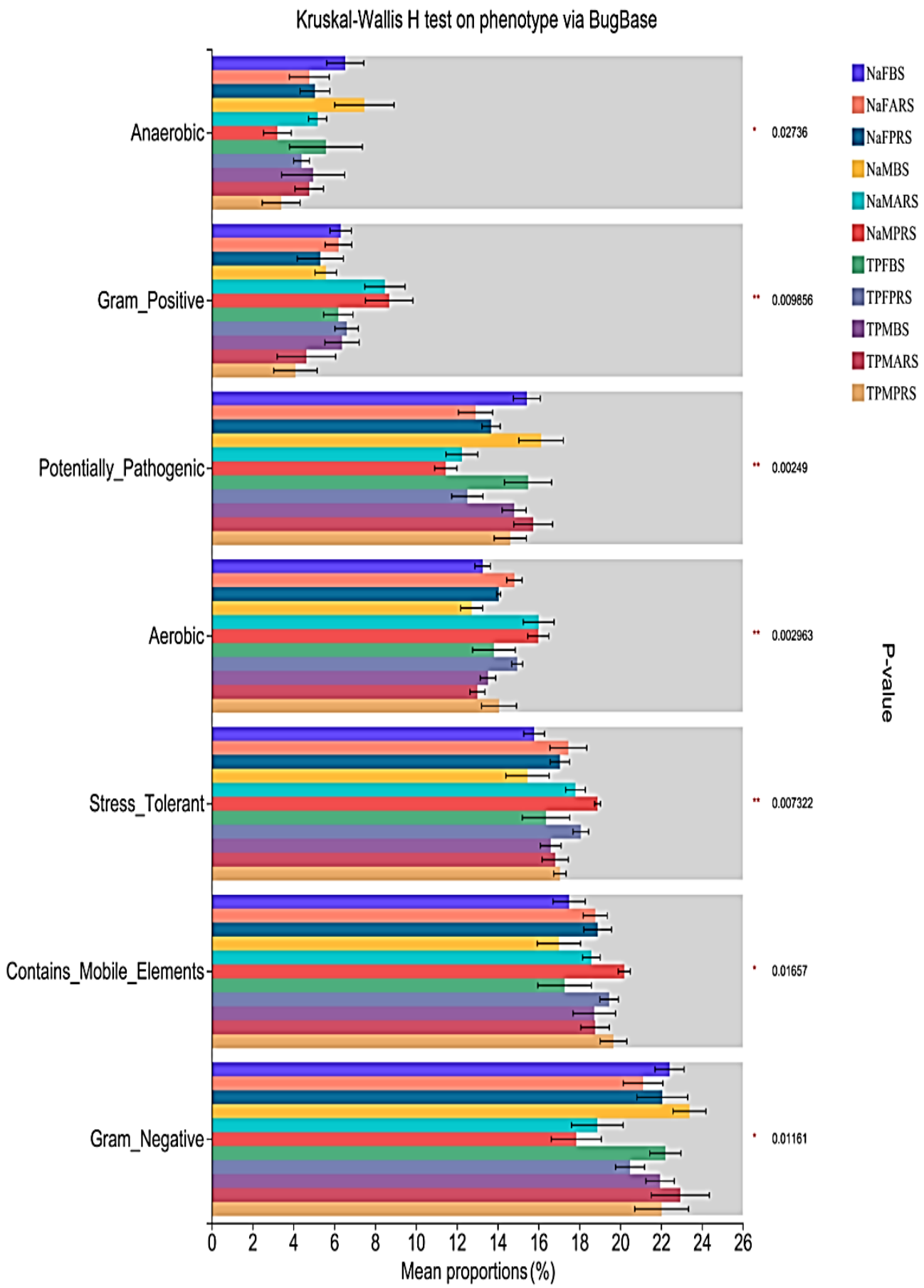

Fig. 6. Bacteriome phenotype predicted by BugBase. Kruskal–Wallis H test followed by Tukey–Kramer post-hoc test was applied to check for significant differences. * indicates $0.01 < p \leq 0.05$; ** indicates $0.001 < p \leq 0.01$. The treatments detail are shown in Table 2.

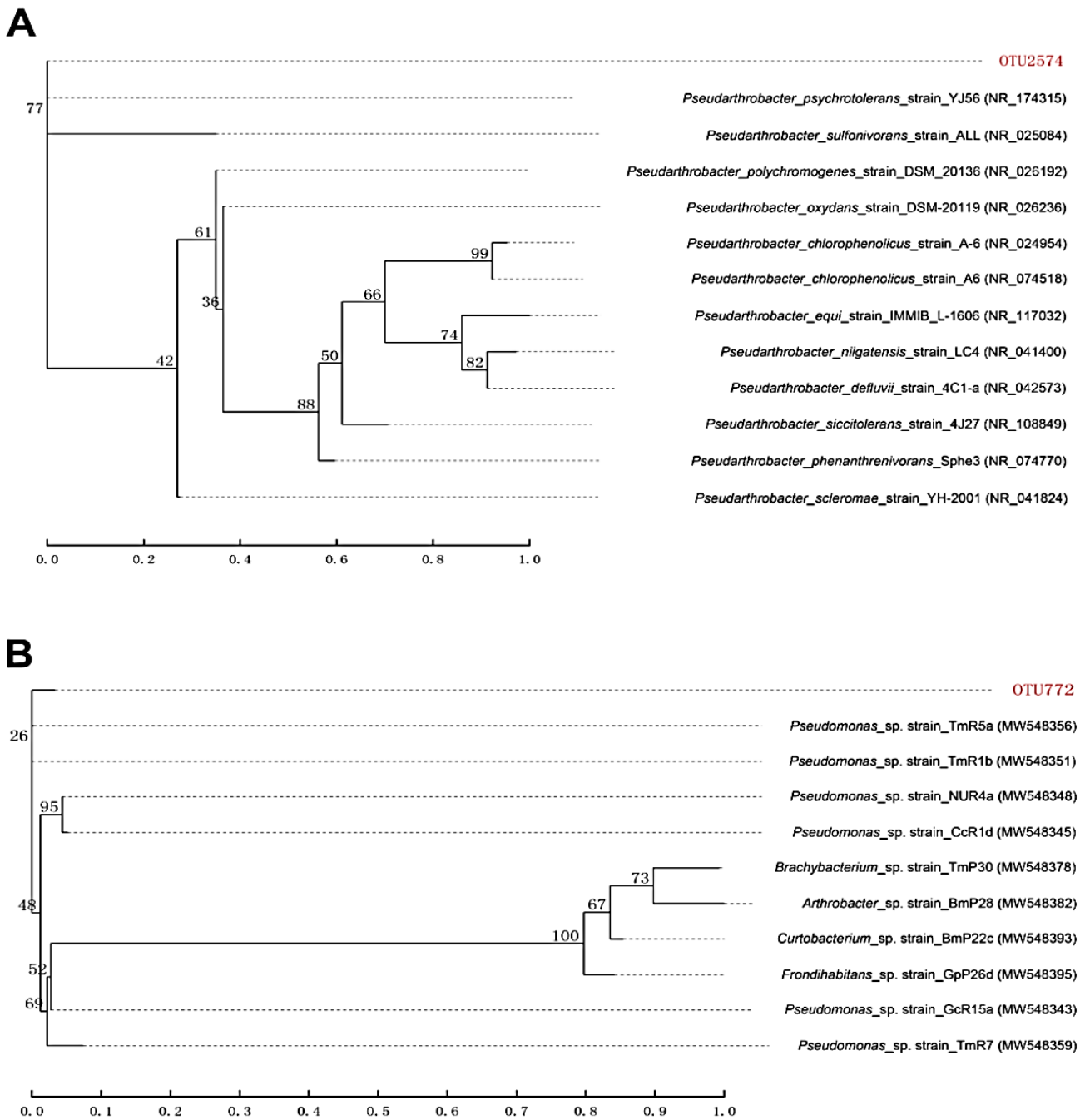

Fig. 7. Maximum-likelihood (ML) phylogenetic tree of two dominant OTUs enriched in the rhizosphere of sweet sorghum N778. (A) ML phylogenetic tree of OTU2574 based on 16S rDNA nucleotide sequences of 12 *Pseudarthrobacter species*; (B) ML phylogenetic tree of OTU772 based on 16S rDNA nucleotide sequences of 6 *Pseudomonas species* and 4 other psychrotolerant bacterial strains reported by Vega-Celedon et al. (Vega-Celedon *et al.*, 2021). Bootstrap values are displayed at nodes of the phylogenetic tree. The number of GenBank accessions of the 16S rDNA sequence is shown in parentheses.

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